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Establishment of Optical Method for Two-dimensional Quantitative Analysis of Synaptic Transmission in Cultured Hippocampal Slice: A Dendritic Layer—specific Persistent Enhancement of Synaptic Strength After Repeated Activation of Protein Kinase A.

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# ABBREVIATIONS

<table>
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<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>CA1</td>
<td>corne d’Ammon 1 (Ammon horn 1)</td>
</tr>
<tr>
<td>CA3</td>
<td>corne d’Ammon 3 (Ammon horn 3)</td>
</tr>
<tr>
<td>CCD</td>
<td>charge coupled devise</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ddFK</td>
<td>1,9-dideoxyforskolin</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory post-synaptic potential</td>
</tr>
<tr>
<td>FK</td>
<td>forskolin</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutylic acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDA</td>
<td>photodiode array</td>
</tr>
<tr>
<td>PKA</td>
<td>cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>PrI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SO</td>
<td><em>stratum oriens</em></td>
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<tr>
<td>SP</td>
<td><em>stratum pyramidale</em></td>
</tr>
<tr>
<td>SR</td>
<td><em>stratum radiatum</em></td>
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<tr>
<td>VSD</td>
<td>voltage-sensitive dye</td>
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要旨
記憶の機構解明は，学術的な興味関心はもちろんあり，痩せや健康を含む様々な記憶疾患の理解や治療に関する知識を得るという上でも非常に重要な研究の一つである。これまで中枢神経系のシナプスには，活動によって伝達効率が増やせると変化する現象が確認されており，これはシナプス可塑性と呼ばれている。記憶現象の細胞レベルでの本質はこのシナプス可塑性にあるとするのが，現代神経科学の共通の理解である。この可塑性現象がニューワシおよびラット海馬（大脳皮質の一部）で実験的に確認されて以来，多くの神経科学者がその機構解明に取り組んできた。

記憶には大きく分けて2つの相がある。瞬時に成立するが一過的に終わる短期記憶と，それに引き続いて成立し数日ないし数週間，あるいは生涯維持される長期記憶である。それに対応するように，シナプス可塑性にも大きく分けて2つの相がある。既存のシナプスにおいてタンパク合成を伴わず瞬時に成立する伝達効率変化，短期可塑性と，タンパク合成を介して新しいシナプスが形成されることによる伝達効率変化，長期可塑性とである。上記のラット海馬のシナプス可塑性（入力繋維の頻回刺激によって伝達効率が上昇するLTP；Long-term potentiation か典型的）は短期可塑性の代表例であり，NMDA型グルタミン酸受容体の活性化によるシナプス部位でのCa流入と，Ca-カルモジュリン依存性タンパクキナーゼの活性化によるAMPA型グルタミン酸受容体の活性化，という分子機構がほぼ確定している。

これに対して，長期可塑性については，短期可塑性における海馬LTPのような優れた実験系がなく，海馬LTPの後期相（刺激1-3時間後以降）で見られるシナプス部の形態変化（軸索末端の分枝，樹状突起の分裂など）がこれに当たるのではないかと想定されて，最近徐々に解析が進み始めた段階にある。しかし，この形態変化が本当に日，週オーダーの長期間持続するのかという問題や，短期のみで終わる可塑性と長期化する可塑性とはどのように選別されるのかといった問題は，その糸口さえつかめていない。これらの問題に答えるには，実際に長期間維持できる実験系の確立と，それを解析する実験方法の開発とが必要不可欠である。

最近，当研究室の富永らは，長期培養したラット海馬切片にアデニル酸シクラーゼ
の活性化剤Forskolin（FK）を繰り返し投与して短期可塑性であるLTPを薬理学的に
繰り返し引き起こすと、数日後からゆっくりと生起し、その後少なくとも3週間維持
される伝達効率の増強が起こることを示した。この増強は重視学的、生化学的指標に
よるシナプス新生を伴うものであった。興味深いことに、FKの単回投与ではいったん
伝達効率が上昇するが、それは24時間以内に消失した。この結果は、LTPの後期相
はそのままでは长期化しないこと、長期化には刺激の繰り返しが必要なことを強く示
唆すると同時に、培養海馬切片が長期可塑性を解析する上での優れた実験系になるこ
とを示している。したがって次に必要なのは、この可塑性現象を生理学的、2次元的
に解析する実験法を開発することである。

私は、膜電位感受性色素（VSD）を培養海馬切片に適用して高速感度二次元撮像
素子によって解析することがその実現になると期待し、本研究を開始した。VSDとは、
細胞活動に伴う膜電位の変化を蛍光または吸光度の変化に変換する色素をいい、実際
これまで新鮮脳切片や株化培養細胞に対して適用されている。VSD測定は、様々な部
位で起こる神経活動を2次元的に同時解析できるという点で、一点でしか記録できな
い電極記録法に比べ有用性が高い。しかしながら、実際VSDを長期培養脳切片に適用
するには、そのそれぞれが本質的に抱える問題（色素の褪色や培養下における各切片
間の細胞数のばらつき等）を解決しなければならなかった。私は、これらの問題点を
一つ一つ解決することで、長期培養標本の神経活動の微細な変化の検出を可能にした。

さらにこの方法を用いて海馬CA1領域の樹状突起層の空間網羅的解析を行い、さき
に富永らが報告したFKの繰り返し投与後の長期的伝達増強が、樹状突起の近位層で
起こっていることをつぶとめた。また、富永らが報告した樹状突起の遠位層でのシナ
プス新生は、機能的伝達増強を伴っていないことを見出した。そこで遠位層における
新生シナプスは、いわゆるサイレントシナプス（構造としては存在するが機能して
いないシナプス）ではないかとの仮説を立て、これを立証するためにさらに解析を進
めたところ、予想通りサイレントシナプスである可能性を強く示唆する結果が得られ
たので、ここで報告する。
ABSTRACT

Experimental approaches to the study of the synaptogenesis underlying long-term memory in the mammalian CNS require a good model system for analysis and a reliable methodology. I propose that the cultured rat hippocampal slice should be the required system because of its stability and preservation of circuitry. For methodology, the optical monitoring of synaptic activity by use of voltage-sensitive dye (VSD) has the potential to be applied to the above system. To accomplish this, however, it needs to overcome the shortcomings of cultured slices and VSD monitoring, the variance in cell number among specimens and signal decay. Here I established a VSD methodology applicable to cultured slices for quantitative analysis. Taking an antidromic spike signal as an internal standard, I compensated for the variance in neuronal number among specimens. The fast signal decay within a single recording was cancelled using a false stimulation recording. The slow signal decay over multiple recordings was compensated with those following repeated antidromic stimuli. The optical signal of postsynaptic potential was isolated from that of the presynaptic spike by an application of receptor antagonists. Employing these techniques, I found a layer-specific enhancement of synaptic transmission in the CA1 area of cultured hippocampal slice following the repetitive activation of adenylyl cyclase. The synaptic transmission in the proximal layer of apical dendrites in the CA1 area was enhanced, confirming the previous result of electrophysiological examination. However, the transmission in the distal layer was not enhanced by the repeated FK-exposure. This result apparently contradicts with the previous immunocytochemical and ultrastructural findings that new synapses were developed in the distal layer as well. So, I hypothesized that the newly-developed synapses in the distal dendritic layer would be the so-called "silent synapses", i.e. synapses apparently mature in morphology but staying inactive. I tested this hypothesis by measuring relative enhancement of EPSP after inducing LTP electrically (silent synapses should readily turn active). The relative enhancement of EPSP was
larger in the distal layer than in the proximal layer, supporting the hypothesis.

I think that the long-term culture of hippocampal slice with the voltage-sensitive dye monitoring of neuronal activity should serve a good model system and methodology for the analysis of long-term synaptic plasticity underlying the brain memory. And I found the interesting aspect of the synaptic plasticity that was the layer specific enhancement of synaptic transmission at CA1 apical dendritic layer in the cultured hippocampal slice.
INTRODUCTION

Brain memory is consolidated through multiple, at least two, phases (Hebb, 1949; Bear et al., 2001). One is the early phase, which is formed within a minute by a modification of transmission efficiency in pre-existing synapses, requiring no protein synthesis. The other is the late phase, which is formed over a passage of hours or days by the formation of new synapses, requiring protein synthesis. The former has been well analyzed using long-term potentiation (LTP) in the hippocampus, an excellent model system for analysis (Bliss and Lømo, 1973; Malenka and Nicoll, 1999). However, the latter is poorly understood mainly because of the lack of a good model.

In our laboratory, Tominaga-Yoshino and her colleagues have recently found a slowly-developing and long-lasting synaptogenesis in cultured hippocampal slices after the repetitive activation of cyclic AMP-dependent protein kinase (PKA), and proposed that this phenomenon should serve as a good model system for the analysis of the late phase of memory (Tominaga-Yoshino et al., 2002). Following repeated exposures to forskolin (FK), an adenylyl cyclase activator, not only the magnitude of population excitatory postsynaptic potential (EPSP) but also the amount of synaptic proteins and the density of immunocytochemically identified synaptic structures increased in the CA1 dendritic area (Fig. 1 and 2). These increases depended on the times and the interval of PKA activation, began ~1 week after the exposure, and lasted for ≥3 weeks. Electron microscopic examination demonstrated an increase in synaptic density and morphological changes (synapse perforation) as well (Fig. 2).

To further characterize this synaptogenesis, especially its geographical aspects, VSD monitoring of EPSP should be useful (Grinvald et al., 1982; Cohen and Lesher, 1986; Salzberg, 1989; Momose-Sato et al., 2001). Monitoring with electrodes, although direct and sensitive, has a limitation in the number of simultaneous manipulations. VSD converts membrane potential to an optical signal (fluorescence or absorbance), and thereby EPSPs occurring locally in the dendritic layers of the slice are
obtained space-exhaustively, if coupled with a 2-dimensional photodetector such as a photodiode array (PDA) (Mochida et al., 2001; Enoki et al., 2002) or a CCD camera (Takashima et al., 1999; Tominaga et al., 2000).

However, in VSD monitoring, the quantitative comparison of data obtained from long-term cultured slices bears two problems, one of which is not critical in acutely prepared slices (Sinha et al., 1995; Otsu et al., 2000). During the prolonged period of culture, a significant number of neurons degenerate and the surviving cells migrate horizontally. To make matters worse, the extent of cell death and migration is not equal among the specimens (not homogeneous even in a single specimen) so that the number of cells related to the optical signal in the region of interest (ROI) varies among specimens. The other problem, which also results from the decreased neuronal number, is a rapid signal extinction (due mainly to the wash-out and bleaching of the dye) during the course of the experiment. To compensate for the variance in neuronal number among the specimens and the signal extinction, it is desirable to standardize the optical signal to convert it to an index for the number of synapses per cell. Here I established a method of standardization: the maximum optical EPSP signal (maximum stimulus is needed to activate all functional synapses existing there) following an orthodromic stimulation was divided by the maximum spike signal following an antidromic stimulation. Since the EPSP signal followed by orthodromic stimuli was contaminated by the signal of presynaptic fiber firing (fiber volley), the latter was subtracted after isolation by the use of glutamate and gamma-aminobutylic acid (GABA) receptor blockers.

In the present report, I developed the methodology coupled with VSD and cultured hippocampus, which is available to analyze the late-phase of memory. Using this methodology, I found a dendritic layer-specific nature of the enhancement of synaptic transmission, which may correspond to the layer-dependent difference in morphological changes reported previously (Tominaga-Yoshino et al., 2002). The
present finding suggests the functional heterogeneity in the newly formed synapses, confirming the usefulness of the cultured hippocampus with the VSD methodology in the analysis of long-term plasticity.
MATERIALS AND METHODS

1. Slice culture of the rat hippocampus

The slice cultures were prepared conventionally (Stoppini et al., 1991). Briefly, the hippocampus was isolated from Wistar rat pups at postnatal day 7, and cut at a thickness of 400 μm. Each slice was placed on the surface of a Millipore filter (Millicell CM) inserted into a 6-well vessel (NUNC). The culture medium (composed of 50% minimal essential medium based on Hank's salt, 25% Hank’s buffer and 25% equine serum; all from Gibco) was changed twice a week. The culture was maintained for 8 days, in which time it stabilized both physically and physiologically (Muller et al., 1993; Gähwiler et al., 1997). Healthy cultures (judged from opacity) were exposed to a new medium containing either 50 μM forskolin (FK) or 50 μM 1,9-dideoxyforskolin (ddFK: a biologically inactive forskolin analog) for 30 min at 34°C. The FK- or ddFK-exposure was repeated 3 times at 24-hr intervals. According to our previous results (Tominaga-Yoshino et al., 2002), the population EPSP increased in size significantly ~1 week after the first application of FK and this state lasted for ≥3 weeks. So I used the cultures 15-23 days after the drug treatment (i.e. 23-31 DIV) for the optical examination (Fig. 1).

2. Immunohistological and cytochemical staining

At 23-31 DIV, the cultured slices were fixed with 4% PFA in PBS, washed with PBS several times, permeabilized with buffer H (PBS containing 1% Triton X-100, 5% Horse serum and 0.05% sodium azide), and incubated with buffer H containing primary antibodies (raised against Neurofilament 200kD, Chemicon; against MAP2, Chemicon). Then the slices were washed with PBS, re-permeabilized with buffer H, and incubated with buffer H containing secondary antibodies (Alexa Fluor 488 goat anti- rabbit IgG, Molecular Probes; Alexa Fluor 594 goat anti-mouse IgG, Molecular Probes). Propidium iodide (PI) staining was carried out by soaking the specimens in
the PBS containing PrI (10μg/mL together with 0.5% Triton X-100) after PFA fixation. Observations were done under an Olympus IX-70 fluorescence microscope or a Nikon-BioRad MRC-1024 laser-scanning confocal microscope.

3. **Instrumentation**

A Nikon inverted microscope (TE-300) was equipped with objective lenses (CFI Plan Apo 4x NA0.2, CFI S Fluor 10x NA 0.5, CFI Plan Apo 40x NA 0.95 and CFI Plan Apo 60x NA 0.95; all from Nikon) and an epifluorescence illuminator (Osram xenon short arc lamp XBO 100W coupled with a Hamamatsu C7085 feedback ripple canceller). The interference filters for excitation and emission were 510-560 nm and >590 nm, respectively. At the height of the real image plane, a Hamamatsu 16 x 16 PDA (C4675-102) was set, the output of which was fed to a Hamamatsu image processor (Argus-50/PDA) though a controller (Hamamatsu C5897). Software for data collection and analysis were provided by Hamamatsu (Argus PDA versions 1.1.8 and 2.0.0). This system provides a 16-bit fluorescence intensity for each of 16 x 16 pixels at a rate of 0.5 msec.

4. **Optical dye-staining**

Immediately before use, a 10 mM stock solution of a styryl VSD RH-414 (Molecular Probes) in DMSO was diluted to 1 mM with an artificial cerebrospinal fluid (ACSF; composed of 126 mM NaCl, 3 mM KCl, 1.25 mM NaHPO₄, 0.5 mM MgSO₄, 2.5 mM CaCl₂, 22 mM NaHCO₃ and 10 mM glucose) bubbled with 95% O₂/5% CO₂. The filter carrying the culture was soaked in the dye solution for 10 min at room temperature. The filter was then placed in the recording chamber set on the microscope, which was perfused with pre-warmed (29°C) ACSF at a rate of 1 ml/min.
5. Optical recording

It is necessary to ascertain the preservation of neural circuits in the present hippocampal cultures. Figure 3 shows the CA1 area of a representative culture stained with an antibody to neurofilament (Chemicon; for staining the Schaffer collateral axons and CA1 pyramidal axons) and an antibody to microtubule-associated protein 2 (Chemicon; for staining the somas and dendrites of CA1 pyramidal cells). The Schaffer collateral axons entered vertically to the apical dendrites of CA1 pyramidal cells as they do in vivo and the CA1 pyramidal cell axons exited vertically toward the external margin also as they do in vivo. This ensures that an orthodromic stimulus can be delivered through an electrode placed at CA3 and that an antidromic stimulus can be applied through an electrode placed at the external margin of the culture. So I placed two bipolar tungsten wire electrodes for stimulation; one in the CA3 pyramidal cell area and the other at the external margin of the culture outside the ROI fixed in the CA1 area.

The scheme and timetable of recordings are depicted in Fig. 4. Ten minutes after placement of the stimulation electrodes, the antidromic (0.3 mA, 250 μsec single pulses) and orthodromic (0.6 mA, 250 μsec) stimuli were delivered at a regular interval of 30 sec (0.033 Hz) for 10 min. This ‘taming’ stimulation was mandatory to obtain a stable optical response. The intensity of the anti- or orthodromic stimuli was determined as ~2x the intensity, at which the obtained response reached the maximum. Then the ‘real’ stimulation was delivered 4 times for the orthodromic paradigm. This was followed by a ‘false’ stimulation with the pulse generator switched off for the correction of fast decay. Next, under continuous perfusion with a cocktail of receptor antagonists (30 μM APV [2-amino-5-phosphonovalerate, Sigma], 40 μM DNQX [6,7-dinitroquinoxaline-2,3-dione, Sigma], 2 μM CGP52432 [Tocris], and 10 μM bicuculline [Sigma] dissolved in ACSF), the sessions of stimulation were repeated in the same manner. Thus I conducted 8 sessions of stimulation for each culture to obtain a set of
8 averaged recordings.

The obtained recordings are a series of two-dimensional images consisting of 16 x 16 pixel channels. For presentation purposes, however, I show here a chart drawing of the average for 8 channels fixed in the apical dendritic layer (stratum radiatum) of the CA1 area (22 μm from the somatic layer [stratum pyramidale]) as shown in hatched boxes in Fig. 3 and 4. All experiments were carried out using 60x objective unless otherwise noted. When 60x objective was used, 1 pixel corresponded to 11 x 11 μm of the specimen, when 40x objective was used it did to 17 x 17 μm of the specimen.

6. Electrophysiological recording

On occasions, I conducted a conventional electrophysiological recording simultaneously with the optical recording. A glass capillary microelectrode filled with ACSF was placed extracellulary in the CA1 stratum pyramidale, and the population EPSP and spike recordings were made through a WPI microelectrode amplifier (Model 707).

7. Animals and Chemicals

All rats used in this study were purchased from Nihon SLC and treated properly following our institutional guidelines for animal welfare. All chemicals were of analytical grade and purchased from Wako Chemical Co. or Nacalai Tesque Inc., unless otherwise noted.
RESULTS

1. The variation of cell number and thickness of the cultured slices

As explained in Introduction, one of the most serious problems in the examinations using the cultured slices is the instability of the neurons. Some of them degenerate and the others migrate horizontally. These lead to the decrease in the cell number through the thickness of specimen, and what is worse, these are variable among the specimens. Actually, confocal microscopic examination showed a considerably large variance in cell densities among the specimens even in those maintained under the same culture conditions, although the mean cell density was unaffected by the FK-treatment (Fig. 5). The thickness of the slice showed an even larger variance among the specimens, although the mean thickness was uninfluenced by the FK-treatment (Fig. 6). These variances eventually produce the difference in VSD signal intensity. To compare quantitatively the enhancement in synaptic strength after the FK-treatment, it is really a heavy obstacle considering that the expected enhancement in synaptic strength is ~20% (Tominaga-Yoshino et al., 2002). The standardization methodology for EPSP-originated fluorescence signals to compensate the variance in the cell number among the specimens is inevitably necessary. To do this, a signal that directly represents the number of cells in the ROI is required.

2. Reliability of optical measurement of voltage signals

Although the reliability of styryl-type VSD in converting the membrane potential change into fluorescence signal is well established (Grinvald et al., 1982; Saggau et al., 1986), it is necessary to test it in the present measurement system by a simultaneous recording of optical and electrical signals. For this test, I used antidromic stimulation, since the response to that is of single component (in orthodromic stimulation, the response consists of multiple components; fiber volley, EPSP and action potential, the signs of which are opposite in the electrical recording).
With changing the intensity of stimulation current, the larger was the current, the larger was the number of activated axons and the larger was the number of cells firing spikes. As shown in Fig. 7, the optical and electrical signals grew in parallel. However, there should be saturation, since the spike signal cannot grow further when practically all neurons existing there fired spikes. As also shown in Fig. 7, this saturation property is reproduced perfectly coincidentally in optical and electrical recordings.

3. Proportionality of optical signals and cell number

The level of raw signal should be influenced by the number of cells existing in the ROI. The larger was the number of cells, the stronger should be the EPSP signal. But, as the action potential generated by a neuron is all-or-none and invariant among cells, the maximum spike signal (the signal when practically all neurons fired spikes) would serve as an index of the neuronal number in the ROI. The larger was the number of cells, the larger should be the maximum spike signal. Then, if the number of synapses per neuron is stable (or in a limited range) among the specimens cultured under the same conditions, the maximum EPSP signal and the maximum spike signal should be proportional, the proportionality constant being the index of synapse number per cell. This was verified and the results are shown in Fig. 8. When seven control (ddFK-treated) cultures having apparently different thicknesses were stimulated, a clear proportionality between maximum EPSP signal and spike signals was obtained. An equivalent discussion should also hold in electrical recordings (see Fig. 8B).

4. Properties of slow signal decay

The optical signal is diminished with time, since it took ~30 min to complete the whole set of examinations. This slow decay of signal was, however, linear so that reconstruction is possible by estimating the decay rate (Fig. 9). But an unexpected feature was found. In a first-order discussion, even if the absolute fluorescence signal
(F) fades due to the bleaching or washout of the dye, the stimulus-induced change of fluorescence ($\Delta F/\Delta F$) would not, as long as the stimulation is maintained constant. But it did fade actually (Fig. 9). The signals for antidromic stimulation and orthodromic stimulation weakened in parallel, suggesting the neuronal origin of this decay property. Although there is no evidence to explain such signal diminution, it may be possible that neurons’ activity in exo- and endocytosis would increase the internalized VSD molecules which cannot sense the surface membrane voltage. Or, the dye incorporated by glial and neuronal membranes would simply differ in retention due e.g. to the difference in lipid environments.

5. **Standardization of EPSP signal with respect to cell number**

    In the present system, the optical signals accompanied by the maximal spike and EPSP were at the maximum $-0.7\%$ in $\Delta F/\Delta F$ (fluorescence change to total fluorescence; a negative sign signifies that membrane depolarization is converted to a decrease in fluorescence), respectively, as shown in Fig. 10. Both the optical and electrical signals of maximal spike and EPSP varied among the specimens cultured in the same conditions due apparently to the variance in the existing cell number (see Fig. 8). Since the range of variance was more than 1.5-fold, the raw EPSP signal could not be adopted as an index of the number of synapses, considering that the expected increase in EPSP following repetitive PKA activation is around 1.5-fold (Tominaga-Yoshino *et al.*, 2002).

    The number of cells, the source of the optical and electrical signals, should be reflected in the maximal size of the population spike signal following antidromic stimulation, since the spike generated by each single neuron is all-or-none and invariant among neurons. The maximal size of the antidromic spike signal should thus be proportional to the number of cells in the ROI. If the number of synapses per cell is supposed to be constant in each of the experimental groups, the maximal size of the
orthodromic EPSP signal should also be proportional to the cell number. So, the
maximal sizes of antidromic spike and orthodromic EPSP should be proportional in
each group and the proportionality constant between them should serve as an index for
the number of synapses per cell (this is merely an index; conversion to the absolute
number is difficult). This conjecture was confirmed (see Fig. 8).

6. Fluorescence decay compensation

The fluorescence signal decayed with time. There were two types of decay;
one occurring rapidly within a single run of measurements, the other occurring slowly
in the course of the examination (it took \(\sim 30\) min to complete the examination). To
correct for the rapid decay, I used the previously described method (Orbach et al., 1985;
Bonhoeffer and Staiger, 1988; Jin et al., 2002), which recorded a decay in a session of
‘false’ stimulation performed immediately after each session of ‘real’ stimulation.
Figure 10 depicts an example, where the fluorescence signal showed a linear decay
upon the ‘false’ stimulation. To compensate for the degree of the slow decay, I used
the fluorescence signal from CA1 pyramidal layer accompanied by antidromic
stimulation (Fig. 10B and 10D). Against expectations, not only the F value (absolute
fluorescence intensity) but also the \(\Delta F/F\) value changed with time. I think this is
probably due to a difference in the dye lifetime between glial cells and neurons, because
the \(\Delta F/F\) values for orthodromic and antidromic stimuli (both are neuron-originated
signals) decreased at the same rate (Fig. 9). If the dyes incorporated by neurons would
be lost more easily than those incorporated by glial cells, the rate of diminution of \(\Delta F\)
(originated predominantly from neurons) should be faster than \(F\) (originated from both
neurons and glial cells), resulting in a decrease in the \(\Delta F/F\) value with time. Whether
this conjecture is correct or not, however, the slow decay in the \(\Delta F/F\) value was linear
with time so that compensation for the slow signal decay was achieved (Fig. 10).
7. **Isolation of EPSP component from optical signal with orthodromic stimulation**

The optical signal obtained in the dendritic region should contain at least two components; the so-called fiber volley which is the action potential generated by the presynaptic (i.e. Schaffer collateral fiber's) terminal and the EPSP generated by the postsynaptic dendrite. To separate these components, I applied a cocktail of receptor antagonists to suppress the postsynaptic components without affecting the presynaptic ones. Digital subtraction between two optical signals (after compensation for decays) before and after the application of antagonists provided a signal, which matched well the EPSP in time course (Fig. 10E and 11).

8. **Spatial difference in EPSP enhancement after repetitive PKA activation**

We have previously reported that the repetitive activation of PKA (whether by applications of FK or of a cyclic AMP analogue) results in an increase in EPSP accompanied by a morphologically-identified synapse formation (Tominaga-Yoshino et al., 2002). With the present optical measurement (incorporating the above-mentioned cell number standardization, fast decay correction and slow decay compensation), the increase in EPSP reflecting the newly formed synapses should be detectable. Furthermore, taking advantage of the space-exhaustiveness of VSD measurements, I should be able to examine whether the enhancement of EPSP would have occurred uniformly throughout the dendritic layers. As shown in the two-dimensional display of standardized EPSP size (Fig. 12), the increase in EPSP was more profound in the proximal layer of the *stratum radiatum* (34-68 μm from the pyramidal cell layer) than in the distal layers (153-187μm). Statistical comparison (Fig. 13) confirmed that tendency.

9. **The possibility that newly developed synapses in distal dendritic layer are "silent"**

Using VSD methodology, I found here a layer-specificity of the synaptic
enhancement. In the CA1 dendritic area, the enhancement was observed limitedly to the proximal layer. In the distal layer, no significant enhancement was observed, although the morphological examinations indicated that the synaptic structures have been increased in number in the FK-treated cultures (see Fig. 2). It is possible that those synapses would be the "silent synapses", i.e. the synapses morphologically existing but not functioning. Silent synapses are generally assumed to be stand-by ones that are ready to be activated by an LTP-inducing stimulus (Isaac et al., 1995; Liao et al., 1995). So, the possibility of silent synapses could be tested by measuring the magnitude of LTP; the more silent synapses are present, the larger should be the magnitude of LTP. If the FK-treated cultures would have a larger number of silent synapses than ddFK-treated cultures, the magnitude of LTP should be larger in the FK-treated cultures. Thus I applied theta-burst stimuli to the cultures. The magnitude of increase in the EPSP-originated signal (representing the magnitude of LTP) from the distal dendritic layer accompanied by the LTP-inducing stimulus was significantly larger in the FK-treated cultures (Fig. 14). The magnitude of LTP in the proximal dendritic layer was also larger in the FK-treated cultures but the increase was not statistically significant. These results support the assumption that the most of the newly developed synapses in the distal dendritic layer of the CA1 area are "silent" that would reserve the next plasticity.
DISCUSSIONS

1. Problems in the application of VSD methodology to cultured brain slice and their solution

For the analysis of long-term synaptic plasticity in mammalian brain, specimens amenable to long-term examination are required. Cultured brain slice is suitable in terms of longevity, the preservation of original circuitry and high accessibility to experimental manipulations. Monitoring of neuronal activity by VSD coupled with high-performance imaging devices developed recently (Grinvald et al., 1982) is promising for the analysis of synaptic plasticity. To combine these tools, however, one has to resolve a critical problem, i.e. the decrease in the number of neurons in a single ROI. The decrease (and its variance among specimens) results in an accelerated decay of optic signal as well as variance in signal intensity.

I here corrected for the fast signal decay by taking mock stimulation recordings and compensated for the slow decay by estimating its rate from repeated antidromic stimulation recordings. I then standardized the variance in cell number using the antidromic spike signal.

2. Possible causes for fluorescence decay

There are two types of decay in the fluorescence signal as reported previously (Bonhoeffer and Staiger, 1988; Jin et al., 2002). The fast decay within a single run of measurement and the slow decay occurring over multiple sessions of recordings. The fast decay would be due to the photobleaching of the dye, since the decay was slowed down by examination with low-power lenses (leading to the weakening of illumination) and suspended during the pause of illumination. The F value in the second run began from the level close to that at end of the first run, when the interval between the runs was short.

The cause of the slow decay is plausibly the washout of the dye, since this
decay continued even if the illumination was shut off, slowed down (though not totally stopped) by shutting off the perfusion, and accelerated by warming the perfusing solution. However, I do not have definitive proofs for the cause.

3. **Layer-specific enhancement of synaptic transmission after repetitive exposure to forskolin**

Recently, we reported that the repetitive application of forskolin induced a slowly-developing long-lasting increase in electrophysiologically monitored EPSP in the CA1 region of the cultured rat hippocampus, which was blocked by a PKA inhibitor (Tominaga-Yoshino *et al.*, 2002). The present study not only confirmed the PKA-dependent synaptic plasticity by the optical recording methodology but found an inhomogeneity of the plasticity among dendritic layers. In that report, we noticed that the ultrastructural changes accompanied by enhancement of EPSP are differed among dendritic layers. The occurrence of “perforated” synapses increased in the proximal layer of the *stratum radiatum*, while the density of “normal” synapses increased in the distal layer. Taking the present results into account, it is possible that the newly developed “perforated” synapses in the proximal layer should have synaptic functionality and contribute to the enhancement of EPSP whereas a fair proportion of “normal” synapses in the distal layer would be silent. This possibility was supported by measuring the magnitude of EPSP signal following the theta-burst stimulus. If the developing synapses in the distal layer were “silent”, they would change to “functional” ones as a consequence of LTP induction (Isaac *et al.*, 1995; Liao *et al.*, 1995). Actually, the increment of EPSP after the theta-burst stimuli was larger in the distal dendritic layer of the FK-treated cultures than in the control cultures.

Quite recently the homeostasis of synaptic strength was evidenced in the intercalated neurons of the guinea-pig amygdala (Royer and Pare, 2003). When one of the synapses of an intercalated neuron was potentiated by a high-frequency stimulus to
an input fiber, the other synapses were depressed. It is then possible to discuss our present results in such a way as: when proximal synapses are enhanced, mid-distal synapses are unchanged and far-distal synapses are depressed homeostatically. Indeed, this tendency was seen in some specimens (the specimen in Fig. 12 is an example). Here I applied FK globally, nevertheless, the changes of synaptic efficacy occurred locally. It is a mere speculation but it might be that the synapses most amenable to enhancement (proximal synapses?) are strengthened preferentially to result in the depression of other synapses even if the stimulus was given globally.
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To make synapses ready for next plasticity
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Fig. 1. Culture of hippocampus. (A) Snapshots of cultured rat hippocampus. Slices were nurtured on membrane cup filter (left). Enlarged image of a slice (right). (B) Schedule of the culture and pharmacological LTP induction. The cultures were grown for 8 days *in vitro* (DIV) until stabilization. At 8, 9 and 10 DIV, forskolin (FK: for induction of LTP) or 1,9-dideoxyforskolin (ddFK: for control) was applied for 30 min every 24h. Slices were further maintained until 23-31DIV and used for experiments.
Fig. 2. Summary of the previous findings on the physiological and morphological changes after the repetitive activation of PKA in cultured rat hippocampus. (A) Schematic representation of examinations. The proximal (red box; <100 μm from soma) and distal (blue box; >150 μm from soma) dendritic layers of the area CA1 are indicated. B, C, D are the layers from which examinations were done. (B) Maximum population EPSP amplitudes from the control and FK-treated cultures (14d after the first drug exposure). Upper panels show representative recordings (Scales: 1mV, 10ms) and lower panel shows a statistical comparison. (C) Morphological features of the proximal dendritic layer. See the increase in 'perforated' synapses in the proximal layer. (D) Morphological features of the distal dendritic layer. See the increase in 'normal' synapses in the distal layer. In C and D, representative electron micrographs are shown in upper panels and statistical comparisons (means ± SEM from 22-51 micrographs) are shown in lower panels. Asterisks indicate statistically significant differences from ddFK (p<0.01). Adapted from Tominaga-Yoshino et al. (2002).
Fig. 3. A cultured hippocampal slice, showing the running patterns of axons and dendrites in the CA1 area. This set of pictures shows a representative slice preparation used for VSD measurement and fixed for immunohistochemical examination at 25DIV. Staining was made using antibodies to neurofilament (NF, green) and microtubule associated protein-2 (MAP2, red). An array of squares in the right most (merged) picture indicates the ROI, the positions of photodiodes from which the optical recording is drawn in the following figures (Figs. 4, 7, 8, 9 and 10). Scale bar represents 25 μm. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.
Fig. 4. Schematized protocol of the examination. (A) The arrangement of electrodes. (B) The actual view of a preparation. An overall view was captured with a 4x objective, in which the ROI was located (square). (C) A magnified view of the ROI captured with a 60x objective. The image was fed to a PDA having 16 x 16 pixels. Eight pixels fixed in the CA1 SR layer (cross-hatched) correspond to the cross-hatched boxes in Fig. 3. (D) A timetable of the experiment. Operations are shown along with the lapse of time (see Methods for detailed descriptions). In each session, the test stimuli were delivered 4 time at 30 sec intervals and thus acquired recordings were digitally averaged. The star signifies the period immediately after the previous operation.
Fig. 5. The variance in cell density. (A) Confocal microscopic images of CA1 pyramidal cell and dendritic layer of the control (ddFK, left) and FK-treated (right) slices stained with propidium iodide. The height of focal plane was fixed at the middle of slice thickness. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Unit of scale is µm. (B) Deviation in the cell number among the specimens. Each dot represents the cell density of each specimen. Although the mean cell density was unaffected by the drug treatment, the cell density was variant among the specimens.
Fig. 6. The variance in slice thickness. (A) Schematic representation for the thickness measurement, illustrating that the uppermost and bottommost layers are those of glial cells whereas the middle portion of the specimen was rich in neuronal cells. (B) Deviation of the thickness among the specimens. Each dot represents the thickness of each specimen defined by a z-axis length between the first and the last neuronal appearance in that specimen. Although the mean thickness was unaffected by the drug treatment, the specimen thickness was widely variant among the specimens.
Fig. 7. The parallelism between the optical and electrical signals using the antidromic stimulation procedure. Signals accompanied by antidromic stimulations delivered from the electrode placed in the CA1 marginal (alveolar) region. Electrical recordings are shown in the top-left (7 traces on 7 stimuli with varied current strengths are superimposed), optical recordings simultaneously obtained from the pyramidal cell layer are shown in the top-right (arrows indicate the timing of stimulation) and the peak values are plotted in the bottom.
Fig. 8. The correlation between the responses to the orthodromic and antidromic stimulations, validating the method of standardization. (A) The correlation in the optical signals. (B) The correlation in the electrical signals. Each point represents the maximal responses from one specimen. The thicker specimen containing the larger number of neurons should produce the larger signal upon an orthodromic stimulus and the larger signal upon an antidromic stimulus. Thus a parallelism should hold between those signals, when the specimens of various thickness were subjected to measurements.
Fig. 9. Slow fluorescence decay in the antidromic and orthodromic stimulus in the optical signal. Not only did the absolute fluorescence intensity (F) weaken with time, but the stimulus-induced change (ΔF/F) also decreased. The left ordinate gives to the ΔF/F values upon antidromic (closed square) and orthodromic stimuli (closed diamond). The right ordinate gives to the relative ΔF/F values (the value obtained in the first session was taken as 100%).
Fig. 10. Correction and standardization to extract the EPSP signal. In the first row, the left panel is the digital average from 4 runs of recording upon maximal orthodromic stimulation (see Fig. 4D). This signal contained a fast decay component and was corrected by subtraction of the false stimulation recording made immediately subsequently. The result is shown in the right panel (A). In the second row, the same correction was made for the recording of maximal antidromic stimulation in the presence of receptor antagonists (B). The amplitude of the spike signal (b) was used for standardization. The antidromic spike signal is obtained from somatic layer from fear that the space constant along the length of dendrite might change after a drug-treatment (see text). In the third and fourth rows, the same correction/standardization was made for the recording of maximal orthodromic and antidromic stimulation in the presence of receptor antagonists (C, D). By this way, the components of presynaptic spike signal (corresponding to a fiber volley signal in the electrical recording) and EPSP were separated. Note the presence of a slow decay, as seen in comparison between the value of b and d. At the bottom, the standardized EPSP signal thus obtained is shown (E). As understood from this calculation process, the ordinate is free of dimension, nevertheless the comparison is valid.
Fig. 11. Extraction of EPSP signal from the recordings of orthodromic stimulation. (A) The optical signals before (open diamonds) and after (open triangles) the application of receptor antagonists. The signals were corrected for fast and slow decays and standardized using antidromic spike signals (as shown in Fig. 10). This is a representative recording from a control (ddFK-treated) specimen. Reasonably enough, the EPSP component developed several milliseconds later than the presynaptic spike component. (B) A comparison between the EPSP signals from control and FK-treated specimens. The time axis was assorted to make the beginning of electrical stimuli as zero.
**Fig. 12.** Spatial distribution of standardized EPSP signal. The stratum radiatum in the CA1 area was subdivided into 2 layers, proximal and distal ones. Each of 256 (16 x 16) pixels overlaid on the raw microscopic image (left panel) indicates the corresponding PDA pixel. The EPSP signal (extracted as explained in Fig. 10) was embedded in each of the respective pixels in middle (a typical control specimen) and right (a typical FK-treated specimen) panels. The peak magnitude of the EPSP was classified into 3 steps, allocated for a pseudocolor code and painted over each of the pixels. In this examination the objective used was 40x.
**Fig. 13.** FK-induced enhancement of EPSP signals in two dendritic layers. The two-dimensional analysis as depicted in Fig. 12 was made for 5 specimens for each of the ddFK-treated and FK-treated groups and statistical comparison was applied separately for the two layers. Decay correction and cell number standardization were applied. Means ± SEM are shown. Asterisk indicates a statistically significant difference in Student’s t-test (p < 0.05). In this examination the objective used was 40x.
Fig. 14. Relative increase in EPSP after LTP induction. (A) Representative optical EPSP signal 5 min before and 30 min after the theta-burst stimulation. (B) The statistical comparison of the relative increase in EPSP signal in the proximal and distal dendritic layers. Note that the larger increase in the distal layer of the FK-treated cultures than in that of the control cultures. Means ± SEM are shown. Asterisk indicates a statistically significant difference in Student's t-test ($p < 0.05$).