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DOCTORAL THESIS

**Involvement of p75^{NTR} signaling pathway in
persistent synaptic suppression coupled with synapse
elimination following repeated LTD induction**

LTD の繰り返し誘発によって生じる長期持続性シナプス
抑圧・廃止現象への p75^{NTR} シグナル経路の関与

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Summary

Synaptic plasticity, especially structural plasticity such as synapse formation and synapse elimination, is thought to be a basis for long-lasting memory. The laboratory I belong to (simply referred to 'we', hereafter) previously reported in stable organotypic cultures of the rat hippocampal slice that the repeated induction of LTD (long-term depression) by application of an mGluR (metabotropic glutamate receptor) agonist led to slowly developing, long-lasting synaptic suppression coupled with synapse elimination. We referred to this phenomenon as LOSS (LTD-repetition-operated synaptic suppression) to discriminate it from conventional single LTD and proposed it as an in vitro model to analyze structural plasticity. Recently the p75NTR signaling possibly activated by proneurotrophins (precursor forms of neurotrophins including NGF and BDNF) has been gaining attention as a novel pathway that regulates neuronal apoptosis as well as synaptic plasticity such as LTD and synapse elimination. In this study, I examined the possible involvement of this signaling in the establishment of LOSS. Application of anisomycin, a protein synthesis inhibitor, indicated the requirement of novel protein synthesis within 6 hours after the induction of mGluR-dependent LTD. This result supports that LOSS is an active process instead of a passive one such as withering due to shortage of trophic factors. Quantification of protein expression level after repeated LTD induction suggested that proBDNF (a potential ligand to p75NTR) is newly synthesized within 6 hours after the induction of LTD. Therefore I treated the cultured slices with antibody that binds to and neutralizes p75NTR following the repeated LTD induction and found that LOSS was blocked by this antibody treatment, supporting the commitment of p75NTR signaling in LOSS

production. In addition, the exogenous application of a cleavage-resistant form of proBDNF led to synaptic suppression and spine elimination similar to LOSS. These results suggest the involvement of the p75NTR signaling pathway for the long-lasting decremental form of synaptic plasticity.

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General Introduction

The nervous system can change in response to the events that an animal has and will later behave differently as a result. This adaptive capacity gives animals the ability to learn and to memorize. The development of views of learning and memory constitutes much of the history of psychology (James 1980, Guthrie 1935). However, the technical advances in neurobiology made it possible to study the cellular and molecular process of learning and memory. Neurobiologists now believe that the capacity for memory is derived from a special case of a more general phenomenon: neuronal plasticity. Many neurons exhibit plasticity, that is, they can change functionally and structurally, often in a lasting way.

The most important discovery which clearly showed that alteration in synaptic efficacy can be correlated directly with behavioral learning is provided by the studies of habituation, sensitization, and classical conditioning in the sea hare Aplysia (Kandel and Taug 1961). Kandel and Spencer (1968) said in their comprehensive review of the neurophysiological basis of learning; “Since persistence is one of the most distinctive features of learning, we believe that analysis of the plastic properties of neurons is a prerequisite for the neurophysiological study of learning”.

Before the neurobiological research of synaptic plasticity in favorable invertebrate preparations, the idea that memory storage in the brain results from activity-dependent changes in synaptic strength had been already introduced by Canadian psychologist Donald Hebb (1949), who proposed that synapses linking two cells could be strengthened if both cells were active simultaneously. According to his theory, synapses respond to stimulation by neurotransmitters in a cumulative manner, and repeated stimulation—for example, by learning—leads to ever-stronger connections

between specific neurons until they become permanent.

The first such Hebbian synapses to be found in the mammalian brain were the connections made by entorhinal perforant path fibers onto dentate granule cells in the hippocampus (Bliss and Lomo, 1973). Brief, high-frequency stimulation of this excitatory pathway elicited a long-lasting enhancement of synaptic transmission, which is now known as long-term potentiation (LTP). Following the demonstration of high frequency stimulation (HFS)-induced LTP in the hippocampal CA1 region *in vitro* (Schwartzkroin and Anderson, 1975), activity-dependent synaptic weakening called long-term depression (LTD) was shown to be induced by low frequency stimulation (LFS) in the same neural pathway (Dudek and Bear, 1992; Mullkey and Malenka, 1992). Fig.1 shows typical type of LTP and LTD in CA3-CA1 synapse of cultured hippocampal slice preparation. LTP was induced by HFS (100Hz 1s), whereas LTD was induced by LFS (1Hz 15min).

LTP and LTD, now assumed to be the cellular basis of learning and memory, has been studied using acutely prepared hippocampal slices as a model system and the cellular mechanisms underlying these phenomena are largely understood (Bliss and Collingridge, 1993; Martin et al., 2000; Malenka and Bear, 2004). According to current understanding, LTP and LTD arise from externalization and internalization, respectively, of AMPA-type glutamate receptors in postsynaptic membrane (Malinow and Malenka, 2002; Kessels and Malinow, 2009). This means that LTP and LTD are the changes of the transmission strength in presently existing synapses.

On the other hand, long-lasting information storage is thought to be a consequence of the restructuring of neuronal circuits realized by a combination of

the generation and elimination of synaptic structures (Bear et al., 2001). However, the cellular mechanisms underlying these structural changes remain unclear, mostly because of the lack of appropriate in vitro model systems. Although a variety of morphological changes in synapses accompanying LTP and LTD in the hippocampus have been reported (Bolshakov et al., 1997; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999; Nagerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004), the longevity of these changes and their contribution to long-lasting memory is controversial (Schrull and Linden, 2007; Wang et al., 2007).

Using stable cultures of rat hippocampal slice, the laboratory I belong to (referred to 'we' in this thesis hereafter) reported previously that repeated but not single inductions of LTP led to the formation of synapses (Tominaga-Yoshino et al., 2002, 2008). The resulted increase in synaptic strength lasted as long as the cultures continued (> 3 weeks). We referred this synaptogenesis as RISE (Repetitive-LTP Induced Synaptic Enhancement) in order to discriminate it from conventional single LTP. . In contrast, the repeated but not single inductions of LTD led to the elimination of synapses (Shinoda et al., 2005).

LTD in the hippocampus induced by electrical stimulation at low frequency or pharmacological activation of mGluR (metabotropic glutamate receptor) is a form of synaptic plasticity that occurs instantaneously in existing synapses (Bear et al., 1996, Gladding et al., 2009). In hippocampal slice cultures, LTD was induced by the application of 50 μ M DHPG, a group I mGluR agonist (Fig. 2A). However, the decrease in the synaptic strength, which is represented by the maximal field EPSP amplitude, returned to the control level 24-27 hours after the DHPG exposure, suggesting that LTD was seen to disappear within 1 day (Fig. 2B). But when the

DHPG exposure was repeated more than 3 times, the suppression of the synaptic strength was observed even 3 weeks later (Fig. 3A, B). This synaptic suppression developed gradually and lasted long after synaptic depression induced by third DHPG exposure returned to the control level (i.e., the level of three-times mock stimulated specimens; shown by an open circle at 2 day in Fig. 3C), indicating that this suppression was not due to the extension of the life time of LTD. Moreover, immunohistochemical and electron microscopic examination demonstrated that a decrease in the density of presynaptic structures and asymmetrical synapses was accompanied by the synaptic suppression (Fig. 4A-D, Shinoda et al., 2005; Kamikubo et al., 2006). Hence, we referred to this phenomenon as LOSS (LTD-repetition-operated synaptic suppression) in order to discriminate it from conventional single LTD. I contributed to these works as an electrophysiologist examining the synaptic responsiveness of the cultured slices (Fig. 2, 3). Upon analyzing the property of RISE and LOSS in detail, we concluded that they stand as good in vitro model systems to analyze activity-dependent structural plasticity occurred in matured synaptic circuits. Here I proceed from phenomenology to mechanistics of LOSS by combining electrophysiological, morphological and biochemical analyses.

The neurotrophins are a family of proteins that are essential for the development of the vertebrate nervous system. Since the discovery of nerve growth factor (NGF) (Levi-Montalcini, 1952), the studies of neurotrophin family have comprised an important part of neuroscience because the influence of neurotrophins spans from developmental neurobiology and degenerative and psychiatric disorders to synaptogenesis and activity-dependent forms of synaptic

plasticity (Chao, 2003). In the mammalian brain, four neurotrophins have been identified: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4. These closely related molecules function by binding to two distinct classes of transmembrane receptor: the p75 neurotrophin receptor ($p75^{NTR}$) and Trk family of receptor tyrosine kinases, which includes TrkA, TrkB and TrkC (Dechant and Barde, 2002; Huang and Reichardt, 2000; Kaplan and Miler, 2000). Each Trk receptor selectively binds a different neurotrophin, whereas $p75^{NTR}$ has a similar affinity for all neurotrophins.

Like other secreted proteins, neurotrophins are initially synthesized in a form of precursors, proneurotrophins (30-35 kDa), which are proteolytically cleaved to produce mature neurotrophins (12-12 kDa) (Seidah et al., 1996). Until recently, only mature neurotrophins are considered biologically active. However, this traditional view was shattered by the finding that proneurotrophins bind with high affinity to $p75^{NTR}$, which for years was thought to be a 'low-affinity' neurotrophin receptor, and induce neuronal apoptosis (Lee et al., 2001; Teng et al., 2005). Therefore, proneurotrophin- $p75^{NTR}$ signaling has recently attracted attention as a possible intercellular signaling pathway that counters the effect of neurotrophin-Trk signaling (Lu et al., 2005; Blöchl & Blöchl, 2007; Martinowich et al., 2007).

In the hippocampus, BDNF is known as the major species of neurotrophin functioning there (Ernfors et al., 1990; Narisawa-Saito and Nawa, 1996). BDNF arises from proBDNF (a precursor of BDNF which possesses a propeptide sequence at its N-terminus). Mature BDNF is a well known intercellular signaling protein that mediates neuronal survival, synaptic plasticity such as LTP and synaptogenesis

through the activation of its high-affinity receptor TrkB (tropomyosin-receptor-kinase), a receptor tyrosine kinase. On the other hand, proBDNF is recently attracting attention as a novel neurotrophic substance possibly functioning by itself separately from BDNF and its effects are almost opposite from those of BDNF. For example, in sympathetic ganglion cell cultures, exogenously applied mature BDNF bound to TrkB to facilitate neuron survival, whereas proBDNF accelerated the occurrence of apoptosis via binding to p75^{NTR} (a molecule previously assumed as a low-affinity receptor for neurotrophins including BDNF) (Teng et al., 2005). In acutely prepared hippocampal slices, exogenously applied proBDNF enhanced the magnitude of LTD induced by a low-frequency stimulus (Woo et al., 2005), whereas exogenously applied BDNF provoked LTP (Figurov et al., 1996). Moreover, the BDNF-TrkB intercellular signaling is thought to affect incremental, long-lasting synaptic plasticity coupled with synaptogenesis (Seil & Drake-Baumann, 2000; Alsina et al., 2001; Taniguchi et al., 2005). Thus it is possible that the proBDNF-p75^{NTR} intercellular signaling pathway might function as a regulatory pathway to affect decremental, long-lasting plasticity coupled with synapse elimination. In fact, peripheral synapses including those in the sympathetic ganglion and the neuromuscular junction have been recently suggested to be pruned through this pathway (Singh et al., 2008; F. Yang et al., 2009).

Here I examined the possible involvement of the p75^{NTR} intercellular signaling pathway in the establishment of LOSS, by 1) examining the dependency of LOSS to protein synthesis; 2) determining the time window for the synthesis of protein(s) essential for the development of LOSS; 3) confirming the production of proBDNF protein in the time window determined above; 4) examining the effect of

anti-p75^{NTR} antibody which masks the effect of proBDNF; 5) examining the effect of exogenously applied proBDNF.

Materials and Methods

Slice culture

The hippocampus was isolated from deeply anesthetized 7-8 days-old Wistar/ST strain rat neonates. The middle portion was sectioned perpendicularly to its long axis into 400 μ m-thick slices with a McIlwain tissue chopper. The slices were placed on a polytetrafluoroethylene membrane filter cup (Millicell-CM; Millipore) and submerged in culture medium (composed of 50% minimal essential medium based on Hanks' salts, 25% Hanks' buffered saline and 25% heat-inactivated horse serum [all from Gibco]). The culture was contained in a moist chamber and maintained at 34°C for 2 weeks until maturation (Muller et al., 1993; Tominaga-Yoshino et al., 2002) with the medium renewed twice a week.

Animals were treated properly according to our School's guideline for animal care and welfare.

Chemical induction of LTD

To induce LTD in cultured slices, an aseptic environment was needed. We have reported that the establishment of LOSS was independent of the method applied for chemical LTD induction (Group I mGluR activation, Na^+/K^+ -ATPase inhibition or mild NMDA receptor activation; Kamikubo et al., 2006). I therefore adopted DHPG (3,5-dihydroxyphenylglycine, an agonist of Group I mGluR; Tocris) as a representative means to induce LTD in this study. The culture was exposed for 10 min to 50 μ M DHPG dissolved in BSS (balanced salt solution; composed of [in mM]: 149 NaCl, 5 KCl, 2 MgSO_4 , 2.5 CaCl_2 , 10 glucose, 10 HEPES-NaOH [pH 7.3]), washed twice with BSS and returned to the culture medium. The LTD induction

was repeated 3 times at 24 hours intervals and the culture was maintained for another 3 weeks (experimental protocol is shown in Fig. 1A). The day of the first LTD induction was reckoned as day 0.

In order to examine possible interference with the establishment of LOSS, the following drugs and chemicals were applied: anisomycin (Wako), a cleavage-resistant proBDNF (B243m-mut; Alomone), anti-p75^{NTR} antibody (AB1554; Millipore) and rabbit IgG (Vector Lab). The dose, onset and period of application are described in each part of Results.

Electrophysiological recording

Synaptic strength was monitored 3 weeks after repeated DHPG application (*i.e.* day 20-21 of the first DHPG application). The piece of filter carrying the cultured slice was transferred to a recording chamber perfused with ACSF (composed of [in mM]: 126 NaCl, 5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 2.0 MgSO₄, 22 NaHCO₃, 10 glucose; saturated with 95% O₂+5% CO₂ [pH7.3]) at 1 mL/min. A tungsten bipolar electrode was placed at the CA3 pyramidal layer, from which test stimuli were delivered every 30 seconds. A glass microelectrode filled with ACSF (1-5 MΩ series resistance) was placed at the CA1 pyramidal layer, from which the population EPSPs and population spikes were recorded. The stimulation current was set at a supramaximal level (usually 0.3 mA) to monitor the response when practically all inputs to the CA1 neurons were activated. This was done so to estimate the change in magnitude of the cells' total inputs. The same monitoring was repeated at more than three CA1 loci. The maximal response obtained was adopted as a representative value for that culture.

It is usual in LTP/LTD studies to monitor the slope of the population EPSP recorded at the dendritic layer of the CA1 area evoked in response to a half-maximal level stimulus delivered at the Schaffer collateral fibers. In cultured slices, however, the slope varied greatly among slices due presumably to the incoherence of the inputs (in slope measurements, the presynaptic action potential arriving to the recording site is presupposed to be coherent). Hence, for the purpose of comparison among many separate cultures like I did here, the aforementioned value has been a better index for synaptic strength (Muller et al., 1993). This strategy has been discussed previously (Kamikubo et al., 2006). In the case of monitoring the induction of LTD within a single slice culture, however, I delivered the half-maximal level stimuli following the convention.

Immunostaining

The cultured slice was fixed with 4% PFA (paraformaldehyde) at 4°C for 48 hours. After washing with PBS (phosphate buffered saline) 3 times and permeabilizing with Buffer H (PBS containing 1% Triton X-100, 5% horse serum and 0.05% NaN₃) for 1 hour, the culture was treated with anti-synaptophysin antibody (Progen; diluted 1:200 in Buffer H) or anti-drebrin antibody (MBL; diluted 1:100 in Buffer H) at 4°C for 48 hour. Then the culture was treated with a secondary antibody (Alexa 546-conjugated anti-rabbit IgG or Alexa 488-conjugated anti-mouse IgG [both from Molecular Probes; diluted 1:200 in Buffer H]) at 4°C for 48 hour, washed 3 times with PBS and mounted onto a glass slide. The proximal apical dendritic layer of the CA1 area of the stained culture was photographed *ad libitum* on an Olympus laser scanning confocal microscope (FV-3000).

Immunopositive punctate structures were counted for 3 separate fields each covering a ~20x20 μm area. The mean of these 3 values was adopted to be a representative value of that culture.

Lucifer Yellow injection

Intracellular injection of fluorescent dye was carried out according to Pace et al. (2002) with slight modifications. At first the culture was lightly fixed with 2% PFA in order to prevent possible injection-induced morphological changes and transferred to a chamber containing 2% PFA placed on the stage of a Nikon epifluorescence microscope. A glass micropipette filled with 1% aqueous solution of lucifer yellow (LY) potassium salt (Molecular Probes) was inserted into the CA1 region of the culture and was pushed in deeper slowly. The impalement of the micropipette in a cell was noticed by the emergence of cell-shaped fluorescence due to the dye leaking from the pipette tip. Then a negative current of 2-3 nA was applied for 5-10 min through a tungsten wire inserted into the micropipette and connected to an iontophoresis programmer (WPI model 701). After the injection was repeated into several cells for a single culture, the culture was fixed firmly with 4% PFA overnight.

Quantification of dendrite complexity and spine density

Neurons filled with LY were viewed with an Olympus laser scanning confocal microscope (FV-3000). To analyze dendritic complexity, each neuron was imaged using a 20x objective and z-sectioned at 2.0 μm steps. Images were then exported to ImageJ software (NIH) in order to reconstruct a

two-dimensional projection of the neuron's entire shape. The reconstructed image was then fed to a Scholl analysis software Neurolucida (MBF bioscience), which calculated the number of intersecting dendrites with the perimeters of a series of concentric circles drawn from the neuron's soma (Scholl, 1953). Analysis was performed in 10 μm steps of radius.

To estimate spine density, the proximal regions of the basal and apical dendrites (~50 μm from soma) of the LY-injected CA1 neuron were imaged using a 60x water immersion objective. Several dendritic segments (~5-30 μm in length) captured in single focal planes were subjected to spine number counting. The criterion for spines was that used by Grutzendler et al. (2002), which identified the dendritic protrusion equipped with a clearly discernible head as a hammerhead spine and that longer than one-third of the dendritic shaft diameter but shorter than 3 times the neck diameter as a stubby spine.

Western Blotting

At the time indicated, the culture was homogenized in cold lysis buffer (composed of [in mM]: 50 Tris-HCl, 150 NaCl, 1 EDTA, 10 Na pyrophosphate, 0.1 phenylarsine oxide, 1% Triton X-100, 1% protease inhibitor cocktail [Roche Diagnostics], 1% phosphatase inhibitor cocktail [Sigma]; pH 7.4). The lysate was centrifuged at 15,000 rpm for 10 minutes at 4 $^{\circ}\text{C}$ and supernatant was collected. Protein concentration of the sample was determined using a BCA protein assay kit (Pierce). The sample was then boiled at 100 $^{\circ}\text{C}$ for 5 minutes in a gel-loading buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% for TrkB and p75NTR and 15% for mBDNF and

proBDNF). 10µg of total protein was loaded in each lane. Proteins were transferred onto a PVDF membrane (Bio-Rad) and blocked with Block Ace (DS pharma biomedical) for 20 minutes at room temperature (RT) after a brief wash with TBS containing 0.05% Tween-20 (TBS-T). The membranes were then incubated for 1 hour at RT sequentially with following primary antibodies. The antibodies were rabbit polyclonal anti-BDNF (Santa Cruz N-20 [lot# G0308T], 1:200), mouse monoclonal anti-p75NTR (Chemicon MAB365, 1:1000) and mouse monoclonal anti-TUJ1 (1:2000), which were diluted in Block Ace, and mouse monoclonal anti-TrkB (BD transduction laboratories, 1:1000), which was diluted in Can Get Signal solution A (Toyobo). After washing with TBS-T 3 times, the membrane was incubated with HRP-conjugated secondary antibodies (GE Healthcare, 1:1000) for 1 hour at RT, followed by several washes with TBS-T for 1 hour, HRP reaction with an ECL-plus kit (GE Healthcare) or a Super Signal West Femto kit (Pierce) and finally exposed to CL-Xposure Film (Thermo Scientific). The image of the membrane was digitalized and the band densities were quantified using an ImageJ software (NIH). For statistical analysis, the band density for each antigen was normalized to that of TUJ1 and was then relativized to that of the control sample (3 × mock-stimulated specimen) prepared for each of sampling times.

Since an antibody specific for proBDNF with high titer is not available, we used the antibody to BDNF for detecting both proBDNF and BDNF. The signals for proBDNF and BDNF were assured so from their molecular weights and the absence in a brain extract sample prepared from BDNF-knockout mouse (kindly provided by Dr. Masami Kojima at National Institute of Advanced Industrial Science and Technology (AIST), Ikeda).

Electron microscopy

Observation with a conventional transmission electron microscope was carried out (Urakubo *et al.*, 2003). The cultured slice was fixed with 2% glutaraldehyde plus 2% paraformaldehyde, post-fixed with 2% osmium tetroxide, embedded in Quetol-812 resin (Nissin-EM, Tokyo, Japan), trimmed for the CA1 apical dendritic region, sectioned (~70-nm thickness) and stained with uranyl acetate/lead citrate (Sigma). The sections were examined on a transmission electron microscope (JEM-1200EX, JEOL, Hitachi, Japan; 5,000 \times at an acceleration voltage of 80 kV). The synapses were enumerated based on the criterion that a presynaptic structure containing ≥ 5 spherical synaptic vesicles was apposed to a distinct postsynaptic density (PSD). Even when the PSD was discontinuous (which would represent the so-called perforated synapse), we regarded that synapse as one, as long as the presynaptic structure was of a single entity. More than four sections were examined (two photographs were taken of each section) and enumerated for each cultured slice and the numbers were averaged over multiple slices and indicated on a per 100- μm^2 area basis. The apparent width of PSD was also measured as one of the indices of synaptic ultrastructure. Since ultrathin sections do not always cut through the center of synapses, the apparent width is not a true one. Nevertheless, this situation is common to all specimen groups, allowing the comparison by average.

Statistical analyses

Statistical comparisons between two independent groups of samples were performed using Welch's modification of the Student's t-test in the case of a normal

distribution or Mann-Whitney's U-test in the case of a non-normal distribution. For comparison among ≥ 3 groups of samples, analysis of variance (ANOVA) in the case of a normal distribution or Kruskal-Wallis' H-test in the case of non-normal distribution was applied and followed by *post-hoc* comparison. The details of the statistical methods of the *post-hoc* test are described in each figure legends. In all figures, means \pm standard errors of the means (SEMs) are indicated. The level of significance is indicated by the asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The number of samples constructing each plot is indicated within each bar (in bar charts) or beside each line (in line charts).

Results

Time window of protein synthesis required for LOSS

Firstly I examined the dependency of LOSS on protein synthesis using the translation inhibitor anisomycin (Fig. 5). This forms a basis for the present study, since LOSS could be a passive process as withering of neurons due to short supply of some factor(s) needed to maintain the cells, if it would not require protein synthesis. At the same time, the determination of time window of protein synthesis should set up a framework for subsequent pursuits of molecular mechanism.

There are reports that LTD induced by mGluR activation was blocked by anisomycin when the inhibitor was applied concomitantly with a LTD-inducing stimulus (Huber et al., 2000). To avoid any equivocation regarding anisomycin's effect on LOSS, we administered the drug after the induction of LTD. As shown in Fig. 5B, anisomycin application immediately after the DHPG application did not interfere with the induction of LTD. Nevertheless, the establishment of LOSS was abolished by the anisomycin treatment when assayed 3 weeks later (Fig. 5C). This result indicates that the establishment of LOSS requires the synthesis of protein(s) distinct from molecule(s) required for the induction of LTD itself. This blockade also occurred when the inhibitor was applied once after the first or third application of DHPG (supplementary Fig.1A), indicating that the protein synthesis is necessary for each LTD induction.

However, anisomycin applied 6 hours after LTD induction failed to block the establishment of LOSS when assayed 3 weeks later (Fig. 5C), indicating that the protein(s) required for the establishment of LOSS had been synthesized in sufficient amount(s) before this time. Anisomycin application that began 1.5 or 3

hours after LTD, however, blocked the development of LOSS (supplementary Fig.1B), indicating that 3 hours was still insufficient for the production of the required protein(s).

The numbers of pre- and postsynaptic structures are depicted in Fig. 6. The presynaptic structure (axon terminal) visualized immunochemically by an antibody to synaptophysin, a synaptic vesicle protein, occurred less frequently when DHPG was applied, whereas this decrease was abolished when anisomycin was applied immediately after DHPG application. The postsynaptic structure (dendritic spine) visualized by an antibody to drebrin, an F-actin-binding protein present in the spine, behaved similarly. These results are in parallel with the electrophysiological examinations shown above (Fig. 4D).

Taken together, LOSS should be an active process accompanied by recruitment of newly synthesized protein(s) that leads to elimination of synaptic structures.

Production of proBDNF after repeated DHPG exposure

What is then the protein(s) required for LOSS. Among many candidates I attended to proneurotrophins, especially to proBDNF and its potential receptor p75^{NTR} because recent findings revealed proBDNF-dependent synaptic elimination in the peripheral nervous system (Singh et al., 2008; F. Yang et al., 2009).

I monitored the cellular content of the molecules related to this pathway by an immunoblotting assay (Fig. 7, 8). Judging from the effective time window of a protein synthesis inhibitor (Fig. 5, 6 and supplementary Fig.1), I prepared the lysate of culture later than 6 hours after the third exposure to DHPG.

As shown in Fig. 7B (shown are the results statistically processed from several runs of immunoblotting, one of which is exemplified in Fig. 7A), the content of proBDNF in the culture was significantly elevated 6 hours after the third exposure to DHPG. This increase returned to the pre-stimulus level within 24 hours, due possibly to its release to the external milieu, its processing to mature BDNF, or proteolytic decomposition. The content of proBDNF remained at its pre-stimulus level 5 days after the third exposure, at which time the process of LOSS establishment should be ongoing (Fig. 5 of Kamikubo et al., 2006).

I also examined the cellular content of proBDNF 1 hour and 3 hours after single or repeated DHPG exposure (Supplementary Fig.2). Because marked increase was not observed either 1 hour or 3 hours after the third exposure, rise of the content of proBDNF is considered to reach a peak around 6 hours later.

The content of mature BDNF in the culture can increase when the production of proBDNF increases. In fact, there was a tendency of increase in the content of mature BDNF 6 hours after the third exposure to DHPG (Fig. 6C), although the increase remained insignificant.

The production of receptors for the proBDNF and mature BDNF was examined as well. The cellular content of p75^{NTR} protein in the culture tended to increase after three exposures to DHPG (Fig. 8B), although the increase did not reach the 95% significance level. The content of TrkB protein was uninfluenced by the exposures to DHPG, (Fig. 8C; shown here is the sum of full length and truncated forms of TrkB).

Although the release of proBDNF to the extracellular milieu has not been revealed, this is in line with the above-mentioned conjecture. Encouraged by this

fact, we treated cultured slices with anti-p75^{NTR} antibody to examine the involvement of p75^{NTR} signaling pathway in the establishment of LOSS.

Abolition of LOSS development by masking the proBDNF receptor p75^{NTR}

I attempted to mask p75^{NTR}, a supposed receptor of proBDNF, by applying anti-p75^{NTR} antibody (AB1554) which had been shown to bind to the extracellular domain of p75^{NTR} and suppress its function (Gehler et al., 2004; Pehar et al., 2006; Sotthibundhu et al., 2008; Koshimizu et al., 2009). As shown in Fig.9, when the antibody was applied for 4 days (at 1:500 dilution; this dose was chosen according to the previous reports; antibody application was begun immediately after the third DHPG exposure), the establishment of LOSS was significantly abolished when assayed 3 weeks later. The control group, to which an equivalent dose of rabbit IgG (1 μ g/mL; according to the manufacturer's protocol) was applied showed no effect. Unexpectedly, when exposure to the antibody was done during and after each of the 3 DHPG applications, an increase in synaptic strength occurred instead of the abolishment of synaptic weakening.

I also examined the effect of p75^{NTR} masking on the density of dendritic spine, a typical structure of excitatory postsynapse. Consistent with the morphological studies already shown in Fig.4 and 6, dendritic spines density was reduced both in apical and basal dendrites of CA1 pyramidal neurons after 3 times exposure to DHPG. (Fig.10) However, the anti-p75^{NTR} treatment prevented the reduction in spine density when applied after third DHPG exposure. The effects of anti-p75^{NTR} are quite similar to that observed in electrophysiological study (see

Fig.9). Judging from both physiological and morphological aspects, LOSS was abolished by masking p75^{NTR}.

Production of LOSS-like synaptic suppression by exogenously applied proBDNF

Next, I tried to determine whether or not proBDNF functions as a ligand for p75^{NTR} in LOSS. It is desired to scavenge the possibly secreted proBDNF by an antibody to it, if it is available. However, it is regrettably not. Therefore, I applied proBDNF exogenously instead to examine whether it could reproduce synaptic suppression similar to LOSS.

Cleavage of the proBDNF amino-terminus 128 polypeptide leads to the mature BDNF, a step known as proBDNF processing. When two arginine residues in the vicinity of the processing site are replaced with neutral residues, proBDNF remains unprocessed (manufacturer's data; cf.

http://www.alomone.com/p_postcards/database/495.htm). I refer to this mutated proBDNF as cleavage-resistant proBDNF (cr-proBDNF).

Thus I applied cr-proBDNF to the stable cultures of hippocampal slice at a dose of 10ng/ml or 100ng/ml (corresponding to 0.19nM and 1.9 nM of dimerized molecule, respectively) for 2 days. To the control group, I prepared two specimens. One is the cultures subjected to heat-denatured cr-proBDNF (boiled at 100 °C for 20 minutes) at a dose of 100 ng/ml and the other is the cultures which were not treated with any drug (i.e. intact cultures). Since I could not find any difference between those two specimens both in electrophysiological and morphological measurement, data from those two specimens were pooled and represented as control. As shown in Fig. 11A and B, the strength of the CA3-CA1 synapse was diminished by the

application of 100 ng/ml cr-proBDNF when assayed 3 weeks later. The application of 10 ng/ml cr-proBDNF was not enough to attenuate synaptic strength significantly.

In order to investigate whether this depressing effect of cr-proBDNF is mediated by p75^{NTTR}, I applied 100 ng/ml cr-proBDNF for 2 days in the presence of anti-p75^{NTTR} antibody. As shown Fig.11B, synaptic strength was significantly enhanced rather than recovered to the control level. This is possibly due to the activation of TrkB receptors because exogenously applied cr-proBDNF still has an ability to bind to TrkB receptor.

Upon 48 hours exposure to 100 ng/ml cr-proBDNF, the density of the spines of CA1 pyramidal neuron's apical dendrite decreased significantly, when examined 3 weeks later (Fig11C,D), whereas the decrease of those in basal dendrite remained insignificant (data not shown). When 100 ng/ml cr-proBDNF was applied concomitantly with anti-p75^{NTTR}, spine density in the apical dendrite increased compared to the control group (Fig.11D). These results indicate that transient exposure to proBDNF is capable of producing LOSS-like synaptic suppression coupled with synapse elimination via p75^{NTTR} signaling.

Independence to changes in neurons' dendritic arborization

It is reasonable to suspect that apoptotic neuronal death or neurons' gross morphological change might occur, if the proBDNF-p75^{NTTR} signaling pathway is activated after 3 inductions of LTD. However, the number of CA1 neurons was seen to remain unaltered (Fig. 8 of Kamikubo et al., 2006). To examine the possible influence to the cells' morphology, I performed Scholl analysis to evaluate

dendritic arborization. This analysis counts the number of neuron's dendrite intersecting with the perimeters of concentric circles drawn from the neuron's soma. A larger number of intersections indicates a higher complexity of dendritic arborization. Area surrounded by the intersection chart and the abscissa represents the total dendrite length.

As shown in Fig. 12, the intersection charts of the CA1 pyramidal cells were indistinguishable between the $3 \times$ mock stimulated (no LOSS) and $3 \times$ DHPG-exposed (LOSS) cultures, indicating that the dendritic arborization as well as dendritic length remained unaffected by repeated exposure to DHPG. Thus I concluded that the density of spines decreased selectively leaving the dendritic morphology intact under these LOSS-producing conditions.

Discussion

Time window for protein synthesis in LOSS

We reported previously that the repeated induction of LTP in stable culture of the hippocampus led to long-lasting enhancement of synaptic strength coupled with synapse formation (Tominaga-Yoshino et al., 2002, 2008), whereas the repeated induction of LTD resulted in long-lasting suppression of synaptic strength coupled with synapse elimination (Shinoda et al., 2005, Kamikubo et al., 2006). We call these novel forms of synaptic plasticity accompanied by structural changes RISE and LOSS, respectively. These apparently symmetric phenomena are not a simple extension of lifetimes of conventional single LTP and LTD (we provide here additional lines of evidence for the distinction of LTD and LOSS; anisomycin susceptibility [Fig. 5, 6] and absence of occlusion [supplementary Fig. 3]). We propose RISE and LOSS as model phenomena to analyze structural plasticity, which is considered to be the cellular basis for memory consolidation.

In this study, I first examined LOSS dependency on protein synthesis, since it is known that memory consolidation process requires protein synthesis (Gold, 2008). When anisomycin, a translation inhibitor, was administered immediately after the application of DHPG, the induction of LTD was uninfluenced while the establishment of LOSS was abolished. However, anisomycin treatment begun 6 hours after DHPG was ineffective, indicating that protein synthesis necessary for the development of LOSS had completed by this time. The presence of a similar time window for protein synthesis was reported in an in vivo study, where protein synthesis in the hippocampus within 3-6 hours after training was necessary for learning acquisition (Igaz et al., 2002).

ProBDNF and p75^{NTR} up-regulation by repeated mGluR activation

What indispensable protein is synthesized in this time window? We attended to the 'yin-yang hypothesis' for neurotrophins and proneurotrophins (Lu et al., 2005). This hypothesis assumes proneurotrophins, previously regarded as mere intermediate products in the synthesis of neurotrophins, have biological activities of their own, especially activities that counteract their mature counterparts.

It is known that BDNF, a species of neurotrophin enriched in the hippocampus, binds to TrkB receptor and plays an important role in the maintenance and differentiation of neurons, the development of the neural network, and induction of LTP (McAllister et al., 1999; Huang and Reichart, 2001; Lu and Figurov, 1997). The involvement of BDNF in RISE has also been suggested (Taniguchi et al., 2005). In contrast, proBDNF is known to be involved in neuronal death, neuromuscular synapse elimination and enhancement of LFS-induced hippocampal LTD through binding to p75^{NTR} (Pang et al., 2004; Teng et al., 2005; Woo et al., 2005; F. Yang et al, 2009). Thus it is reasonable to assume the involvement of proBDNF-p75^{NTR} signaling in the establishment of LOSS, which can be described as a 'yin-yang' relationship with RISE.

Firstly I monitored by immunoblotting the cellular contents of proBDNF, mature BDNF, TrkB and p75^{NTR} 6 hours, 24 hours, 5 days after the first and third exposure to DHPG (Fig. 4). It would appear that 6 hours is the time necessary to synthesize the sufficient amounts of required proteins, 24 hours is the time that the protein synthesis is over, and 5 days is the time when LOSS is developing as revealed in Fig. 3. The content of proBDNF was significantly increased 6 hours after the third

exposure to DHPG. The content of mature BDNF tended to increase as expected, since its precursor form is increased. But this increase remained statistically insignificant (at the significance level of 95%). I examined proBDNF up-regulation in a separate experiment using an enzyme-linked immunosorbent assay (ELISA) with an antibody against the prodomain of proBDNF, which was developed on a trial basis by Dr. Masami Kojima at National Institute of Advanced Industrial Science and Technology (Supplementary Fig. 4). The proBDNF concentration was apparently overestimated in this ELISA system probably because of insufficiency of the specificity of the antibody. Nonetheless, this result is in line with the aforementioned result that proBDNF was up-regulated 6 hours after repeated mGluR activation.

In the receptor side, the expression of TrkB was kept uninfluenced at any time-points after the DHPG exposure (either the first or the third exposure), whereas the expression of p75^{NTR} tended to increase after the exposure to BDNF (either the first or the third exposure). It is well known that proneurotrophin or p75^{NTR} is up-regulated in pathological conditions such as Alzheimer's disease (Pedraza et al., 2005), brain injury (Beattie et al., 2002) and retinal dystrophy (Srinivasan et al., 2004). In contrast, the proBDNF up-regulation elicited by repeated induction of mGluR-dependent LTD which is shown here can be regarded as physiological stimulation. Taking the expression levels of those molecules in total, the assumption that proBDNF-p75^{NTR} intercellular pathway is involved in the establishment of LOSS is supported.

Group I mGluR-mediated signaling and proBDNF/BDNF expression

Group I mGluRs (mGluR1/5) are G-protein-coupled receptors. Through G α q, they activate phospholipase C β (PLC β) and are positively coupled to PKC activation and intracellular Ca $^{2+}$ release triggered by inositol-1,4,5-triphosphate (IP₃) (Nakanishi et al., 1998). Indeed, potent PKC inhibitor Go-6983 (1 μ M) applied concomitantly with DHPG prevented the establishment of LOSS (Supplementary Fig. 5). Although repeated PKC inhibition seemed to affect cell viability as seen in the reduction in the absolute size of maximal field EPSP amplitude, this result suggests that LOSS is mediated by PKC activation. In contrast, it should be noted that DHPG-induced LTD in the CA1 region does not require PKC activation (Schnabel et al., 1999, 2001)

In addition to PLC β cascade, group I mGluRs are assembled with other synaptic proteins by their adaptor protein Homer to form submembranous signaling complexes (Fagni et al., 2000). Upon activation of mGluR1/5, these signaling molecules lead to the phosphorylation of transcription factors (CREB and Erk-1) and thereby facilitate the expression of immediate early genes such as c-Fos (Yang et al., 2004). Immediate early gene products serve as third messengers in a stimulus-transcription cascade to initiate the expression of the late response genes including neurotrophins (Mao et al., 2008). Recently, Viwatpinyo and Chongthammakun (2009) reported that the activation of mGluR1/5 by DHPG increased BDNF mRNA in rat glioma cells and that this up-regulation began 4 hours after DHPG exposure and obtained its peak level at 12 hours. Although my findings showed that cellular content of proBDNF protein was enhanced by

repeated DHPG exposure, not by a single DHPG exposure, both reports reveal the linkage between mGluR activation and proBDNF/BDNF expression.

Possible involvement of p75^{NTR} signaling in establishment of LOSS

I administered antibody that binds to the extracellular domain of p75^{NTR} in order to inhibit its function (Pehar et al., 2006). This should interfere with proBDNF at the receptor level, if proBDNF acts as a ligand for p75^{NTR}. I initially applied the antibody to the cultured slice for 6 days from the first DHPG exposure to prevent entire p75^{NTR} signaling which is activated by repeated DHPG exposure. Contrary to my expectation, not inhibition of synaptic weakening but rather synaptic strengthening was occurred (Fig. 9). This synaptic strengthening seems to be a RISE-like phenomenon because the density of dendritic spine increased simultaneously (Fig. 10). I next applied the anti-p75^{NTR} for 4 days from the end of the repeated DHPG exposure and found that LOSS was abolished in this experimental schedule.

One can expect that anti-p75^{NTR} may increase the synaptic density in proportion to the length of its treatment regardless of repeated mGluR activation. However, this possibility should be ruled out, because in the control group (3×mock group), anti-p75^{NTR} treatment did not lead to increases in synaptic strength and spine density. The reason why the inhibition of p75^{NTR} for a longer period led to synaptic enhancement can be explained as follows. There is evidence that proBDNF binds to TrkB and drives trophic signals in cells that did not express p75^{NTR} (Fayard et al., 2005). In addition, as shown in Fig. 7, mature BDNF tended to be up-regulated by repeated mGluR activation. Therefore, it is plausible that

both proBDNF and mature BDNF produced and released during repeated DHPG exposures activated TrkB coincidentally and shifted the balance from synapse elimination to synapse formation. In any case, these results indicated the involvement of p75^{NTR} in the establishment of LOSS.

Zagrebelsky et al. (2005) reported that a transgenic mouse line expressing no p75^{NTR} had hippocampal neurons equipped with higher density of dendritic spines than wild type mouse, whereas another line expressing excess amount of p75^{NTR} had hippocampal neurons with lower dendritic spine density. These results, indicating the p75^{NTR} pathway as a negative regulator of spinogenesis, are consistent with the present results.

Possible involvement of proBDNF in establishment of LOSS

In order to examined whether proBDNF actually lead to synapse elimination via p75^{NTR}, I administered cr-proBDNF to the hippocampal slice cultures for 2 days. 10 ng/ml cr-proBDNF was insufficient to suppress synaptic strength, whereas 100 ng/ml (1.9 nM) cr-proBDNF led to a reduction both in synaptic strength and spine density when assayed 3 weeks later (Fig. 11). Although this dose was sufficient to cause apoptosis in a PC12 cell line excessively expressing p75^{NTR} (Fan et al., 2008), no decrease in CA1 neuron density was seen in this study (Supplementary Fig. 6). In dissociated hippocampal cell cultures as well, application of a different species of cleavage-resistant proBDNF at a similar dosage caused a decrease in spine density but no cell death (Koshimizu et al., 2009). It appears that CNS neurons are less apoptosis-susceptible to proBDNF than PNS cells. Next, I applied 100 ng/ml cr-proBDNF concomitantly with anti-p75^{NTR} and

found that synaptic strength and spine density was increased (Fig. 11). This result is plausible, because cr-proBDNF used in this study is also shown to bind to and activate TrkB in PC12 cells expressing TrkB (manufacturer's data; cf. http://www.alomone.com/p_postcards/database/541.htm), though the affinity for it is thought to be much lower than that for p75^{NTR}. It is therefore apparent that proBDNF regulates synaptic efficacy via p75^{NTR}.

However, the LOSS mimicry of exogenously applied proBDNF and the abolition of LOSS by p75^{NTR} masking do not necessarily mean the involvement of endogenous proBDNF in the establishment of LOSS, since some unspecified endogenous substance may have activated p75^{NTR} and exogenously applied proBDNF may have mimicked the substance's effect. Although the quantification of the cellular contents of proBDNF supports our hypothesis (Fig. 7, 8), there is no proof for its release.

Matsumoto et al. (2008) claimed that all proBDNF molecules are processed within a cell and not released to the extracellular milieu. In rebuttal, J. Yang et al. (2009) argued that at least some proBDNF is released from the cell and is cleaved by extracellular proteases including plasmin, meaning that proBDNF can work by its own. In this context the fact that mRNA of BDNF is transported to dendrite to be translated locally upon synaptic activity (Tongiorgi et al., 1997) is important. Since dendrite is devoid of Golgi complex, BDNF synthesized in dendrite has to be released in the form of proBDNF and processed extracellularly. Although the absolute amount of proBDNF produced locally in dendrite would be small in comparison to that in cell soma, the effect at synaptic sites should be large. A transgenic mouse line lacking dendritically transported mRNA of BDNF showed

higher density of dendritic spines than wild-type mouse, suggesting a crucial role of proBDNF release in spine density regulation (An et al., 2008).

Nevertheless, the argument will not be settled until a method for chemically quantifying extracellularly present proBDNF is achieved. This requires overcoming its high absorption to the extracellular matrix, rapid processing and rapid non-specific proteolysis. (In systems with low absorption and breakdown, such as dissociated cell cultures, direct quantification of released proBDNF has been done in success [Nagappan et al., 2009].)

To argue strictly, even if the production and release of proBDNF after the third DHPG exposure would have been shown, the involvement of proBDNF in the LOSS establishment should remain inconclusive, since proneurotrophins other than proBDNF or mature neurotrophins (which bind to p75^{NTR}, though with low affinities) or some yet unidentified molecules can also be candidate ligands. Ideally, one would specifically scavenge extracellular proBDNF, if any, using an antibody or a receptor-body, but no such scavenging substance is currently available.

Zagrebelski et al. (2005) showed that transgenic mice expressing excess p75^{NTR} had hippocampal neurons of decreased dendritic arborization. This observation appears contradictory to our proBDNF-p75^{NTR} hypothesis for LOSS, since LOSS was not accompanied by the change in dendritic arborization (Fig. 12). However, the difference might have resulted from a constitutive up-regulation of p75^{NTR} in the transgenic mouse in contrast to its transient activation in LOSS.

Considering all the data in this study, I created a model of cellular mechanisms underlying DHPG-LTD and DHPG-LOSS (Fig.13).

In vitro model system for structural plasticity

Morphological changes accompanied by LTP and LTD are often regarded as models for structural plasticity. However, LTP and LTD evoked in acutely prepared hippocampal slices, first established by Schwartzkroin and Wester (1975) and used widely thereafter, have serious limitations regarding lifetime (at maximum several hours). So it remains to be examined whether LTP and LTD as well as the morphological changes related to LTP and LTD can last longer than several hours. It has been observed that LTP evoked in the brain *in vivo* lasted as long as 2 weeks, when monitored through an electrode implanted in the unanesthetized animal (Bliss and Gardner-Medwin, 1973). However, in the brain *in vivo*, the activation of neural circuit is at the animal's disposal so that the experimenter cannot definitely claim whether the single LTP evoked by the experimenter lasted this long by itself. Nevertheless, since behavioral learning requires the repetition of training the morphological changes coupled with RISE and LOSS (synapse formation and elimination) produced after the repeated induction of LTP and LTD, respectively, should be hopeful targets for cell biological analysis of memory consolidation.

Figures and Legends

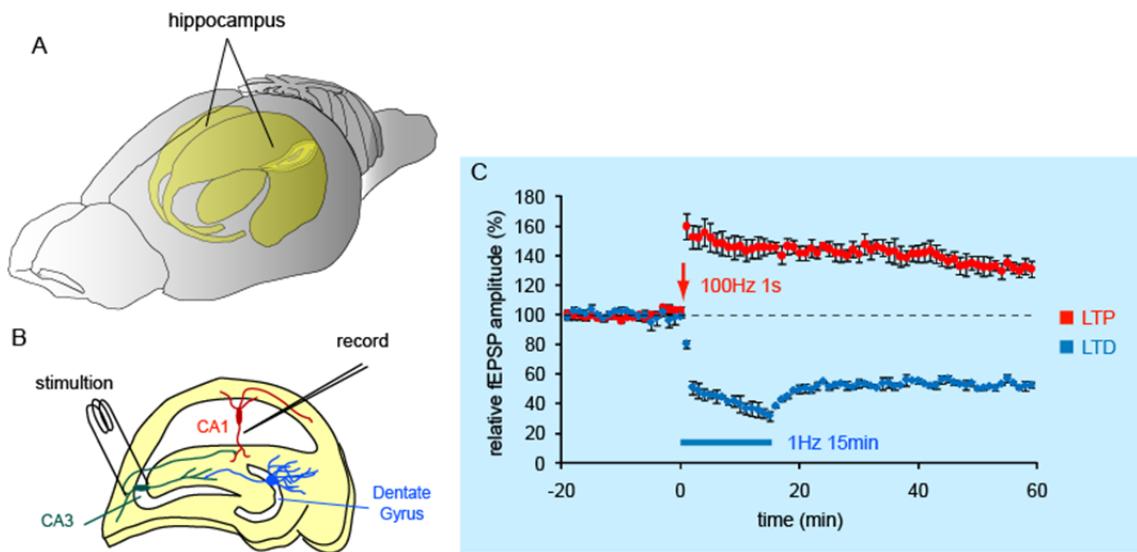


Fig.1

Electrical stimulation induced-long term potentiation (LTP) and long-term depression (LTD) in the hippocampus. (A) The three-dimensional organization of the hippocampal system in the rat brain. The hippocampus is C-shaped structure located in the inner part of the telencephalon. (B) Experimental configuration of the extracellular recording in the organotypic slice culture of rat hippocampus. CA3 pyramidal neurons were stimulated by bipolar tungsten electrode placed in the somatic layer of the CA3 region and the field excitatory postsynaptic potential (field EPSP) was recorded in somatic layer of the CA1 region. (C) LTP was induced by high frequency stimulation (100Hz 100pulses, red arrow) and LTD was induced by low frequency stimulation (1Hz 900pulses, blue bar).

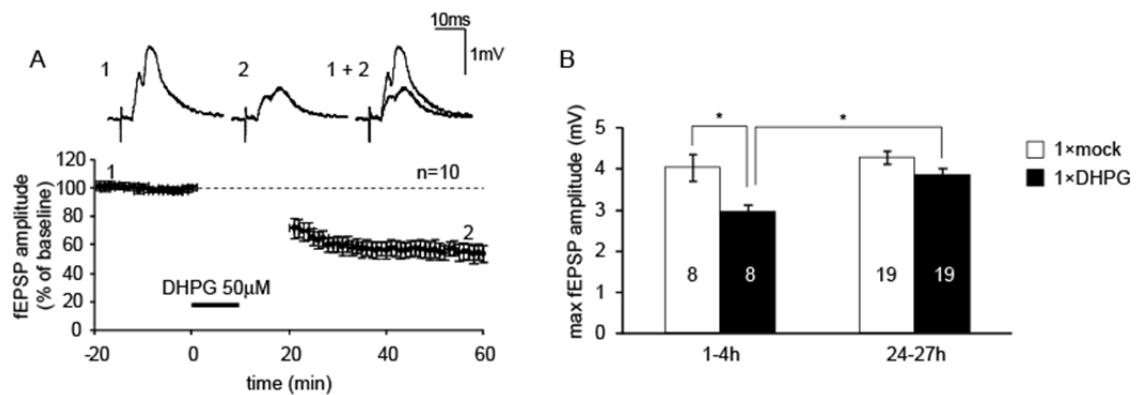


Fig. 2

Group I mGluR-dependent LTD did not last longer than 24h. (A) Application of 50 μM DHPG, a group I mGluR agonist induced LTD in cultured rat hippocampal slice (application period is indicated by a bar). Test electrical stimulus was applied at 0.05 Hz (once in 20s) and mean field EPSP amplitude binned for each minute was plotted. Data were normalized against the average of the 20 baseline traces. The representative traces of field EPSP shown atop are those recorded at time-point 1 and time-point 2. The amplitude of field EPSP at 60min was $53.7 \pm 5.9\%$ ($n=10$) of the pre-exposure baseline. (C) Maximal field EPSP were recorded immediately (1-4h) and 1 day (24-27h) after exposure to 50mM DHPG for 10min (open column) or mock stimulation (filled column). LTD was recovered to the control level within 1 day after DHPG exposure. Asterisks indicate statistical difference between bracketed pairs. * $P < 0.05$ (Two-way ANOVA followed by Scheffe's post hoc test).

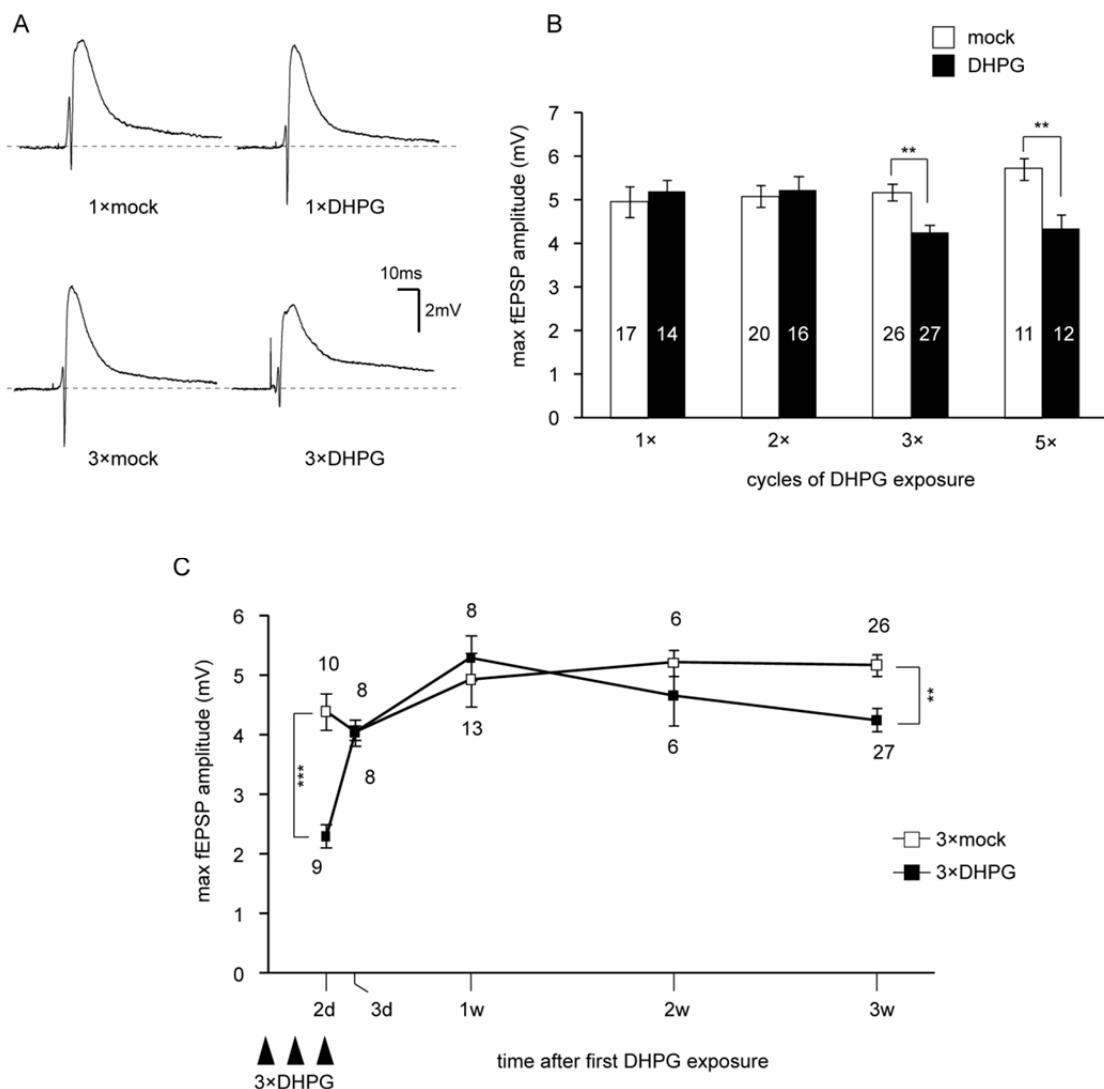


Fig. 3

Repeated exposure (≥ 3) of cultured slices to DHPG led to a slowly developing synaptic suppression which lasted for at least 3 weeks. (A) Representative maximal field EPSP traces recorded 3 weeks after single or 3 times DHPG exposure. (B) Maximal field EPSP amplitude was evaluated 3 weeks after repeated DHPG exposure at a 1-day interval. More than 3 times LTD-inducing stimuli were required for the synaptic suppression. ** $P < 0.01$ (two-tailed Welch's t test). (C) Time course of the synaptic suppression induced by 3 times exposure to DHPG. Maximal field EPSP amplitudes were recorded 2 days, 3 days, 1 week, 2 weeks and 3 weeks after first DHPG exposure. It should be noted that the synaptic suppression induced by third DHPG exposure observed at 2d was return to the same level as control and thereafter synaptic strength began to decline. ** $P < 0.01$, *** $P < 0.001$ (two-tailed Welch's t test).

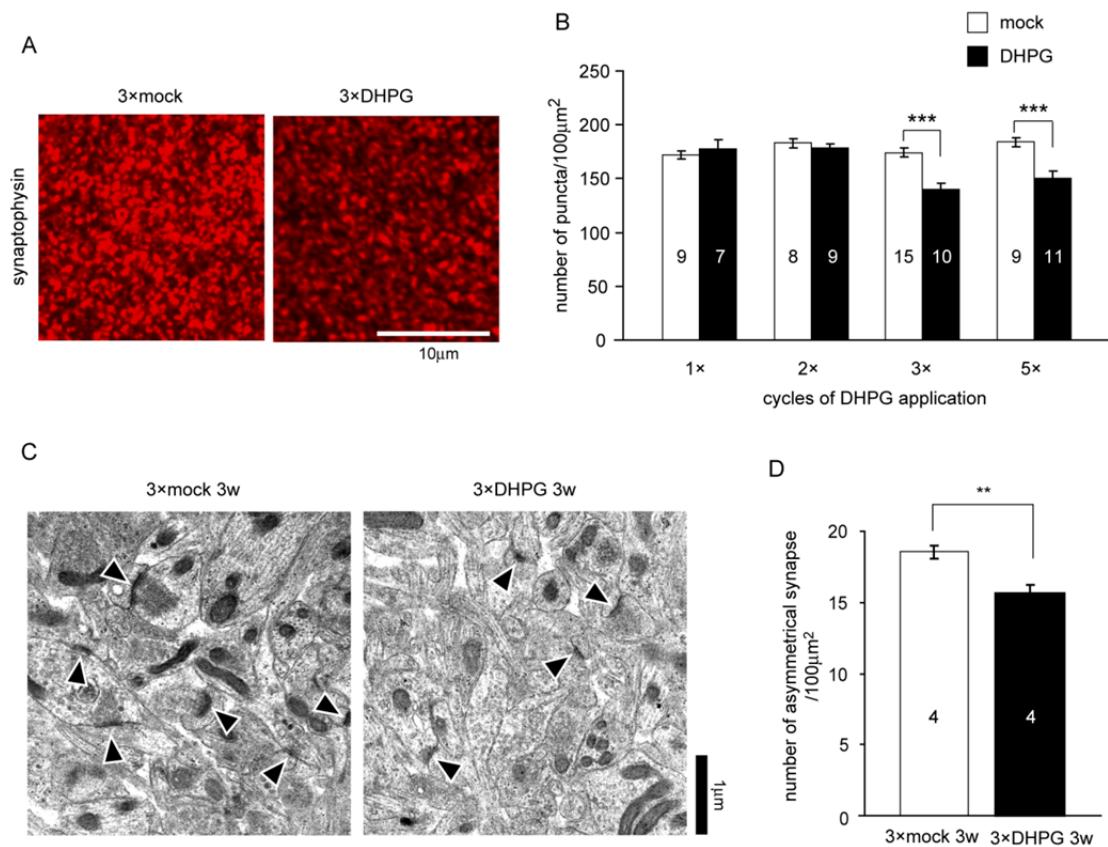


Fig. 4

Synaptic density also reduced 3 weeks after repeated (≥ 3) DHPG exposure. (A) Representative micrographs of synaptophysin-immunoreactive puncta, a morphological index of presynaptic structure, taken in the CA1 apical proximal dendritic layer. (B) Density of synaptophysin- immunoreactive puncta was evaluated 3 weeks after repeated DHPG exposure at a 1-day interval. These results are completely consistent with those of electrophysiological recording shown in Fig.3 B. *** $P < 0.001$ (two-tailed Welch's t -test). (C) Representative electron micrographs taken in the CA1 region of the cultured slice which were exposed to 3 times DHPG or mock stimulation. Arrowheads indicate asymmetrical synapses. (D) Mean density of asymmetrical synapse was quantified. The number within each column indicates the number of cultures examined (not the number of images for enumeration of synapses). The values do match those in B. ** $P < 0.01$ (two-tailed Welch's t -test).

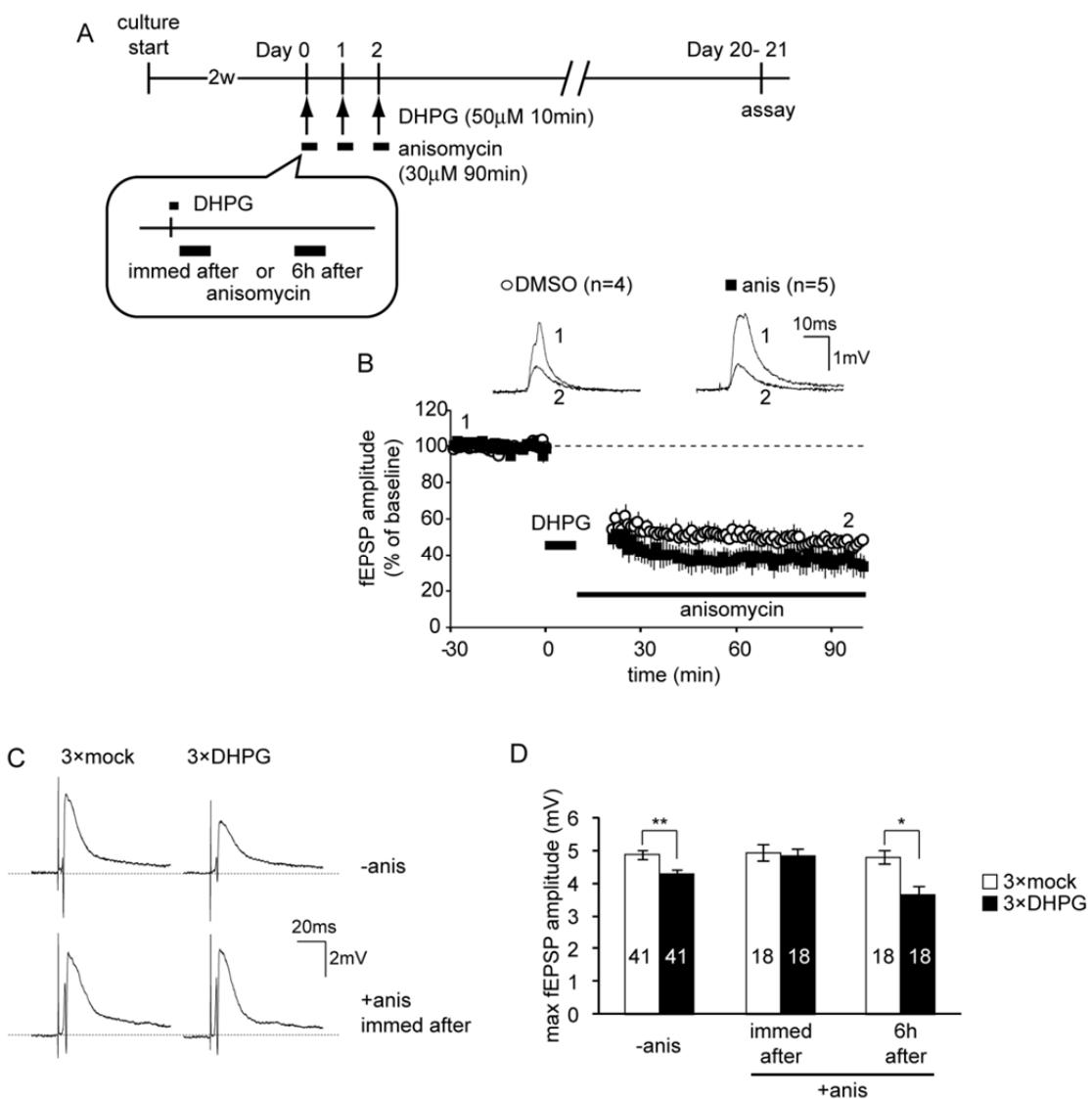


Fig. 5

LTD-repetition-operated synaptic suppression, or LOSS, dependency on protein synthesis in hippocampal slice cultures. (A) Timeline of the examination. Anisomycin (30 μ M, 90 min) was applied immediately after or 6 hours after exposure to DHPG (50 μ M, 10 min). (B) Independence on protein synthesis of LTD induced by exposure to DHPG. Anisomycin application (30 μ M) beginning immediately after exposure to DHPG (50 μ M) did not influence the induction and maintenance of LTD. At 90 min after anisomycin application (indicated by digit 2), the amplitude of field EPSP was $36.9 \pm 7.53\%$ ($n=5$) of the pre-exposure baseline (recorded at the time-point indicated by digit 1), whereas that of the anisomycin's vehicle control specimen (dimethylsulfoxide of the equivalent dose was applied) was $46.6 \pm 2.87\%$ ($n=4$). The sample traces of field EPSP shown atop are those recorded

at time-point 1 and time-point 2. (C) Representative traces of maximal field EPSP recorded 3 weeks after DHPG exposures. (D) Statistical comparison of maximal field EPSP amplitude. LOSS was abolished by anisomycin application immediately after each DHPG exposure, whereas anisomycin application beginning 6 hours after each DHPG exposure had no effect on the establishment of LOSS. *P < 0.05, **P < 0.01 (two-tailed Welch's t-test).

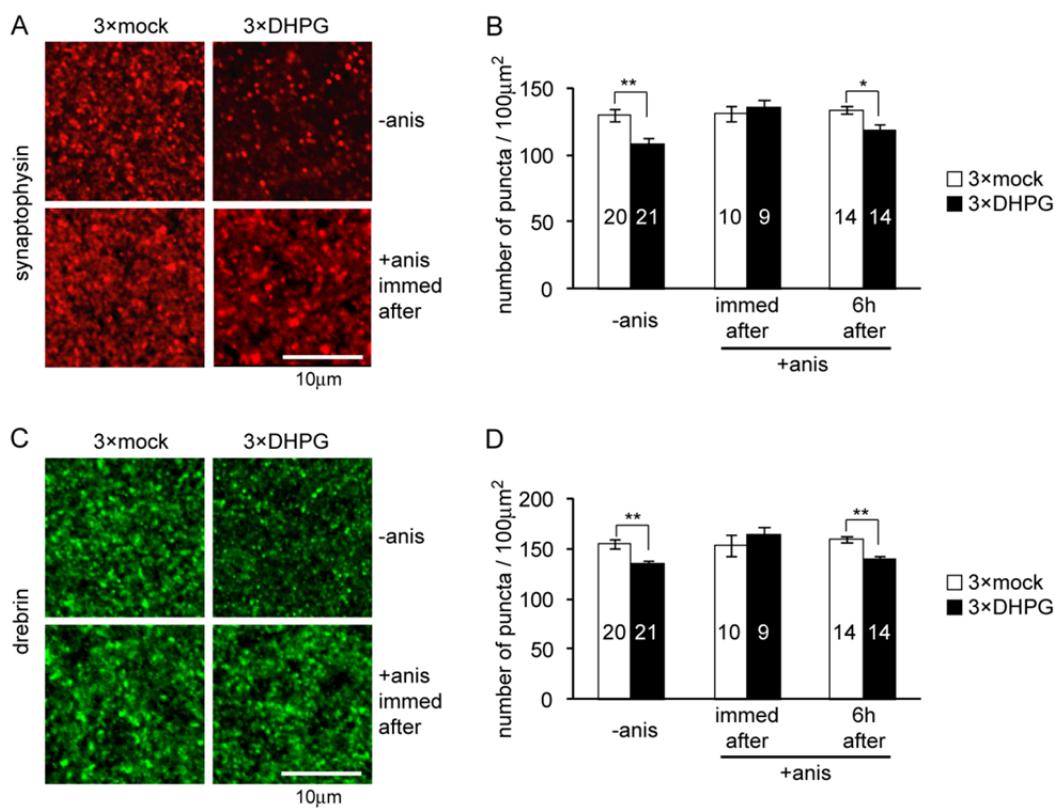


Fig. 6

LTD-repetition-operated synapse elimination, dependency on protein synthesis in hippocampal slice cultures. The experimental protocol is the same as that in Fig. 4. (A) Representative photographs of punctate structures immunopositive to synaptophysin, a presynaptic marker protein. (B) The reduction in the density of the presynaptic puncta was abolished by anisomycin treatment within 6 hours after each DHPG exposure. (C) Representative photographs of punctate structures immunopositive to drebrin, a postsynaptic marker protein. (D) The reduction in the density of the postsynaptic puncta was also abolished by anisomycin treatment within 6 hours after each DHPG exposure. *P < 0.05, **P < 0.01 (two-tailed Welch's t-test).

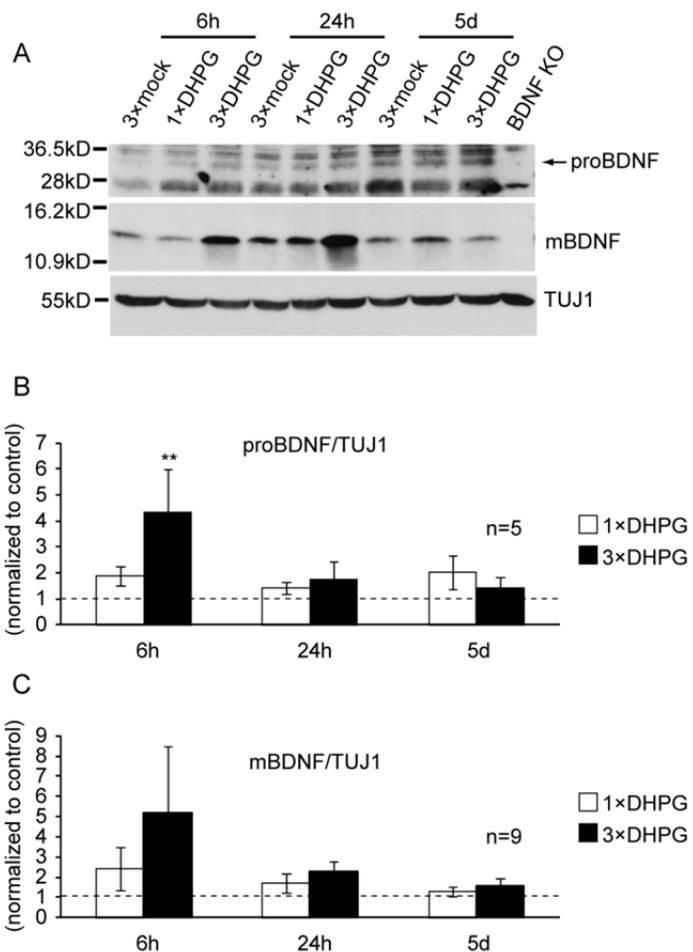


Fig. 7

The tissue contents of proBDNF and mature BDNF in hippocampal slice cultures sampled 6 hours, 24 hours and 5 days after single or repeated exposure to DHPG.

(A) Representative immunoblotted membranes. Note that bands of proBDNF (32 kDa) and mature BDNF (14.2 kDa) are absent in BDNF knock-out mice lysate. (B) Statistical comparisons of densitometried bands of proBDNF. As explained in the text, the band density corresponding to proBDNF was normalized to that of TUJ1 for each lane and then relativised to the normalized proBDNF band density of the control sample (i.e. 3 × mock-stimulated culture). The number of independent examinations is indicated to the right. **P < 0.01 (Kruskal-Wallis test followed by Scheffe's post hoc test). (C) Statistical comparisons of densitometried bands of mature BDNF. The data was treated the same as in B. Although mature BDNF tended to increase 6 hours after 3 × DHPG, there is no statistical significance. P=0.196 (Kruskal-Wallis test).

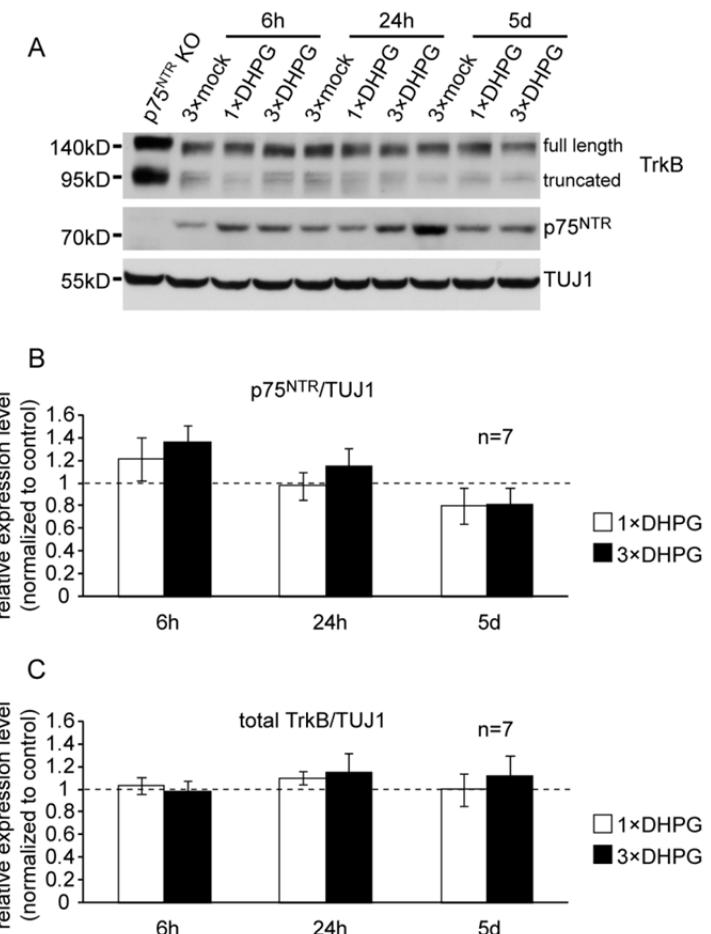


Fig. 8

The tissue contents of p75^{NTR} and TrkB in hippocampal slice cultures sampled 6 hours, 24 hours and 5 days after single or repeated exposure to DHPG. (A) Representative immunoblotted membranes. TrkB has two forms, full length (140 kDa) and truncated form (95 kDa). Band of p75^{NTR} (75 kDa) is absent in p75^{NTR} knock-out mice lysate. (B, C) Statistical comparisons of densitometried bands of p75^{NTR} and TrkB (sum of full length and truncated forms). The data was treated the same as in Fig.7.

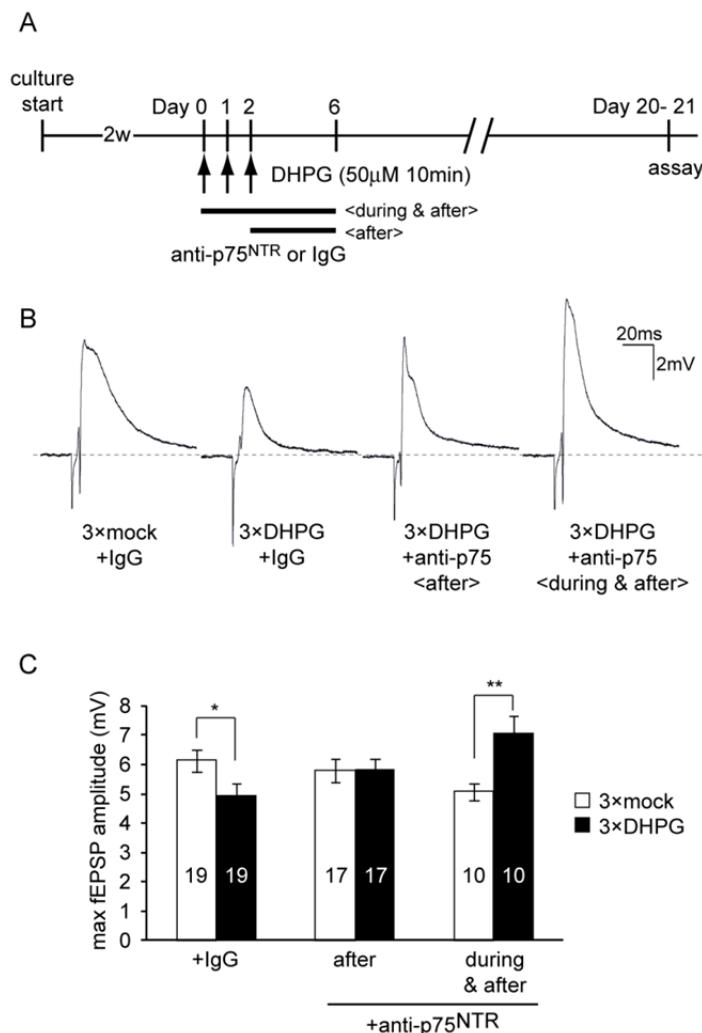


Fig. 9

Abolishment of LOSS by p75^{NTR} antibody applied immediately after the third exposure to DHPG. (A) Timeline of the examination. Cultured slices were treated with neutralizing antibody against p75^{NTR} during and after 3 DHPG exposures or only after the third DHPG exposure. (B) Representative field EPSP traces recorded 3 weeks after repeated exposure to DHPG in the presence of anti-p75^{NTR} antibody (experimental) or rabbit IgG (control). (C) Synaptic strength as assayed 3 weeks after DHPG exposure. Note that the synaptic suppression was abolished when antibody was applied after third DHPG for 4 days, whereas the antibody treatment during and after repeated stimulation caused rather a synaptic enhancement. *P < 0.05, **P < 0.01 (two-tailed Welch's t-test).

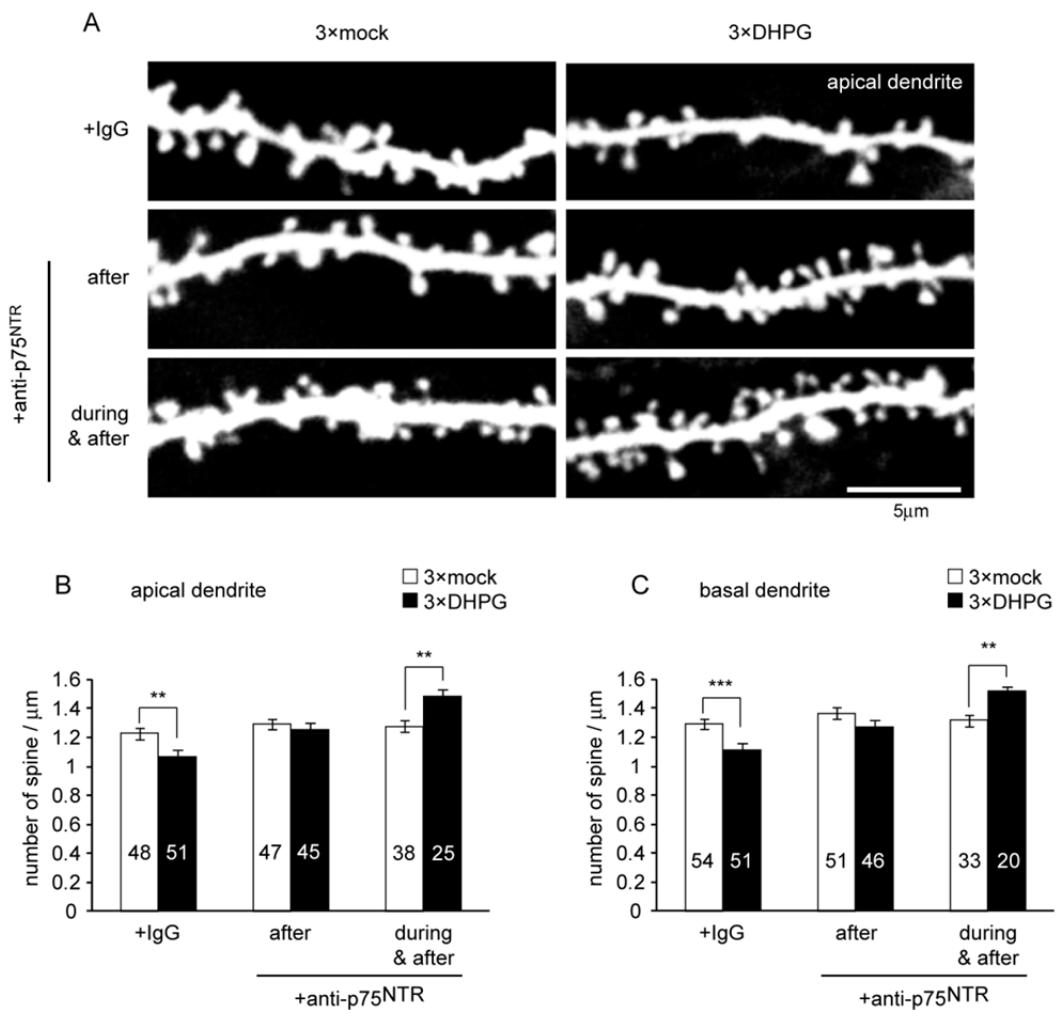


Fig. 10

Abolishment of spine elimination by p75^{NTR} antibody applied immediately after the third exposure to DHPG. (A) Representative micrographs of CA1 pyramidal neuron's apical dendrite segments. (B, C) Spine densities in apical and basal dendrites. Dendritic spines were eliminated by repeated DHPG exposure. Note that the anti-p75^{NTR} treatment abolished the spine elimination or even converted it to enhancement similarly to the result of electrophysiological measurement in Fig. 9. Numbers indicated within the columns are those of examined dendritic segments. The numbers of examined neurons are 13 (3 × mock + IgG), 13 (3 × DHPG + IgG), 11 (3 × mock + anti-p75 <after>), 12 (3 × DHPG + anti-p75 <after>), 9 (3 × mock + anti-p75 <during & after>) and 8 (3 × DHPG + anti-p75 <during & after>). *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Welch's t-test).

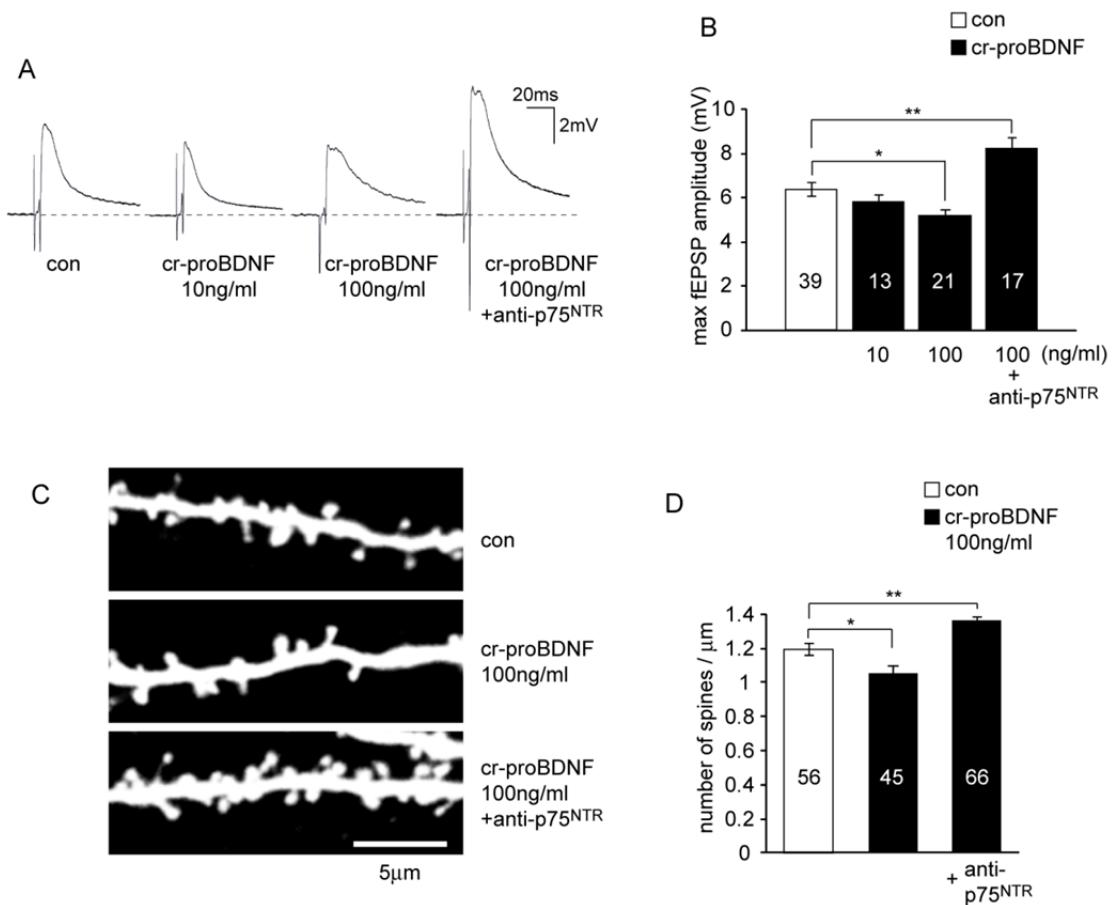


Fig. 11

Weakening of synaptic strength and elimination of dendritic spines of the CA1 pyramidal neuron 3 weeks after 2 days exposure to cleavage-resistant proBDNF at a dose of 100 ng/ml. (A) Representative traces of maximal field EPSP recorded 3 weeks after cr-proBDNF treatment. (B) Application of 100 ng/ml cr-proBDNF reduced maximal field EPSP amplitude, whereas when given concomitantly with anti-p75^{NTR} exerted an opposing effect. *P < 0.05, **P < 0.01 (one-way ANOVA followed by Dunnett's post hoc test). (C) Representative micrographs of CA1 pyramidal neuron's apical dendrite segments in cr-proBDNF-treated slice. (D) The effects of cr-proBDNF (100ng/ml) on spine density in the apical dendrite highly matched to that observed in synaptic strength. Numbers indicated within the columns are those of examined dendritic segments. The numbers of examined neurons are 16 (control), 12 (cr-proBDNF 100 ng/ml) and 23 (cr-proBDNF 100 ng/ml + anti-p75^{NTR}). *P < 0.05, **P < 0.01 (one-way ANOVA followed by Dunnett's post hoc test).

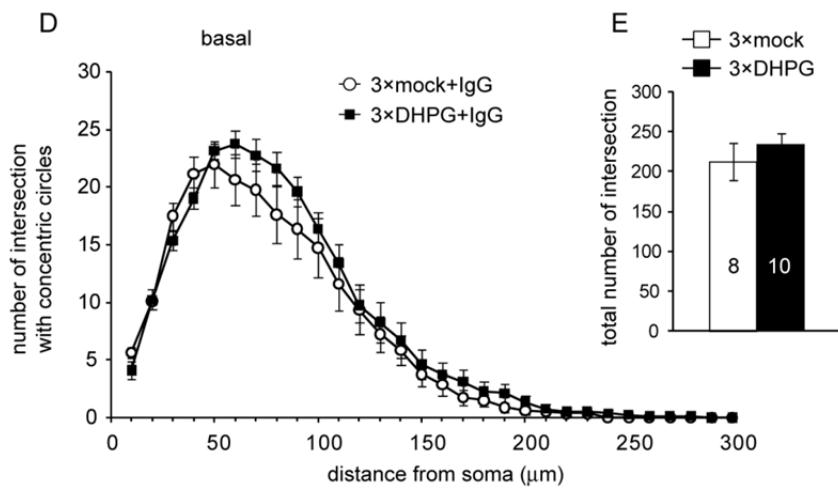
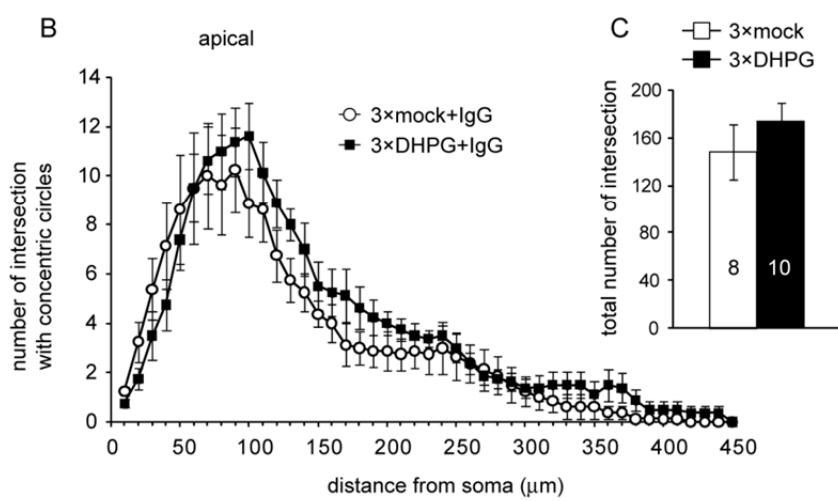
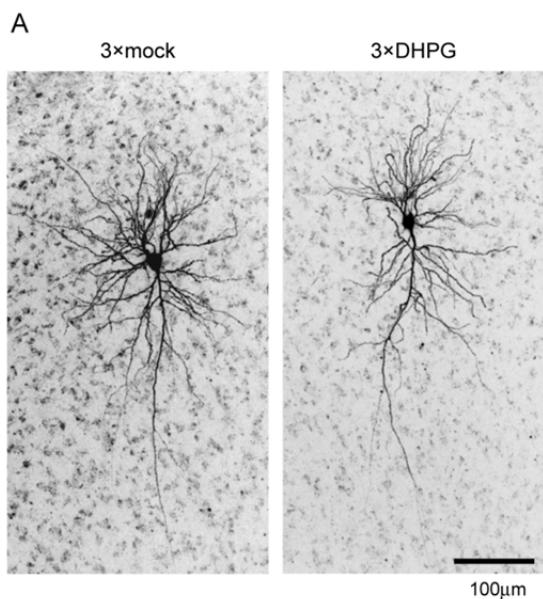


Fig. 12

Dendritic complexity of CA1 pyramidal cells unaffected by repeated exposure to DHPG. (A) Gross cell morphology. Serial images of Lucifer Yellow-injected cells were obtained by confocal microscopy and stacked to form a two-dimensionally projected image. (B, D) The number of intersections of apical (B) and basal (D) dendrites with concentric circles drawn from cell's soma at 10 μm steps of radius. The area surrounded by the curve and the abscissa serves as an index for total dendritic length. (C, E) The total number of intersections, which serves as an index for dendritic branching.

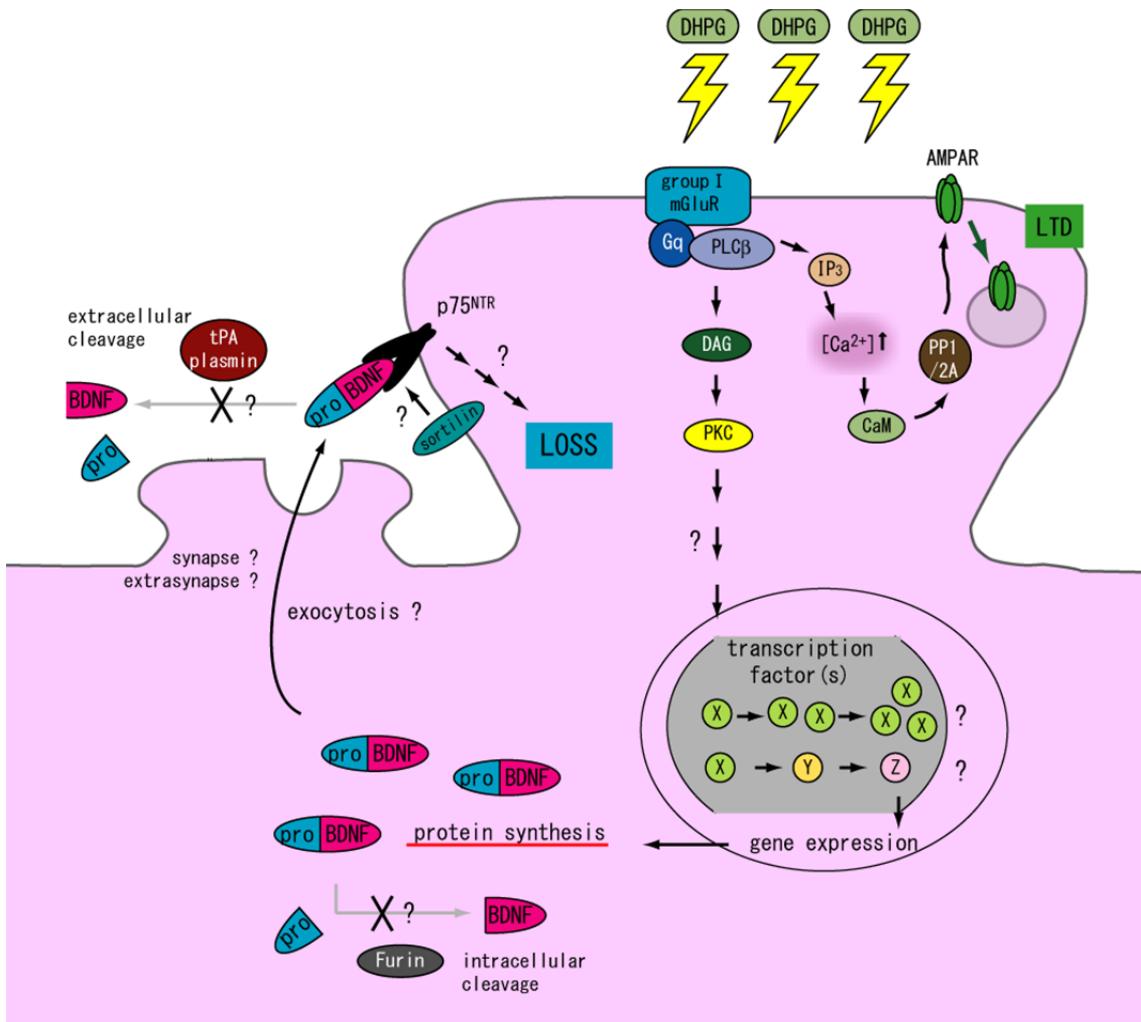
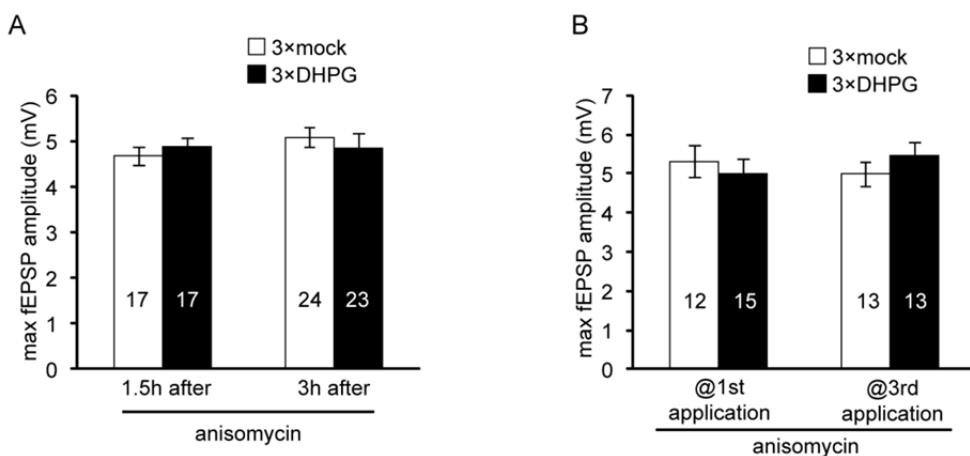


Fig.13

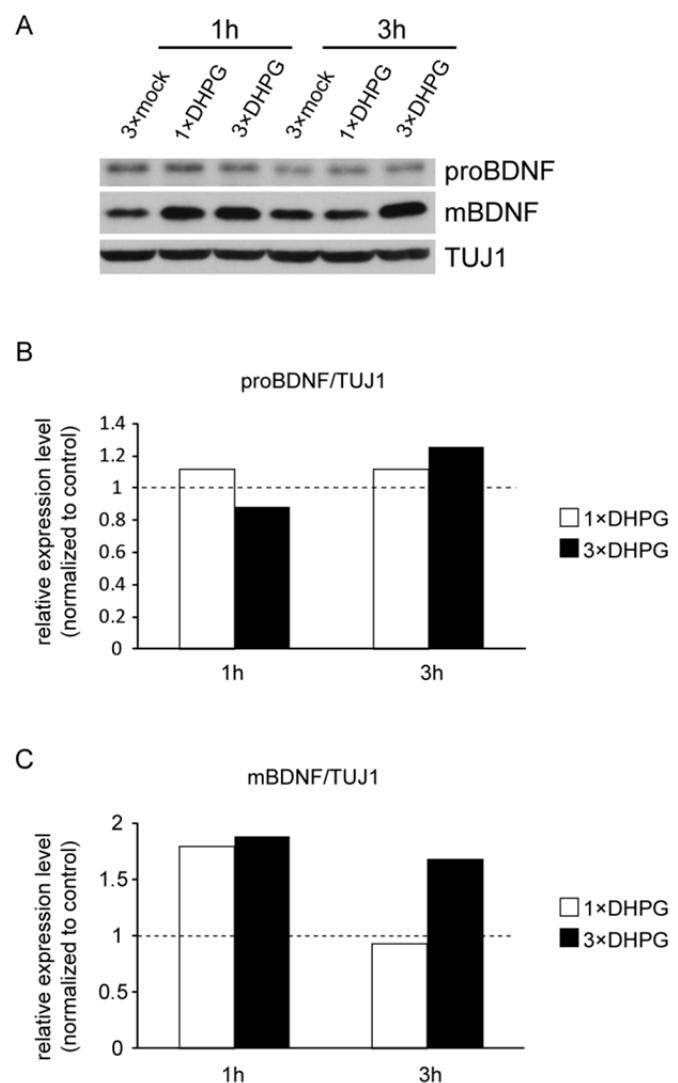
Model of LTD and LOSS, which is induced by single and repeated DHPG exposure, respectively. Upon activation of group I mGluR by DHPG, phospholipase C β (PLC β) is activated via G protein. The downstream signaling of PLC β is divided into two broad pathways. In inositol-1,4,5-triphosphate (IP₃) pathway, release of intracellular Ca²⁺ store mediates calcium-calmodulin (CaM) activation, which in turn activates protein phosphatases. PP1 in particular dephosphorylates the intracellular domain of AMPA receptor and then leads to the internalization of it. This signaling pathway is considered a molecular mechanism underlying the induction of DHPG-LTD. On the other hand, diacylglycerol (DAG) is coupled to PKC activation. Because of the requirement of PKC activity for the establishment of LOSS (Supplementary Fig.5), this pathway is thought to mediate LOSS signaling. However, for the establishment of LOSS, this pathway has to be activated more than three times, that is, there seems to be a kind of threshold. To explain the

threshold for LOSS, here I propose two models, where repeated mGluR activation regulates specific gene expression that is critically involved in LOSS. One possibility, still being nothing but speculation, is that single mGluR activation merely leads to insufficient activation of transcription factor 'x', whereas repeated (≥ 3) mGluR activation make the level of 'x' to exceed the threshold to trigger the gene expression critical for LOSS. Another possibility is that 'x' is relayed to other transcription factor 'y' if second mGluR stimulation comes while 'x' is still active and third mGluR stimulation similarly relay 'y' to 'z', which then triggers the gene expression. In either case, there is no doubt that repeated DHPG exposure facilitates particular set of gene expression and following protein synthesis. Among many proteins to be up-regulated, proBDNF is one of the most important secretive factors. An et al., 2008 suggested that (pro)BDNF mRNA is widely distributed from soma to dendrite and that translation product with functional importance in dendrite is almost proBDNF. Therefore, increased proBDNF in LOSS-inducing neuron is also expected to be released from dendritic region, especially from synaptic site. Extracellularly secreted proBDNF acts by binding to p75^{NTR}, which is located in dendritic spine head in CA1 pyramidal neuron (Woo et al., 2005), and then leads to spine elimination or LOSS although details of p75^{NTR} downstream signaling is still an open question. There are other important questions to be addressed. For example, how repeated mGluR activation controls the intracellular and/or extracellular conversion of proBDNF to mature BDNF is a question of significant importance in understanding activity-dependent synapse elimination and formation. If proBDNF to mature BDNF conversion is not inhibited, repeated mGluR activation may increase the level of mature BDNF rather than proBDNF and therefore stimulate synapse formation by way of a trophic effect of TrkB activation. In addition, it is necessary to investigate the role of sortilin, a co-receptor for proBDNF and p75^{NTR} in LOSS induction, because it is well known that p75^{NTR} makes a trophic effect if it acts by itself.



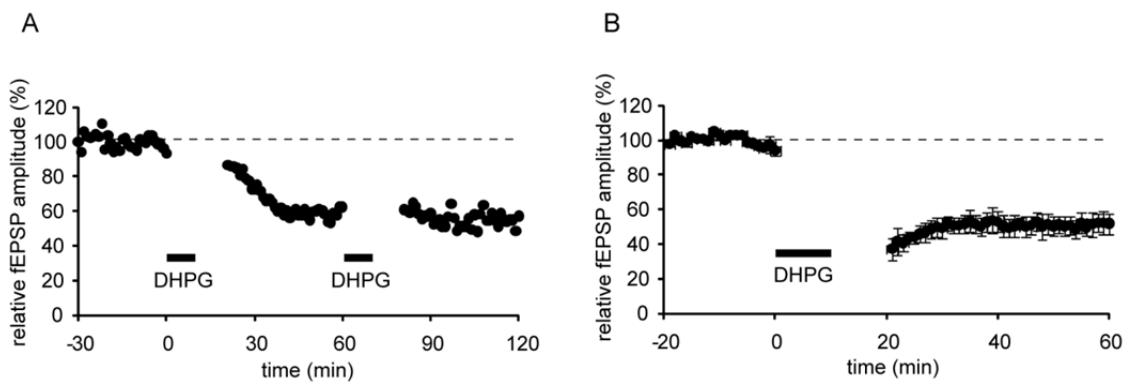
Supplementary Fig. 1

(A) Requirement of protein synthesis for each DHPG exposure. Single time application of anisomycin after either the first or third DHPG exposure abolished the establishment of LOSS. Field EPSP amplitude was monitored 3 w after the repeated DHPG exposure. (B) Time window of the synthesis of the protein(s) necessary for to establish LOSS. Anisomycin application for 90 minutes beginning 1.5 or 3 hours after each exposure to DHPG abolished the establishment of LOSS when monitored 3 weeks later. Taken together with the results of anisomycin application beginning 6 hours after DHPG exposure (Text Fig. 5, 6), the synthesis of the necessary protein(s) is completed between 3 and 6 hours.



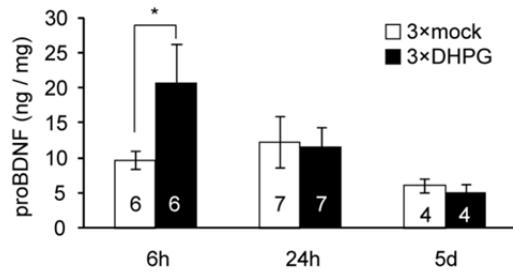
Supplementary Fig. 2

The tissue contents of proBDNF and mature BDNF in hippocampal slice cultures sampled 1 hour and 3 hours after single or repeated exposure to DHPG.



Supplementary Fig.3

Additional evidence for the distinction of LOSS and LTD. Absence of occlusion of LTD after the establishment of LOSS. When LTD was once induced by the application of 50 μ M DHPG, the second application of 50 μ M DHPG fails to induce further LTD (A). However, LTD could be induced by 50 μ M DHPG after LOSS had been established (B).



Supplementary Fig.4

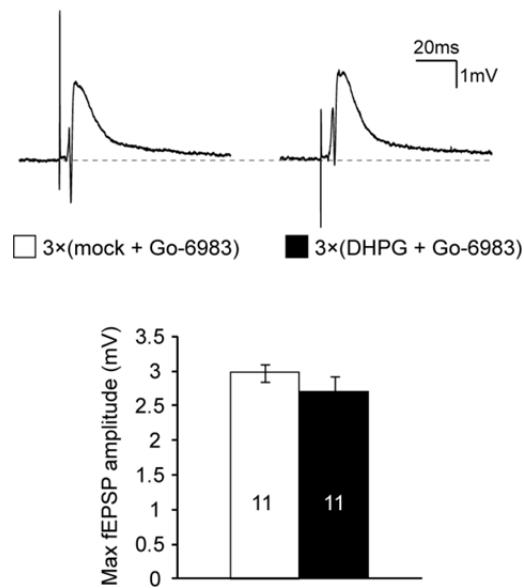
ELISA quantification of proBDNF produced.

Level of proBDNF protein, per milligram of total protein mass, was measured 6 hours, 24 hours and 5 days after 3 DHPG exposures. *P < 0.05 (one-tailed Welch's t-test).

Supplementary Method

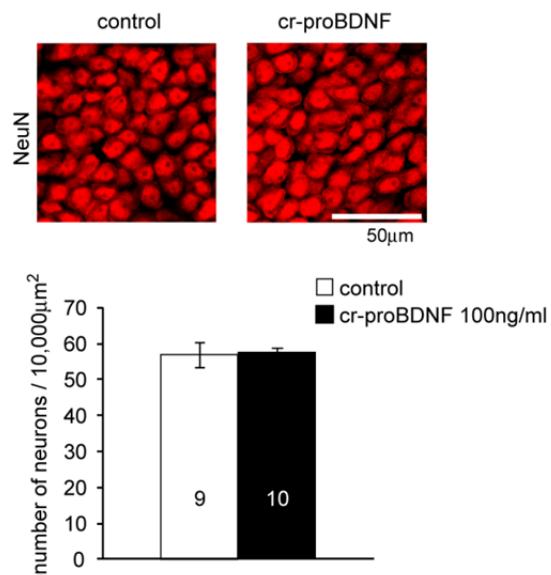
Enzyme-Linked ImmunoSorbent Assay (ELISA)

Hippocampal lysate was prepared in the same way as described for western blotting and diluted with lysis buffer to a total protein concentration of 1.0 mg/ml. 96-well plates (NUNC) were coated overnight at 4°C with an antibody raised in chicken against the prodomain of proBDNF, which was diluted in carbonate coating buffer (25mM Na₂CO₃, 25mM NaHCO₃). The plates were then blocked with Block Ace for 1 hour. The plates were subsequently incubated for 2 hours with samples and standards, washed, and incubated for another 2 hours with a rabbit anti-proBDNF antibody. The plates were washed thoroughly and incubated with an anti-rabbit horseradish peroxidase-conjugated antibody (1:1000) for 1 hour. Each reaction was developed using TMB One solution (Promega) for 15 minutes. The reaction was stopped with 1 N HCl, and the sample absorbance was measured at 450 nm with an ELISA plate reader (Bio-Rad). Standard proBDNF protein was kindly provided by Dr Masami Kojima.



Supplementary Fig.5

Contribution of PKC signaling pathway in the development of mGluR-dependent LOSS. Cultured slices were treated with PKC inhibitor Go-6983 for 90 minutes begun at 30 minutes before each 10 minutes' DHPG exposure (or mock stimulation). 3 weeks after the repeated stimulation, maximal field EPSP amplitude was measured.



Supplementary Fig.6

The application of cr-proBDNF at a dose of 100 ng/ml did not affect neuronal density in CA1 region of the hippocampal slice culture when measured 3 weeks later.

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- Egashira Y, Tanaka T, Soni P, Tominaga-Yoshino K, Ogura A. Involvement of p75^{NTR} signaling pathway in persistent synaptic suppression coupled with synapse elimination following repeated LTD induction. (submitted)
- Kamikubo Y, Egashira Y, Tanaka T, Shinoda Y, Tominaga-Yoshino K, Ogura A. Long-lasting synaptic loss after repeated induction of LTD: Independence to the means of LTD induction. Eur. J. Neurosci., 24, 1606-1616. (2006)
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- Egashira Y, Kojima M, Tominaga-Yoshino K, Ogura A. Possible involvement of proBDNF-p75NTR signals in LTD-repetition Operated synaptic suppression, a form of long-lasting synaptic plasticity. The 36th Congress of the International Union of Physiological Science, Program# P1PM-9-6 (Kyoto International Conference Center, Kyoto) Jul. 28, 2009
- Oe Y, Egashira Y, Shinoda Y, Ogura A, Tominaga-Yoshino K. Repetitive-LTP-induced synaptic enhancement (RISE) established locally by local induction of LTP. Society for Neuroscience 38th Annual Meeting, Program# 541.16 (Washington Convention Center, Washington DC) Nov. 18, 2008
- Shinoda Y, Tanaka T, Kamikubo Y, Egashira Y, Tominaga-Yoshino K, Ogura A. Repeated activations of mGluR led to a long-lasting synaptic elimination in dissociated culture of the rat hippocampal neurons. Society for Neuroscience 36th Annual Meeting, Program# 135.6 (Georgia World

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Japanese Scientific Meetings and Symposiums

- 江頭良明
mGluR 依存的 LTD の繰り返しによって生じるシナプス廃止への proBDNF-p75NTR シグナルの関与
生理学若手研究者フォーラム（順天堂大学医学部、東京）2009年7月4日、口頭発表
- 江頭良明、小島正己、原とも子、富永・吉野恵子、小倉明彦
LTD の繰り返し誘発による長期シナプス抑圧／廃止現象への proBDNF-p75NTR シグナルの関与
第32回日本神経科学大会 Program# P2-b27（名古屋国際会議場、名古屋）2009年9月17日、ポスター発表
- 大江祐樹、江頭良明、篠田陽、小倉明彦、富永・吉野恵子
局所的な繰り返し LTP による長期シナプス強化を局所的に成立させる
第31回日本神経科学大会 Program# P2-a04（東京国際フォーラム、東京）2008年7月10日、ポスター発表
- 江頭良明、田中庸弘、上窪裕二、篠田陽、富永・吉野恵子、小倉明彦
繰り返し LTD 後の長期シナプス減弱の発現に必要なタンパク合成の時間枠
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- 江頭良明、田中庸弘、上窪裕二、篠田陽、富永・吉野恵子、小倉明彦
mGluR 依存性 LTD の繰り返し誘発後の長期シナプス減弱はタンパク質合成を必要とする
第29回日本神経科学大会 Program# P3-022 (国立京都国際会館、京都) 2006年7月21日、ポスター発表
- 田中庸弘、江頭良明、上窪裕二、篠田陽、富永(吉野)恵子、小倉明彦
繰り返し LTD 誘発後の長期シナプス廃止にはタンパク質合成が必要か
第83回日本生理学大会 Program# 3P 2-130 (群馬県民会館・前橋商工会議所、前橋) 2006年3月30日、ポスター発表
- 上窪裕二、江頭良明、田中庸弘、篠田陽、富永(吉野)恵子、小倉明彦
培養器内における長期持続性シナプス減弱
第28回日本神経科学大会 Program# P1-052 (パシフィコ横浜、横浜) 2005年7月26日、ポスター発表

Reviews

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