The characteristics of LTP induced in hippocampal slices are dependent on slice-recovery conditions

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In area CA1 of hippocampal slices which are allowed to recover from slicing “in interface” and where recordings are carried out in interface, a single 1-sec train of 100-Hz stimulation triggers a short-lasting long-term potentiation (S-LTP), which lasts 1–2 h, whereas multiple 1-sec trains induce a long-lasting LTP (L-LTP), which lasts several hours. Moreover, the threshold and the features of these LTP depend on the history of the neurons, a phenomenon known as metaplasticity. Here, where all recordings were performed in interface, we found that allowing the slices to recover “in submersion” had dramatic metaplastic effects. In these conditions, a single 1-sec train at 100 Hz induced an L-LTP which lasted at least 4 h and was dependent on protein synthesis. Interestingly, this type of metaplasticity was observed when the concentration of Mg2+ used was 1.0 mM but not when it was 1.3 mM. The LTP induced by four 1-sec trains at 100 Hz was similar whatever the incubation method. However, the signaling cascades recruited to achieve that pattern were different. In the interface–interface paradigm (recovery and recording both in interface) the four-train induced LTP recruited the PKA signaling pathway but not that of the p42/44MAPK. On the contrary, in the submersion–interface paradigm the four-train induced LTP recruited the p42/44MAPK signaling pathway but not that of the PKA. To our knowledge this is the first example of metaplasticity involving the recruitment of signaling cascades in LTP.

Long-term potentiation (LTP) of synaptic transmission is believed to play an important role in encoding memories in neuronal networks (Bliss and Collingridge 1993; Martin et al. 2000). This phenomenon was first discovered on an in vivo preparation (Bliss and Lomo 1973) but the foremost improvements in deciphering its mechanisms were obtained on the in vitro brain slice preparation (Collingridge et al. 1983; Lynch et al. 1983; Frey et al. 1993; Nguyen et al. 1994; Abel et al. 1997; Giese et al. 1998; Kandel 2001).

There are two different techniques to maintain brain slices alive for hours (Dingledine et al. 1980). In the interface-slice preparation, slices are partially submerged in artificial cerebrospinal fluid (ACSF) with the top surface of the slice exposed to a humidified atmosphere of 95% O2 and 5% CO2. In this case the slice gets oxygen that is diffused through the very thin film of liquid covering the slice. In the submerged-slice preparation, slices are completely submerged in ACSF. In this case, the oxygen supply to the slice is provided by the oxygen dissolved in the ACSF. Researchers use one technique or another depending upon the technical constraints of their experiments. For instance, experiments where imaging measurements are performed using a confocal microscope require the use of the submerged-slice preparation. However, these two different techniques can have different effects on cell physiology. For instance, it was recently found that phosphorylation of alpha calcium/calmodulin-dependent kinase II (αCaMKII) at T286, a phosphorylation playing a key role in LTP (Giese et al. 1998), was transiently increased in slices maintained in submersion but was persistently decreased in slices maintained in interface chambers (Ho et al. 2004).

Moreover, there are two different steps in each experiment. After slicing, slices must be left undisturbed (recovery period) for 1.5 h before starting the recordings (recording period). Recovery and recording are not necessarily carried out in the same type of chamber (submersion or interface). For instance, in some studies slices recover in interface and are subsequently tested in a submersion chamber whereas in other experiments recovery and recording are made in submersion. Synaptic plasticity is sensitive not only to the induction stimulus but also to what occurred earlier in the synapse. The fact that synaptic plasticity is dependent on the history of the synapse is known as “metaplasticity” (Abraham and Bear 1996; Abraham and Tate 1997). For instance, inhibition of LTP can be elicited by priming stimulation below threshold for LTP or LTD induction (Christie and Abraham 1992; Huang et al. 1992). Application of the mGluR agonist 1S,3R-aminocyclopentanedicarboxylic acid (ACPD) leads to a facilitation of LTP induction and persistence, as elicited later by a tetanus normally just above threshold for LTP induction (Cohen and Abraham 1996). It is even possible that a stimulation, which alone has no effect on synaptic plasticity, inhibits the late phase of LTP, a phenomenon called “silent metaplasticity” by Woo and Nguyen (2002). Thus, it could be that the type of chamber used for having the slices recovered (submersion or interface) would influence the characteristics of the LTP subsequently induced in interface conditions. Here we found this to be the case.

Results

Facilitation of induction of long-lasting LTP by recovery in submersion
When the experiments were performed in ACSF containing Mg2+ at a concentration of 1 mM, the way in which the slices were allowed to recover (submersion or interface) had a dramatic influence on the duration of the LTP induced by a single train of high-frequency stimulation (100 Hz, 1 sec) (Fig. 1 A,B).

When both recovery and recording occurred in interface, one train elicited an initial potentiation of the slope of the field excitatory postsynaptic potential (fEPSP) reaching 177 ± 9% (mean ± SEM). Four hours later, it was reduced to 111 ± 7% of
The duration of the LTP, measured by the time at which the increase of the fEPSP slope ceased to be different from the baseline, was 1 h 30 min (Fig. 1B). By contrast, when slices first recovered in submersion and were subsequently studied in the interface condition, a single train induced a strong and long-lasting LTP. The initial potentiation was of 268 ± 20% over the baseline, a level not significantly different from pre-induction level (P = 0.09). The duration of the LTP, measured by the time at which the increase of the fEPSP slope ceased to be different from the baseline, was 1 h 30 min (Fig. 1B). By contrast, when slices first recovered in submersion and were subsequently studied in the interface condition, a single train induced a strong and long-lasting LTP. The initial potentiation was of 268 ± 20%. Over the next 10 subsequent min it diminished to 199 ± 14% and then remained nearly stable, reaching 189 ± 19% 4 h after LTP induction, a value significantly higher than that measured in the interface-interface paradigm (111 ± 7%, P < 0.05).

This effect was not related to a change in the general excitability of the neurons. The amplitudes of the baseline fEPSPs were similar in both conditions (1.3 ± 0.1 mV after recovery in submersion and 1.4 ± 0.1 mV after incubation in interface, P = 0.6), as were the input-output curves. The width of the fEPSPs were also similar across the two conditions (9.1 ± 0.4 msec in the submersion-interface paradigm vs. 9.2 ± 0.6 msec in the other paradigm, P = 0.9).

Surprisingly, the difference induced by the incubation method was not observed when the experiments were carried out in a medium where the concentration of Mg²⁺ was 1.3 mM (Fig. 1C). In such a medium, the LTP measured 4 h after induction was not different from baseline, whether recovery occurred in interface (120 ± 14%, P = 0.47) or in submersion (100 ± 9%, P = 0.18). This shows that a small modification in the concentration of Mg²⁺ can have a great influence on certain biological responses.

Properties of the facilitated L-LTP

We then studied the properties of the L-LTP elicited, surprisingly, by a single train in the experimental conditions used here (submersion-interface/Mg²⁺: 1 mM). When slices were bathed in a solution containing cycloheximide (CHX), an inhibitor of protein synthesis, from 30 min before to 45 min after LTP induction, the late phase of potentiation was hindered (Fig. 2A). Under the influence of cycloheximide (80 µM), the LTP measured 4 h after induction was reduced to 135 ± 16% (mean ±SEM), a level different from that measured in control slices (P < 0.05) and not different from the basal level (P = 0.11).

The long-lasting LTP induced by a single train in our experimental conditions was thus dependent on protein synthesis. However, it was not dependent on the signaling pathway of PKA (Fig. 2B) but on that of ERK (or p42/44MAPK) (Fig. 2C). When slices were submitted to KT5720 (1 µM), an inhibitor of PKA, the LTP triggered by one train was 162 ± 21% 4 h after induction, a level not different from that measured in control slices (P = 0.35) and different from baseline (P < 0.05). By contrast, when slices bathed in a medium containing U0126 (20 µM), an inhibitor of MEK, which is the kinase that activates ERK specifically, the LTP measured 4 h after induction (133 ± 16%) was significantly lower than the control LTP (P < 0.05) and not significantly different from the baseline (P = 0.11).

Signaling pathways involved in the facilitated L-LTP

In a subsequent series of experiments, we explored the potential role of different signaling pathways in the dramatically increased duration of the LTP induced by one single train after allowing the slices to recover in submersion. With such a goal in mind, slices were submitted to different pharmacological agents during the recovery period (Fig. 3). It was found that the positive metaplasticity induced by the recovery in submersion was impaired when p38MAPK was inhibited by SB203580 (10 µM), by MCPG (500 µM), an inhibitor of metabotropic glutamate receptors, and when Cs (1 mM) was applied. Four hours after its induction, LTP was as low as 115 ± 13%, 113 ± 13% and 115 ± 12% when the slices were submitted to the influence of SB203580; MCPG; and Cs, respectively. Each of these three levels were lower than that of control LTP (P < 0.05).

By contrast, no de novo protein synthesis was involved in the metaplasticity phenomenon described above. Protein syn-
thesis was inhibited by cycloheximide (40 µM) during the recovery period. In these conditions, LTP was not different from control value 4 h after induction (158 ± 19%, $P = 0.24$). Similarly, bathing the slices during recovery in a medium containing either APV (50 microM), an inhibitor of the NMDA receptor, or U0126 (20 µM), an inhibitor of MEK, did not suppress the type of metaplasticity shown in Figure 2. Four hours after induction, the mean LTP was lower than that of the control (149 ± 25% for APV and 132 ± 26% for U0126) but these differences were not statistically significant ($P = 0.22$, $P = 0.09$).

Influence of recovery in submersion on the recruitment of signaling pathways in LTP

It is known that distinct signaling cascades can be recruited in function of the different induction paradigms. Thus, we decided to investigate whether a change in the recovery procedure (interface vs. submersion) could modify the recruitment of the various signaling cascades in a paradigm using a stronger induction stimulus, such as four trains separated from each other by an interval of 5 min (Fig. 4).

Figure 2. The L-LTP induced by a single train of HFS in a slice that had recovered in submersion is dependent on protein synthesis and on ERK (p42/44MAPK) but not on PKA. (A) Time course of fEPSP slope 30 min before and 4 h after a 1-sec train in the control situation (●) and in presence of cycloheximide (CHX), an inhibitor of protein synthesis (○). The potentiation was significantly reduced 4 h after the train when cycloheximide was present around the time of induction (black bar). (B) Time course of fEPSP slope in presence of KT5720, an inhibitor of PKA (○). The potentiation was not different from the control (●). (C) Time course of fEPSP slope in presence of U0126, an inhibitor of MEK (○). The potentiation 4 h after the train was significantly lower than the control potentiation (●). All the experiments presented in this figure were carried out at a Mg$^{2+}$ concentration of 1 mM.

Figure 3. The metaplastic phenomenon induced by the in-submersion recovery of the slices (L-LTP induced by one train) is blocked by an inhibitor of p38MAPK (SB203580), an inhibitor of metabotropic glutamate receptors (MCPG) and cesium but not by inhibitors of MEK (U0126), of NMDA receptor (APV) and of protein synthesis (CHX). (A) Time course of fEPSP slope 30 min before and 4 h after the train of HFS in the control situation (●) and when SB203580 was applied during the recovery period in submersion. The potentiation of the fEPSP slope was significantly reduced under the influence of SB203580. Inset: examples of fEPSP recorded in one experiment just before (a, c) and 4 h after (b, d) induction in the control situation (left) and when SB203580 was applied (right). Traces a and b were recorded in control situation; traces c and d were recorded after the slice had recovered in submersion in presence of SB203580. (B) Percentage of potentiation of the fEPSP slope 4 h after induction in the control situation (black column) and after application of different drugs during the recovery period occurring in submersion (white columns). The number of experiments ($n$) is indicated on the top of each column. The level of potentiation measured 4 h after induction was significantly lower than in the control situation when slices were submitted to the action of SB203580, MCPG or cesium. An asterisk points to a statistically significant decrease. NS = not statistically different. All the experiments referred to in this figure were carried out at a Mg$^{2+}$ concentration of 1 mM.

Learning & Memory 273
www.learnmem.org
Because lowering the concentration of Mg\(^{2+}\) to 1 mM was proven to be essential for the metaplasticity phenomenon described here, all the experiments in this section were performed using this concentration of Mg\(^{2+}\).

When recovery occurred in interface, the L-LTP induced by four trains was dependent on the PKA signaling pathway and not on the p42/44MAPK pathway. On the contrary, when recovery took place in submersion, the L-LTP was sustained thanks to the p42/44MAPK cascade, which is blocked by U0126, in the presence of APV (218 \(\pm\) 4% vs. 94 \(\pm\) 10% in submersion when submitted to U0126 (118 \(\pm\) 10% \(\text{P} < 0.05\)). All the experiments gathered in this figure were performed at a Mg\(^{2+}\) concentration of 1 mM.

### Discussion

Synaptic plasticity is sensitive not only to the “state” imposed by extra factors at the moment of LTP induction (Gelinas and Nguyen 2005), but also to the state created by certain factors prior to the induction (Abraham and Bear 1996; Abraham and Tate 1997). Here, we found that prior incubation in either submersion or interface conditions affected the nature of LTP subsequently induced in interface.

1. When the recovery of the slice occurred in submersion, a single train of 100-Hz stimulation triggered an L-LTP instead of producing an S-LTP.
2. The L-LTP induced either by four electrical trains or application of FSK/IBMX recruited different signaling cascades in function of the type of recovery.

### Increased persistence of LTP

The increase in persistence of LTP induced by prior incubation in submersion was not blocked by APV but it was by MCPG. Thus, contrary to a classic metaplastic effect (i.e., stimulation of a synapse at frequencies ranging from 5 to 30 Hz causes a dramatic reduction in subsequently evoked LTP), which is mediated by
NMMA receptors (Huang et al. 1992; O’Dell and Kandel 1994; Zhang et al. 2005), ours involved an MCPG-sensitive receptor.

The known role of metabotropic glutamate (mGl) receptors in LTP is complex. MCPG, an mGl receptor antagonist, blocks LTP induction (Bashir et al. 1993). However, this inhibitory effect of MCPG can be suppressed by a 1-sec train stimulation applied hours earlier (Bortolotto et al. 1994). In this type of metaplasticity, the molecular switch involved is also set by the activation of an mGl receptor since it is antagonized by MCPG. However, since the potent broad spectrum (mGl1-8) mGl receptor antagonist LY341495 blocks the setting of the molecular switch but not the induction of LTP per se, the receptors involved in these two effects must be different (Fitzjohn et al. 1998). MCPG has been found to block the setting of the molecular switch by activating the mGl5 receptor whereas it prevents LTP induction by turning on a not yet identified mGl receptor (Bortolotto et al. 2005). Moreover, LTP persistence can be increased by previous activation of group I mGl receptors by the selective agonist DHPG (Cohen et al. 1998; Raymond et al. 2000). Here, we found that the persistence of the 1-sec train triggered LTP induced by the recovery of the slices in submersion was suppressed by MCPG. The precise receptor involved remains to be identified. It could be one of the known mGl receptors expressed in the CA1 region and blocked by MCPG, which are mGl1 and mGl5 receptors (Shigemoto et al. 1997; Mannaioni et al. 2001) or the not yet identified MCPG-sensitive mGl receptor.

When applied during the recovery period, cesium prevented the metaplasticity induced by the incubation conditions. Cesium is known to block several types of potassium channels: delayed rectifiers (Kv), K+ channels, big and small Ca++-activated K+ channels (BK and SK), inward rectifier K+ channels (Kir) (Hille 1992). Each of these K+ channels tends to dampen neuronal excitability. Moreover, cesium also blocks Ih channels (Hille 1992). Like Kir channels, Ih channels open at negative potentials. Unlike Kir, however, they are as permeable to Na+ as to K+ ions. Ih channels also influence neuronal excitability but not in the same way as K+ channels. Because they let through an inward current when open, the effect of Ih channels is to initiate slow depolarization if the membrane potential has become very negative. Facing the multiple blocking patterns cesium can take, it is difficult to specify its way of acting on our metaplasticity effect. However, it cannot operate by inducing change in neuronal excitability still persistent at the time of LTP induction. Indeed, at that time, the amplitudes of the baseline fEPSP are similar, as are the input-output curves across the two conditions (submersion or interface). A change in synaptic inhibition seems not to be involved either, as indicated indirectly by the fact that the width of the fEPSPs is equal across the two conditions.

The signaling pathway through which SB203580, an inhibitor of p38MAPK was able to block our metaplastic effect is not known. However, interestingly, p38MAPK is known to be activated via mGl receptors (Thomas and Huganir 2004).

**Importance of Mg++ concentration**

It is remarkable that the concentration of Mg++ used is critical in permitting the metaplastic effect described here. It develops when Mg++ concentration is 1.0 mM but not when it is 1.3 mM.

All the more surprising is that a drastic decrease of Mg++ concentration has the opposite effect. After a transient 30-60 min removal of Mg++, no LTP can be elicited for 2-3 h (Hsu et al. 2000). It seems that this suppression is mediated by the Ca++ influx through the NMDA receptors and through the voltage-dependent Ca++ channels (VDCC) activated in the seizure-like activity induced by the Mg++-free condition. In our observations, NMDA receptors are certainly not involved, as application of APV during the recovery of the slices does not suppress the effect. Mg++ concentration of 1 mM is important in our paradigm but is not a general condition for observing metaplasticity. While some metaplasticity phenomena were shown in slices bathed in ACSF containing 1.0 mM Mg++, others were observed in presence of Mg++ at a concentration of 1.3 mM (Raymond et al. 2000; Woo and Nguyen 2002).

**Recruitment of signaling cascades**

It was already known that different induction protocols recruited different signaling cascades (Woo et al. 2003). In mice, for instance, LTP induced by a single high-frequency train is independent of p42/44MAPK (Winder et al. 1999). In contrast, LTP induced by multiple high-frequency trains, theta frequency, or forskolin requires p42/44MAPK (English and Sweatt 1997; Winder et al. 1999; Patterson et al. 2001; Rosenblum et al. 2002). Here, we described a first example of metaplasticity involving the recruitment of signaling cascades. In LTP induced by four electrical trains it is possible that a role is played by the pathway through which the intracellular concentration of Ca++ increases. The LTP triggered after recovery in interface is strictly NMDA-dependent; that induced after incubation in submersion is only partly NMDA-dependent. However, other factors must also play a role. Indeed, when FSK and IBMX are applied, the way to increase the
intracellular concentration of the secondary messenger (cAMP) involved is the same across the two conditions.

Characteristics of the used submersion procedure

Here we found that the conditions of incubation (submersion or interface) had a profound influence on the LTP triggered when the slice was maintained in interface. It would have been interesting to see what kind of LTP was generated in the submerged condition after recovery in submersion or interface. However, this experiment could not have been done in symmetrical conditions because of the way the submersion was done in this work. Actually, just after slicing, the slices were placed and maintained in an interface chamber. To obtain submersion conditions, the level of perfusion liquid was raised a few millimeters above the grid. At this stage, it cannot be excluded that our submersion should be done with caution.

The second finding of this paper concerning the recruitment of the signaling cascades has more conceptual implications. The cell’s use of this or that particular signaling cascade to do something at a given moment (LTP in this case) depends on the history of that cell.

Implications

Our results demonstrate that it is possible to dramatically increase the sustaining of LTP by allowing the slices to recover in submersion instead of in interface. Of course, this finding does not question the invaluable aspect of the acute brain slice preparation in LTP and memory research. However, it draws attention to the fact that comparing results obtained in interface and in submersion should be done with caution.

Materials and Methods

Animals

Male C57BL/6 mice, aged 6–10 wk (Charles River) were used for all the experiments, which were carried out in accordance with National Institutes of Health regulations for the care and use of animals in research and with local ethics committee guidelines.

Electrophysiology

Transverse hippocampal slices (400-µm thickness) were prepared as described by Nguyen and Kandel (1997). The hippocampus was isolated and sliced with a McIlwain chopper. Slices were allowed to recover at 28°C for 1.5 h either in submersion or in interface. The rate of flow of the perfused liquid was 2 mL/min in submersion and 1 mL/min in interface. After this recovery period all the recordings were made in an interface chamber (FST) at 28°C. Baseline recording started 30 min later and lasted 30 min. Then LTP was induced either electrically or chemically. Slices were perfused with ACSF with the following composition: 124 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.2 mM NaH₂PO₄, 2.4 mM CaCl₂, either 1.3 or 1 mM MgSO₄, 10 mM glucose. The ACSF was aerated with 95% O₂ and 5% CO₂. Extracellular fEPSPs were recorded with a glass microelectrode (2–5 Mohm, filled with ACSF) positioned in the stratum radiatum of area CA1. A bipolar nickel–chromium stimulating electrode was used to elicit fEPSPs by stimulation of the Schaffer collateral fibers. Stimulation intensity (0.08 msec pulse duration) was adjusted to elicit fEPSP amplitudes that were around 40% of maximum size. Basal synaptic transmission was assessed by stimulating Schaffer collaterals once per minute at this test stimulation intensity. Slices that showed maximal fEPSPs <2 mV were rejected.

LTP was induced electrically by applying either a single 1-sec train (100 Hz, at test strength) or four 1-sec trains (100 Hz, test strength) spaced 5 min apart. LTP was also induced chemically by applying 50 µM forskolin combined with 30 µM IBMX for 15 min. 2-amino-5-phosphonopentanoic acid (APV, Acros organics) was prepared as a concentrated stock solution in H₂O. Cycloheximide (CHX, Sigma), anisomycin (ANL, Sigma), forskolin (FSK, Alomone Labs), and IBMX (Sigma) were all prepared as concentrated stock solutions in dimethyl sulfoxide (DMSO) and then diluted to 0.01% in ACSF to give their final concentrations.
Slice recovery and metaplasticity of LTP

Data analysis

For each slice, the fEPSP slopes were normalized against the average slope over the 30 min before LTP induction. To determine whether or not the normalized fEPSP of a group of slices submitted to the same experimental conditions was significantly potentiated (P < 0.05), the baseline percentages measured just before the treatment (4 h unless otherwise specified) after the end of the treatment were compared using a paired Student’s t-test. The statistical significance (P < 0.05) of the difference in increase of the fEPSP measured 4 h after two different treatments applied to two distinct groups of slices was assessed using a Student’s t-test.

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