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# Persistent LTP without triggered protein synthesis

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ABSTRACT

Protein synthesis is believed to be involved in stabilizing synaptic plasticity. Effects lasting longer than about 2–3 h are considered to require synthesis of new proteins, implying a functional separation between early (E) and late (L) components. However, the issue of constitutive vs. new protein synthesis is still unclear, especially in young animals. Here, we examined the effects of two protein synthesis inhibitors, anisomycin and emetine, on long-term-potentiation (LTP) in CA1 area of hippocampal slices from 12- to 20-day-old rats. Either drug was applied from −30 min to +30 min with respect to LTP induction, a time window previously reported to be critical. However, the LTP remained stable under the entire recording period of 4 h (anisomycin), or 8 h (emetine). Proper preparation of emetine solution was evidenced by the fact that, in separate experiments, prolonged treatment with emetine gradually blocked baseline responses. Although no corresponding effect was observed with anisomycin, the drug was judged to be potent by its ability to inhibit yeast growth. The ability of anisomycin to inhibit protein synthesis was further confirmed by radiolabeling experiments assessing the degree of leucine incorporation. Our data suggest that LTP up to at least 8 h is not dependent on triggered protein synthesis but can be attained by utilizing proteins already available at induction time.


## 1. Introduction

Long-term potentiation (LTP) is an important form of synaptic plasticity believed to be involved in mammalian learning and memory. The main form of LTP is NMDA-dependent and obeys Hebb's rule of association (Collingridge et al., 1983; Harris et al., 1984; Wigström and Gustafsson, 1986). In analogy with memory, LTP is composed of different temporal phases. Most commonly, a division is made between an early and a late phase, based on the need for *de novo* protein synthesis to establish LTP that lasts longer than a few hours (Stanton and Sarvey, 1984; Frey et al., 1988; Otani et al., 1989; Huang and Kandel, 1994). It is conceived that the early phase of LTP (E-LTP), which lasts for up to about 3 h, relies on post-translational modifications of pre-existing proteins. The following, later phase (L-LTP) is believed to depend on protein synthesis triggered by the LTP-inducing stimulation. Additionally, the late phase has been shown to be related to activation of protein kinase A (PKA) although an intermediate phase of LTP may also exist, which depends on PKA but does not require protein synthesis (Winder et al., 1998).

An essential issue is whether the division of LTP into phases, based on protein synthesis requirements, is coupled to a corresponding difference in expression mechanisms in terms of separate synaptic modifications. Indirect evidence for a change of expression mechanism comes from experiments showing that saturation of LTP prevents further LTP at 2 h but not at 4 h after the first induction (Frey et al., 1995). It has also been proposed that synapses that express E-LTP are equipped with a "tag" that allows them to capture the essential proteins later on and so attain L-LTP properties (Frey and Morris, 1997; Barco et al., 2002). This hypothesis can explain how proteins synthesized in, e.g. the soma can be directed to the proper synapses in the dendrites and so providing "input specificity". Little is known about the nature of these tags and about the difference between synapses before and after capturing the proteins. Other works suggest that the proteins necessary for L-LTP may also be synthesized locally via available mRNA at synaptic sites (Steward and Levy, 1982; Steward, 1994; Aakalu et al., 2001; Ostroff et al., 2002), providing an alternative explanation for the specificity, but without providing any clue to the changes that synapses undergo in the transition from E-LTP to L-LTP.

In recent work in our laboratory, the issue of the E-LTP to L-LTP conversion was addressed and LTP was examined with respect to

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the relative involvement of changes in AMPA- and NMDA-receptor mediated responses at two different times after LTP induction, 1 h and 4 h (Dozmorov et al., 2006). The study demonstrated an AMPA/NMDA potentiation ratio of near 2:1, being the same at both time points and so failing to disclose a difference in expression between E-LTP and L-LTP. It can be noted that the mentioned study was based on work in young animals, aged 2–3 weeks, whereas in most other studies on E-LTP vs. L-LTP, animals older than 5–7 weeks were used. It might be expected that protein metabolism be different in young animals, including synthesis as well as degradation. Using this reasoning as a starting point, we wondered whether persistent LTP might be based on existing proteins under conditions of a plentiful supply. This question is of principal interest in view of the predominating model that the necessary proteins for LTP are synthesized on demand as a result of the inducing stimulation. We conjectured that using young animals should optimize chances to observe the hypothesized sufficiency for constitutive proteins in persistent LTP. In fact, in 2- to 3-week-old rats, we found that applying protein synthesis inhibitors around induction time did not affect any part of LTP for recording times of 4–8 h after induction. Two different inhibitors were used, anisomycin and emetine. Control experiments verified that the negative results were not due to inefficient application of drugs. Our work suggests that, within the limitations of the current experimental protocol, persistent LTP is possible without triggered protein synthesis, using proteins that are constitutively synthesized and present all the time.

## 2. Material and methods

### 2.1. Preparation

Experiments were performed on 12- to 20-day-old Sprague–Dawley rats. The animals were decapitated after initial isofluran (Forene) anesthesia, the hippocampus was dissected out and transverse 400 μm thick slices were prepared by either a vibratome or tissue chopper. All possible steps were taken to minimize animal suffering. The procedures conformed to the guidelines of the Swedish Council for Laboratory Animals and were approved by the Local Ethics Committee of Gothenburg University. The slices were allowed to recover for about 1 h at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 26 $NaHCO_3$, 1.0 $NaH_2PO_4$, and 10 D-glucose, and equilibrated with 95% $O_2$, 5% $CO_2$. Slices were transferred as needed to a submerged-type recording chamber with the same ACSF composition except for 2.5 mM $CaCl_2$ and 1.3 mM $MgCl_2$, and a temperature of 30–31 °C.

### 2.2. Slice electrophysiology and analysis

Field excitatory postsynaptic potentials (fEPSPs) were recorded from the apical dendritic layer in area CA1 using glass micropipettes filled with 1–3 M NaCl (resistance 3–8 MΩ). Stimulation was delivered as 100 μs negative constant current pulses using monopolar tungsten electrodes (0.1 MΩ). Two stimulation electrodes were placed in the dendritic layer on opposite sides of the recording electrode. Pulses were delivered every 10 s or 30 s, alternating between electrodes in order to get fEPSP measurements of both inputs. Stimulus strengths (20–40 μA) were adjusted between the pathways to initially evoke similar responses, as measured by the recording electrode. The LTP-inducing stimulus was either a train of high frequency stimulation, HFS (100 impulses at 100 Hz), or theta-burst stimulation, TBS (10 bursts of four pulses at 100 Hz, repeated with a burst frequency 5 Hz). For LTP induction, three such HFS or TBS were given, normally separated by 20 s. HFS was used in emetine experiments whereas anisomycin experiments used either HFS or TBS. All comparisons between groups were balanced with respect to the induction protocols involved. In some cases, TBS were separated by 15 min. Drug application started 30 min before the first HFS/TBS and was maintained until 30 min after the last one, implying that total time of application was normally 60 min, and in some cases 90 min. The duration of drug application is consistent with the reported critical time for new protein synthesis being 15–30 min after LTP induction (Stanton and Sarvey, 1984; Otani et al., 1989; Scharf et al., 2002).

Signals were amplified, filtered, digitized and transferred to a PC computer for on-line and off-line analysis. The fEPSPs were estimated as initial slope starting after the presynaptic volley or as integrated amplitude relative to the prestimulus baseline, yielding essentially the same results. LTP was quantified by comparison with the control input, which was not subjected to tetanization. This tends to eliminate unspecific drug effects or changes associated with declining slice viability. Statistical comparisons were made using Student's *t*-test. Results are expressed as mean ± S.E.M. Anisomycin and emetine were purchased from Sigma–Aldrich (St. Louis, MO, USA); CNQX and AP5 were from Tocris (UK).

### 2.3. Yeast assay

Yeast strain W303-1A of *Saccharomyces cerevisiae* species was used to test an effect of anisomycin on yeast growth. Yeast cells were routinely grown in medium containing 1% yeast extract and 2% peptone supplemented with 2% D-glucose as a carbon source (YEPD). Plate growth assays were performed by pregrowing the cells in YEPD liquid medium. Cells were resuspended in the same medium to an optical density ($OD_{600}$) of 1.0. A 10-fold serial dilution of this culture was made and 5 μl of each dilution was spotted onto agar YEPD plates. While one YEPD agar plate was used as control the other was supplemented with 25 μM anisomycin. Yeast growth was monitored after 2–3 days at 30 °C.

### 2.4. Leucine incorporation

The level of inhibition of protein synthesis in slices was measured by incorporation of leucine into trichloracetic acid (TCA) precipitable macromolecules (Lipton and Heimbach, 1977). Four slices were used for testing in each experiment, thus being subjected to the protein synthesis inhibitor (group A); another four slices were used as control, leaving protein synthesis intact (group B). Slices were randomly assigned to group A or group B. Treatment was carried out in a set of submerged-type chambers with oxygenated ACSF at 30–31 °C. Both groups of slices were treated by tritiated leucine (Amersham, Buckinghamshire, UK), final concentration 0.5–1 μCi/ml, incorporation of which was terminated by washing out the isotope in ACSF and placing slices in NaOH (5 mM). After protein purification, leucine incorporation was measured in a scintillation counter. Percentage inhibition of leucine incorporation produced by drug treatment was calculated by comparing counts in treated slices with those of control slices.

## 3. Results

### 3.1. Test of anisomycin on LTP

To test the effect of the protein synthesis inhibitor anisomycin, experiments were run with the drug present during LTP induction (by HFS or TBS), interleaved with the same number of control experiments exposed to the same protocol but without the drug. Anisomycin was delivered in ACSF at a concentration of 25 μM