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# Long-Term Potentiation in Cultures of Single Hippocampal Granule Cells: A Presynaptic Form of Plasticity

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## Summary

**We have explored the mechanisms of mossy fiber long-term potentiation (LTP) at autapses in single-cell cultures of guinea pig hippocampal dentate granule cells. L−AP4-sensitive, but not insensitive, cells responded to a brief tetanus with a sustained potentiation in the synaptic responses. The induction of this LTP appeared identical to that observed in hippocampal mossy fiber synapses in situ, in that it required a rise in presynaptic $Ca^{2+}$ and activation of protein kinase A. Its expression also appeared to be presynaptic and was due, at least in part, to events that occurred after the entry of $Ca^{2+}$ and to the switching on of previously silent release sites.**

## Introduction

In the hippocampus, two distinct forms of synaptic plasticity have been identified (Nicoll and Malenka, 1995). The N-methyl-D-aspartate receptor-(NMDAR) dependent form of long-term potentiation (LTP) occurs at excitatory synapses throughout the brain. While the induction of this form of LTP requires a rise in postsynaptic $Ca^{2+}$, the site of expression remains controversial (Bliss and Collingridge, 1993; Larkman and Jack, 1995; Nicoll and Malenka, 1995). The synapses formed by mossy fibers onto CA3 pyramidal cells exhibit an LTP that is entirely independent of NMDARs (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990). In earlier studies, considerable disagreement existed regarding the site involved in the induction of this form of LTP. Previous results from our lab (Zalutsky and Nicoll, 1990, 1992) supported a presynaptic locus for induction, while results from others (Derrick and Martinez, 1994; Jaffe and Johnston, 1990; Johnston et al., 1992; Williams and Johnston, 1989) favored a postsynaptic locus. There was also disagreement on whether this form of LTP is expressed pre- or postsynaptically (Staubli et al., 1990; Yamamoto et al., 1992; Zalutsky and Nicoll, 1990). However, more recent evidence favors a model in which both the induction (Castillo et al., 1994; Ito and Sugiyama, 1991; Katsuki et al., 1991; Langdon et al., 1995) and the expression (Weisskopf and Nicoll, 1995; Xiang et al., 1994) of mossy fiber LTP are presynaptic.

Much of the confusion that has existed in this field can be attributed to the complexity of the circuitry in the CA3 region of the hippocampus (Claiborne et al., 1993; Johnston et al., 1992; Weisskopf and Nicoll, 1995). Thus, it would be of considerable value if it were possible to demonstrate this form of LTP in the absence of this complex circuitry. The proposal that mossy fiber LTP is both induced and expressed presynaptically predicts that it should be possible to elicit LTP at these synapses by activating a single granule cell, the axons of which form mossy fibers in situ, and that the type of cell contacted by these axons should be irrelevant.

To test these predictions, we have taken advantage of the observation that when a single neuron is grown on a small island of glial cells, the neuron forms synapses onto itself, referred to as autapses (Segal and Furshpan, 1990). This single cell culture system has proved extremely valuable in elucidating basic mechanisms involved in synaptic transmission. Thus, the demonstration of LTP at autapses made by dentate granule cells would not only confirm the simple nature of this form of LTP, hypothesized from the considerably more complex slice preparation, but would also provide a powerful system for dissecting the molecular mechanisms involved in this form of synaptic plasticity. Therefore, we have examined the possibility of establishing LTP in single granule cells in culture.

## Results

### Selective Expression of LTP in L−AP4-Sensitive Cells

We initially cultured neurons from the dentate gyrus of the rat. These cells were found to form autapses, and in approximately 30% of cells, tetanic stimulation did result in an APV-resistant sustained enhancement of the EPSC. However, based on visual inspection, we were unable to distinguish granule cells from nongranule cells. This posed a significant problem, since to study mossy fiber LTP in culture it was essential to have a means of reliably identifying granule cells. Toward this end, we took advantage of a pharmacological finding in guinea pig slices, in which the metabotropic glutamate receptor agonist L−AP4 blocks the release of glutamate from mossy fiber synapses, but has no effect on the associational/commissural synapses made by the axons of CA3 pyramidal cells (Lanthorn et al., 1984; Yamamoto et al., 1983). Therefore, we cultured neurons from the dentate region of guinea pig embryos. While we were still unable to visually identify granule cells, the neurons could be unambiguously divided into two functionally independent groups. Figure 1 shows examples of photographs of two neurons in culture. Neither neuron has features similar to those of granule cells in situ, and both neurons formed autapses. However, there was a striking difference between these cells in terms of the sensitivity of their synaptic responses to L−AP4. In one cell, the NMDAR-mediated EPSC was greatly reduced (Figure 1A2), while in the other, L−AP4 had no effect (Figure 1B2). These results show that it is possible to establish autapses on dentate neurons in culture, and that it is also possible to separate these cells into two groups, based on the ability of L−AP4 to inhibit their synaptic responses. We also found that synaptic responses of L−AP4-sensitive cells were inhibited by the

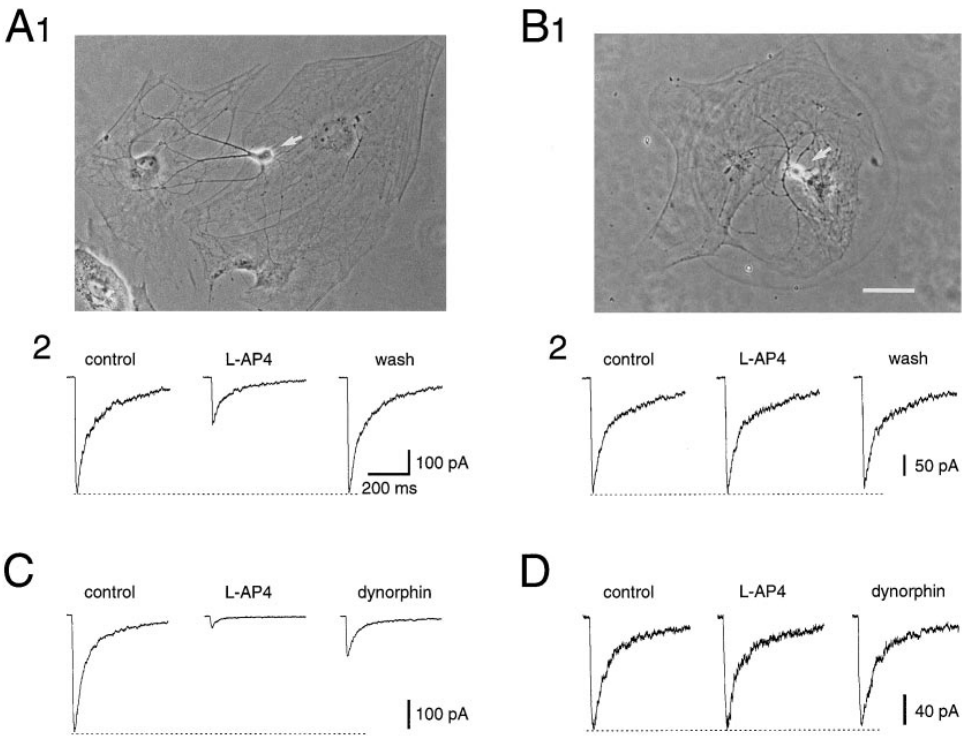


Figure 1. L−AP4 Divides Cultured Dentate Neurons into Two Functionally Independent Groups

(A1) Photograph of a L−AP4-sensitive neuron (white arrow). (A2) The autaptic NMDAR EPSCs recorded from this neuron. L−AP4 (30 μM) reduces the NMDAR EPSC to 40% of control in this neuron.

(B1) Photograph of a L−AP4-insensitive neuron (white arrow). Scale bar: 50 μm.

(B2) In this neuron, the NMDAR EPSC is not affected by application of L−AP4 (30 μM). Both neurons are 13 days old.

(C) The synaptic input of L−AP4-sensitive neurons was also inhibited by the opioid peptide dynorphin (300 nM).

(D) The synaptic input of L−AP4-insensitive neurons was not affected by dynorphin. All records are averages of 8–14 responses. Responses in (A)–(D) are from four different neurons. Holding potential: −70 to −85 mV.

opioid peptide dynorphin, similar to mossy fiber synapses in guinea pig slices (Weisskopf et al., 1993). Dynorphin was inactive on L−AP4-insensitive cells and also inactive in cultured rat dentate neurons (cf. Salin et al., 1995). For simplicity, we will refer to the L−AP4-sensitive neurons as granule cells and their synapses as mossy fiber synapses, although it is clear that the target of these synapses differs from those in situ. The L−AP4-insensitive cells will be referred to as nongranule cells.

In most cells in this study, the small size of the autaptic current made it difficult to separate clearly the fast AMPAR-mediated component of the EPSC from the unclamped action potential used to evoke the synaptic response. Since all autaptic responses contained a slower component mediated by NMDARs, in most experiments, the charge transfer of the NMDAR EPSC was used to monitor synaptic strength.

Initially, we examined the consequence of repetitive high frequency synaptic activation on the strength of synaptic transmission. Figure 2A shows a typical experiment in which after establishing the presence of autapses and their sensitivity to L−AP4, depolarizing voltage pulses (1 ms duration, 50 Hz for 1 s applied four times) were given in the presence of the NMDAR antagonist D-AP5. It is clear that the synapses in this cell were sensitive to L−AP4, and that the size of the synaptic response increased following the tetanus and remained elevated for the duration of the experiment. Figure 2B shows an identical experiment carried out on a cell that had no sensitivity to L−AP4. In this case, only a brief potentiation was seen following the tetanus.

A number of such experiments were performed and the summary of these is shown in Figure 2C. Cells sensitive to L−AP4 showed a large potentiation immediately after the tetanus, followed by a sustained potentiation of approximately 2-fold. The potentiation was seen in 18 out of a total of 23 granule cells. In contrast, the L−AP4-insensitive cells showed a much smaller potentiation after the tetanus that lasted only a few minutes. A long-lasting potentiation (>10 min) was not observed in any of the 18 nongranule cells. Thus far, all of the experiments reported were done in the presence of CNQX. However, a similar D−AP5-resistant LTP could also be elicited when the AMPAR EPSC was monitored (n = 12) (Figure 2D).

**Mechanisms of Induction**

The induction of LTP in single granule cells was unaltered by a number of glutamate receptor antagonists, including the NMDAR antagonist AP5 (n = 23), the AMPAR antagonist CNQX (n = 7), and the metabotropic glutamate receptor antagonist MCPG (n = 2). In addition, the opioid receptor antagonist naloxone had no

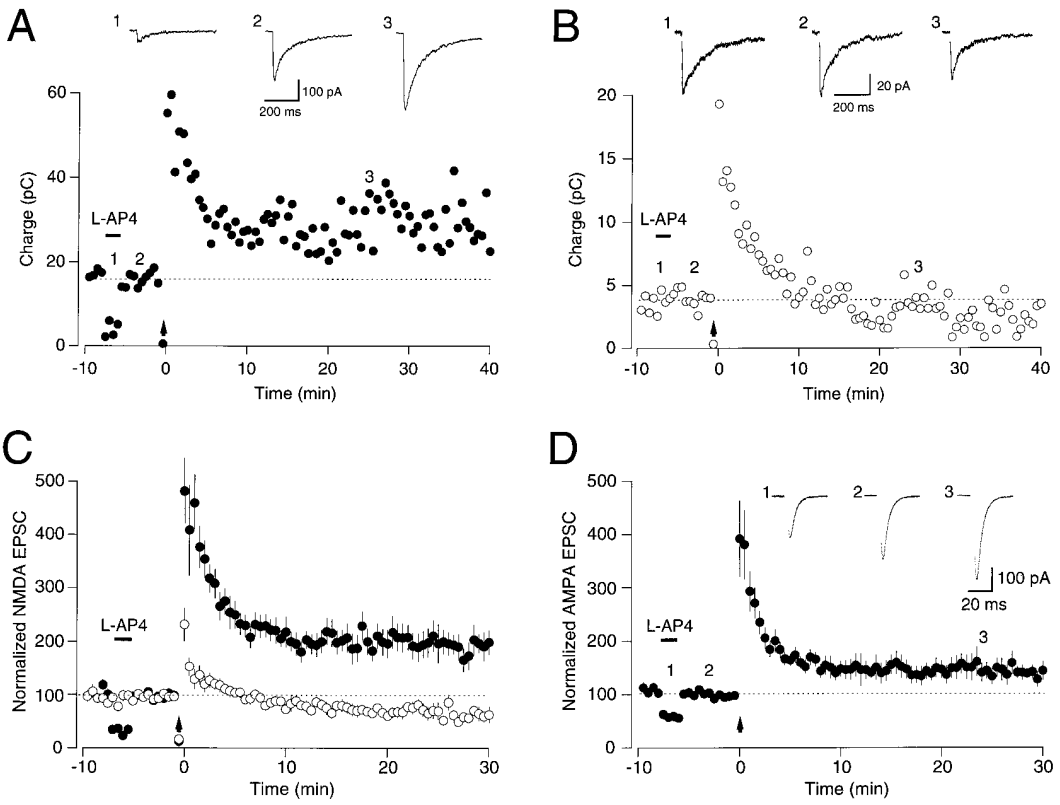


Figure 2. LTP of Autaptic EPSCs in Cultured Dentate Neurons

(A) Example of LTP of autaptic NMDAR EPSCs induced by tetanic stimulation (arrow) in a L−AP4-sensitive cell. Tetanic stimulation was given in the presence of 1 mM $Mg^{2+}$ and 50 μM D-AP5. Top traces: averaged NMDAR EPSCs in L−AP4 (30 μM) (1), control (2), and 25 min after tetani (3). Holding potential: −80 mV.

(B) Tetanic stimulation does not elicit LTP in a L−AP4-insensitive cell. Top traces: averaged NMDAR EPSCs in L−AP4 (30 μM) (1), control (2), and 25 min after tetanic stimulation (3). Holding potential: −85 mV.

(C) Summary graphs of autaptic NMDAR EPSCs from 23 L−AP4-sensitive cells (closed circle) and 18 L−AP4-insensitive cells (open circle). Same protocol as illustrated in (A) was used (error bars represent mean ± SEM).

(D) LTP of autaptic AMPAR EPSCs in 12 L−AP4-sensitive cells. Inset: averaged AMPAR EPSCs in L−AP4 (30 μM) (1), control (2), and 25 min after tetanic stimulation (3).

effect on LTP (n = 2). It was not possible to determine if postsynaptic depolarization is required for LTP in the single cell cultures, because activation of the autapses necessitated depolarizing the cell. Therefore, for these experiments we resorted to multicell cultures. Cells were recorded with 10 mM BAPTA added to the pipette solution. Synaptic NMDAR responses to focal stimulation were tested for L−AP4 sensitivity, and then in the presence of AP5, the input was tetanized while holding the postsynaptic cell in voltage clamp. As can be seen in Figure 3, normal LTP was induced under these conditions (n = 4). Thus far, the results suggest that neither glutamate receptors, a rise in postsynaptic $Ca^{2+}$, nor depolarization of the postsynaptic membrane are required for the induction of this form of LTP.

At the crustacean neuromuscular junction, long-term facilitation, which shares many of the properties of mossy fiber LTP, is unaltered by removal of extracellular $Ca^{2+}$ (Wojtowicz and Atwood, 1988). To determine if there was any $Ca^{2+}$ dependence to the present phenomenon, experiments were performed on single granule cells in which the tetanus was given while synaptic transmission was blocked by the removal of extracellular $Ca^{2+}$ (Figure 4A). After returning to the normal extracellular $Ca^{2+}$ concentration, the synaptic responses returned to baseline values and exhibited no LTP. A subsequent identical tetanus, now given in the presence of $Ca^{2+}$, evoked substantial LTP. A summary of a number of experiments (closed circles, n = 7; open circles, n = 8) is shown in Figure 4B, and establishes that the induction of this form of LTP is entirely dependent on the presence of $Ca^{2+}$ in the extracellular medium.

To address more directly the role of $Ca^{2+}$ in LTP, we filled our pipettes with high concentrations of BAPTA or EGTA to buffer $Ca^{2+}$ in the presynaptic terminal. Loading single cells with BAPTA (10 mM) resulted in the complete blockade of transmitter release within 20 min (data not shown). In contrast, loading single granule cells with the slower acting chelator EGTA (100 mM) had much less effect on transmitter release, and in this condition, a tetanus did not elicit LTP, but instead generated only a brief potentiation lasting a few minutes (Figure 5).

### Role of cAMP

In the slice preparation, evidence has been obtained that suggests a role for cAMP in mossy fiber LTP (Huang

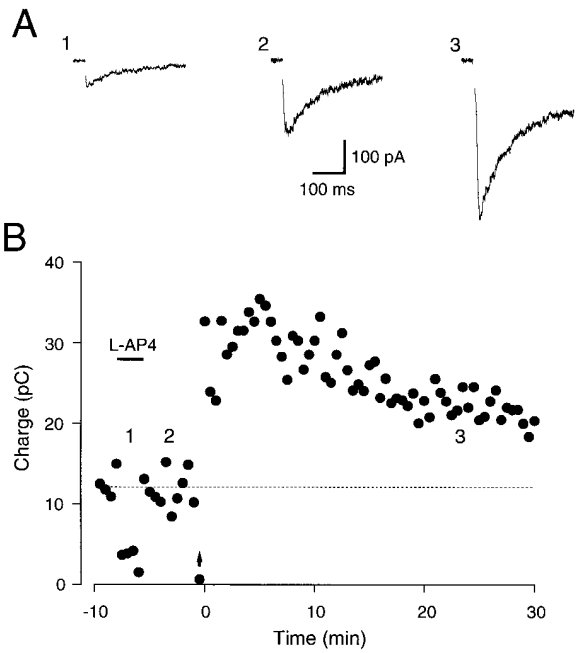


Figure 3. LTP Does Not Require a Rise in Postsynaptic $Ca^{2+}$ or Membrane Depolarization

(A) Example of NMDAR EPSCs recorded in a neuron in a multicell culture and evoked by extracellular stimulation of an L−AP4-sensitive input. (1) shows the inhibition by L−AP4, (2) shows the control response before tetanic stimulation and (3) shows the response 23 min after tetanic stimulation. The whole-cell pipette solution contained 10 mM BAPTA.

(B) After establishing the sensitivity of the input to L−AP4, the input was tetanized in the presence of 50 μM D-AP5 and while the postsynaptic membrane potential remained voltage clamped at −80 mV.

et al., 1994; Weisskopf et al., 1994). Therefore, we have tested the effects of the diterpene forskolin, an activator of adenylyl cyclase, on the autaptic responses. Figure 6 shows that in an L−AP4-sensitive cell, a brief application (3 min) of forskolin (30 μM) causes a long-lasting enhancement in synaptic transmission. Results from 10 granule cells are summarized in Figure 6B (closed circles). Also illustrated on this graph is the effect of forskolin on synaptic responses recorded from nongranule cells (n = 14) (open circles). In these cells, only a transient enhancement was observed.

To test whether increases in cAMP and activation of protein kinase A (PKA) play a direct obligatory role in the LTP at granule cell autapses, we examined the effects of Rp-8CPT-cAMPS (100 μM), a regulatory site antagonist of protein kinase A. In the presence of this antagonist, a tetanus elicited only a brief potentiation of the synaptic responses in granule cells (n = 9) (Figure 7A). This result suggests that PKA plays an essential role in this form of LTP. However, it has recently been reported that like forskolin, a brief application of 8-Br-cGMP to rat hippocampal CA1/CA3 cells in culture causes a long-lasting presynaptic enhancement of synaptic transmission (Arancio et al., 1995). To determine whether cGMP contributes to the LTP observed in single granule cells, we applied 8-Br-cGMP at a higher concentration (100 μM) than that found to be effective in the previous study. In none of the five cells did we observe any effect of 8-Br-cGMP on synaptic transmission (Figure 7B).

**Mechanisms of Expression**

The results presented thus far strongly suggest that the induction of this form of LTP is presynaptic. To examine its site of expression, we took advantage of the fact that all of the synapses on the recorded cell are derived from that cell. This means that all of the recorded miniature EPSCs (mEPSCs) will come from synapses that have been activated by the tetanus. Thus, we recorded the size and frequency of these events in the presence of tetrodotoxin (TTX) before and after inducing LTP. Figure 8A shows the results from one experiment. In basal conditions, the frequency of mEPSCs was approximately 0.2 Hz and the amplitude distribution showed a skewed distribution toward larger events, as is typical in central neurons (Stevens, 1993). After the tetanus (given in the absence of TTX), the frequency increased approximately 2- to 3-fold (Figure 8A2), while the mean amplitude (Figure 8A3) and amplitude distribution (Figure 8A4) of the mEPSCs was entirely unchanged. A summary of the data from a number of cells (n = 9) is shown in Figure 8B, and confirms that an LTP-inducing tetanus causes a large and sustained increase in the frequency of mEPSCs, but no change in their amplitude.

To address the possibility that LTP might be due to the turning on of previously silent synapses, as has been suggested for NMDAR-dependent LTP (Isaac et al., 1995; Liao et al., 1995), we took advantage of the use-dependent irreversible NMDAR antagonist MK-801. After establishing that the cell was L−AP4-sensitive (Figure 9A, closed circles), MK-801 was applied, and synaptic stimulation was repeatedly given at low frequency until the synaptic response had entirely disappeared. In the presence of AP5, the standard tetanus was then given and the AP5 and MK-801 were washed out. This resulted in the immediate reappearance of a synaptic response that remained for the rest of the experiment. While it could be argued that the recovery of the EPSC simply reflected reversibility in the MK-801 block of NMDARs, this does not appear to be the case. First, we repeated the exact same experiment in nongranule cells (Figure 9A, open circles) and found that following the tetanus, there was no recovery of responses after the tetanus. Second, in a separate set of experiments with exogenous application of NMDA, we were unable to reverse the MK-801 blockade in the time frame of the experiment.

These results clearly suggest that mossy fiber LTP is due in part to the turning on of silent synapses. However, this switch could reflect a postsynaptic upregulation of NMDARs at previously active synapses, or alternatively, it could reflect the turning on of release sites that were functionally silent prior to the tetanus. To distinguish between these two alternatives, we first applied NMDA in the presence of MK-801, to block all functional NMDARs on the granule cell. Following the washout of MK-801, a tetanus failed to restore any synaptic response (Figure 9B). Thus, it would appear that upregulation of NMDARs cannot explain the previous results, and that a presynaptic mechanism underlies the turning on of silent synapses.

**Discussion**

There is now general agreement that in the hippocampus, there are at least two forms of LTP, one that is

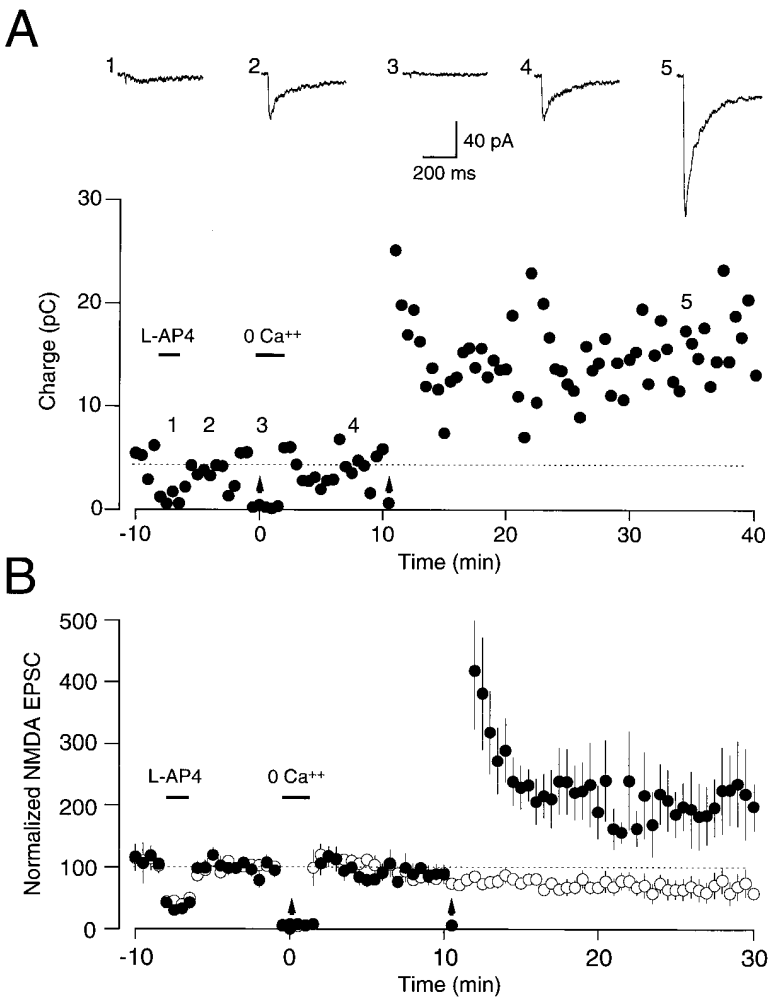


Figure 4. LTP of Autaptic EPSCs Requires Extracellular $Ca^{2+}$

(A) Example of a L−AP4-sensitive cell showing that tetanic stimulation in the presence of 0 $Ca^{2+}$, 4 mM $Mg^{2+}$ and 50 μM D-AP5 (first arrow) does not enhance synaptic strength. In the same cell, subsequent tetanic stimulation in the presence of 3 mM $Ca^{2+}$, 1 mM $Mg^{2+}$, and 50 μM D-AP5 (second arrow) induces LTP. Top traces: averaged NMDAR EPSCs in L−AP4 (30 μM) (1), control (2), in the absence of $Ca^{2+}$ (3), 5 min after tetanic stimulation given in the absence of $Ca^{2+}$ (4), and 25 min after tetanic stimulation given in the presence of $Ca^{2+}$ (5). Holding potential: −80 mV.

(B) Summary of experiments like that in (A) (n = 7, closed circles); open circles summarize cells (n = 8) in which a second round of tetanic stimulation was not given.

dependent on the activation of NMDARs, and one that is independent. The latter form is found at synapses made by mossy fibers, the axons of dentate granule cells, onto CA3 pyramidal cells, and while the preponderance of evidence suggests that the induction and expression are presynaptic (for review, see Nicoll and Malenka, 1995), contradictory views exist (Derrick and Martinez, 1994; Johnston et al., 1992). This lack of unanimity is due in part to the complex circuitry of the CA3 region. The goal of the present study was 2-fold: first, we wished to determine if it were possible to create in cultures of single granule cells an LTP similar to that described at mossy fiber synapses in the slice preparation. Second, having once established LTP in single-cell cultures, we wished to use this simplified system to probe the mechanisms of this form of LTP.

To accomplish these goals, it was first necessary to grow single granule cells on islands of glial cells. We were unable to identify granule cells in our cultures morphologically, and therefore used the selective sensitivity of mossy fiber synapses of the guinea pig to the presynaptic inhibitory action of L−AP4 to separate granule cells from nongranule cells. Further evidence supporting the supposition that the L−AP4 cells were granule cells was the finding that these cells were also sensitive to dynorphin and were associated with an LTP that is indistinguishable from that generated by the synapses made by granule cells on CA3 pyramidal cells in the slice, i.e., mossy fiber synapses. In marked contrast, L−AP4-insensitive cells never exhibited this form of LTP. Thus, the action of L−AP4 provides a reliable pharmacological test in the guinea pig for identifying dentate granule cells.

Using this cell culture system, we have confirmed and extended many of the findings originally made in slice preparations on mossy fiber LTP. The induction of LTP in cultured granule cells was independent of the activation of a variety of transmitter receptors, including NMDARs, AMPARs, mGluRs, and opioid receptors. Since activation of autapses required depolarizing the cell that receives the synapses, it was not possible in the single cell cultures to determine if postsynaptic depolarization is required for LTP induction. Therefore, we used multicell cultures and found that with L−AP4-sensitive inputs, voltage clamping the postsynaptic cell during the tetanus had no effect on the induction of LTP. In these same experiments, BAPTA was present in the postsynaptic cell, strongly suggesting that neither postsynaptic depolarization nor a rise in postsynaptic $Ca^{2+}$ is necessary. Since at autapses BAPTA was shown to diffuse to distant synaptic terminals and block transmitter release, a process that requires high local concentrations of $Ca^{2+}$, we are confident that somatic and dendritic $Ca^{2+}$ transients were also blocked in these experiments.

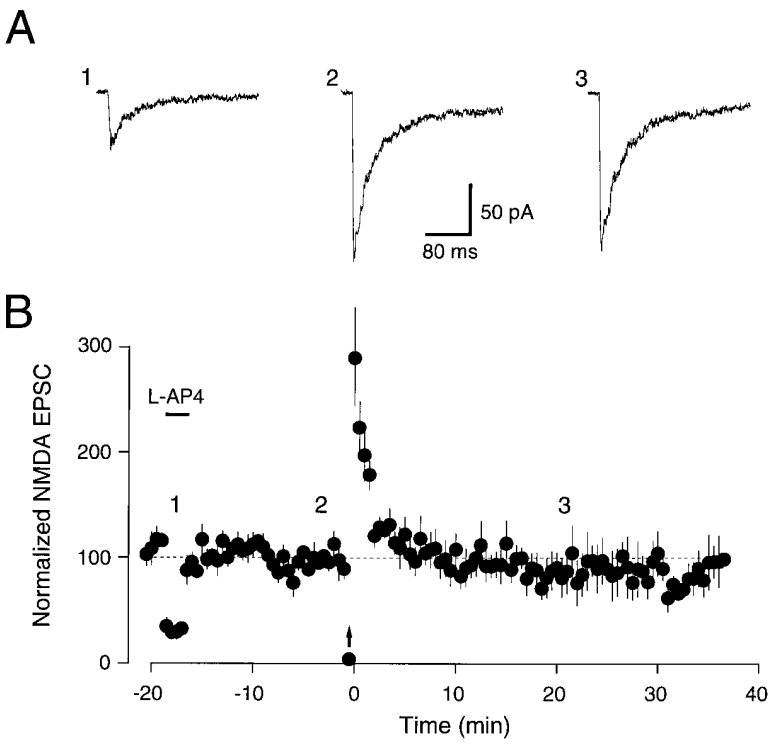


Figure 5. Buffering $Ca^{2+}$ in the Presynaptic Terminal Prevents LTP

Cells were loaded with 100 mM EGTA. After allowing sufficient time for EGTA to diffuse to the synaptic terminals, the standard tetanus was applied.

(A) Example of NMDAR EPSCs recorded in the presence of L−AP4 (30 μM) (1), before tetanic stimulation (2), and 20 min after tetanic stimulation (3).

(B) Summary graph (n = 5) showing that tetanic stimulation gave only a short-lasting enhancement of the NMDAR EPSC, but no LTP.

LTP was dependent on the presence of $Ca^{2+}$ in the extracellular medium and was blocked by loading the terminals with the $Ca^{2+}$ buffer EGTA. The latter finding rules out the possibility that a $Ca^{2+}$-dependent depolarization is required for LTP. Therefore, these results indicate that a tetanus-induced rise in presynaptic $Ca^{2+}$ is necessary to generate this NMDAR-independent form of LTP at granule cell autapses.

It is suggested by two lines of evidence that, as in the slice preparation (Huang et al., 1994; Weisskopf et al., 1994), this rise in presynaptic $Ca^{2+}$ activates a calmodulin-sensitive activation of adenylyl cyclase, and the consequent rise in cAMP is required for LTP. First, forskolin caused a long-lasting enhancement in granule cells, but only a small transient enhancement in nongranule cells. Second, a blocker of the regulatory site of protein kinase A prevented the LTP. It has been proposed that mossy fiber LTP requires the coactivation of noradrenergic fibers, as well as mossy fibers (Hopkins and Johnston, 1988; Huang and Kandel, 1996). The fact that an LTP can be recorded in single granule cells that remains stable for the duration of the recording (up to 50 min) would seem to rule out the necessity for a coactivated noradrenergic input, and favors a model in which the direct activation of a Ca/calmodulin-sensitive adenylyl cyclase is sufficient (Weisskopf et al., 1994).

The ability to elicit mossy fiber LTP in single granule cell cultures offered a major advantage for studying the expression mechanisms involved in this form of synaptic plasticity. Both the NMDAR- and the AMPAR-mediated component of the EPSC expressed LTP, a finding that is consistent with either a presynaptic expression mechanism or with a postsynaptic upregulation of both classes of receptor. Since all of the synapses impinging onto the cell are formed by one cell, i.e., the same cell from which the synaptic responses are recorded, the preparation is ideally suited for the analysis of any changes in the properties of mEPSCs during tetanus-induced LTP. We have found that LTP is associated with a marked and sustained increase in the frequency of AMPAR mEPSCs, but no change in their amplitude distribution. Increases in mEPSC frequency have also been reported to occur, following NMDAR activation in CA3/CA1-cultured neurons (Arancio et al., 1995; Malgaroli and Tsien, 1992). These findings are consistent with a presynaptic expression mechanism, although it does not exclude the postsynaptic all-or-none upregulation of clusters of AMPARs at previously silent synapses, a mechanism analogous to that proposed for NMDAR-dependent LTP (Isaac et al., 1995; Liao et al., 1995).

To examine the possible involvement of "silent synapses" in more detail, we took advantage of the use-dependent irreversible NMDAR antagonist MK-801. After complete blockade of the evoked NMDAR EPSC by MK-801, a tetanus resulted in the appearance of an NMDAR EPSC that remained for the duration of the experiment. In terms of classical quantal analysis, this reappearance of synaptic responses is equivalent to an increase in the parameter n, which classically represents the number of release sites or "active zones." However, this apparent change in n could again be explained either by a postsynaptic upregulation of NMDARs or alternatively, by the switching on of presynaptic release sites that prior to the tetanus were silent. To distinguish between these alternatives, we coapplied NMDA and MK-801 to block all functional NMDARs on the cell. Tetanic stimulation in this condition now failed to restore any synaptic response, indicating that at these synapses, LTP does not cause an upregulation of NMDARs; rather, it is due, at least in part, to the switching on of previously silent release sites.

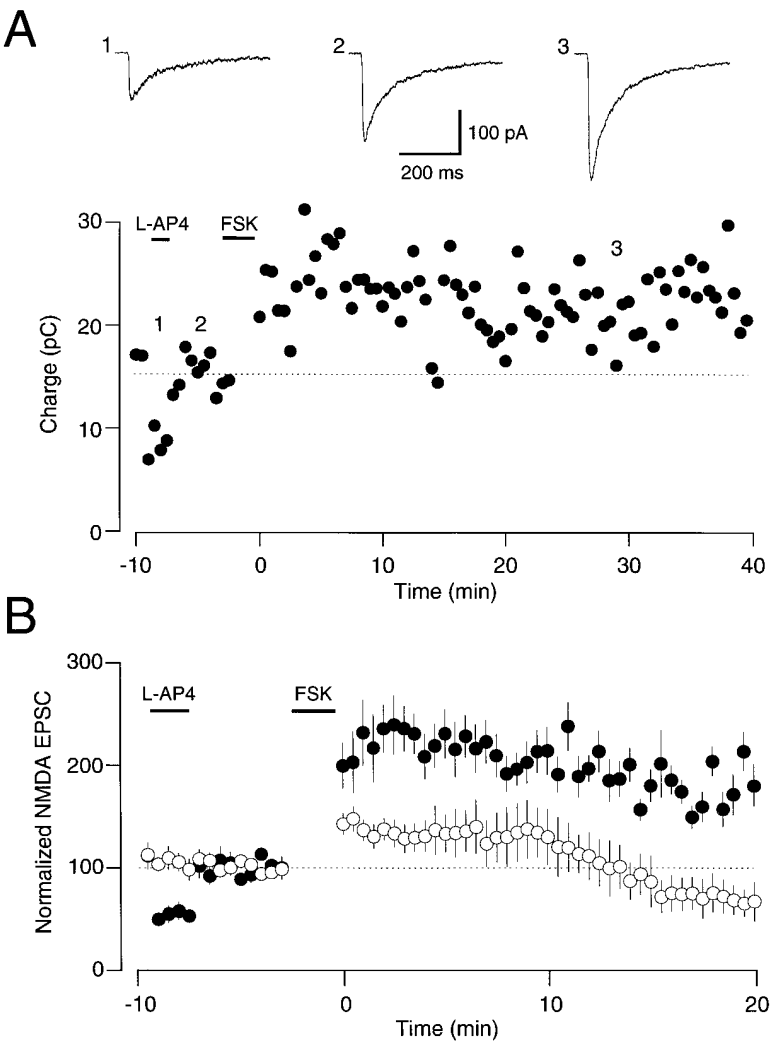


Figure 6. Forskolin Induces a Long-Lasting Potentiation of Autaptic EPSCs in L−AP4−Sensitive Cells

(A) Example of a L−AP4-sensitive cell, in which forskolin (FSK, 30 μM) was applied for 3 min and induces a long-lasting enhancement. Top traces: averaged NMDAR EPSCs in L−AP4 (30 μM) (1), control (2), and 30 min after application of forskolin (3). Holding potential: −80 mV.

(B) Summary graphs of effects of forskolin in L−AP4-sensitive cells (n = 10; closed circle) and L−AP4-insensitive cells (n = 14; open circle).

Previous results in the slice (Staubli et al., 1990; Weisskopf and Nicoll, 1995; Xiang et al., 1994; Zalutsky and Nicoll, 1990) suggest that mossy fiber LTP is also associated with an increase in the probability of neurotransmitter release, p. The increase in frequency of mEPSCs accompanying LTP indicates that this enhancement cannot result simply from an increase in $Ca^{2+}$ influx during the action potential, but must reflect a lasting change in some step downstream to $Ca^{2+}$ entry. A fundamental issue that remains to be elucidated is how cAMP exerts these long-lasting presynaptic effects. Candidate proteins that are known to be phosphorylated by PKA are the synapsins (Greengard et al., 1993) and rabphillin-3A (Fykse et al., 1995). The synapsins do not appear to be critically involved in mossy fiber LTP, since no deficit in the LTP recorded in mutant mice lacking both synapsin I and II could be detected (Spillane et al., 1995). The role of rabphillin-3A, which binds to the synaptic vesicle proteins rab3A and rab3C (Südhof, 1995), is poorly understood and remains an interesting candidate.

It is of interest to compare the present results to those obtained at the crayfish neuromuscular junction, where cAMP is thought to mediate long-term facilitation, a presynaptic form of plasticity. Quantal analysis suggests that long-term facilitation is associated primarily with an increase in n (Wojtowicz et al., 1988), and EM studies have shown that there is an increase in the number of presynaptic dense bodies or active zones (Wojtowicz et al., 1994). It has also been proposed that ''latent'' connections become functional during LTP at inhibitory synapses on the Mauthner cell (Charpier et al., 1995).

In summary, we have succeeded in demonstrating in single-cell cultures of hippocampal granule cells a form of LTP that is indistinguishable from that observed at mossy fiber synapses in the slice preparation. Such a demonstration confirms the apparent simplicity of mossy fiber LTP, originally proposed from experiments on slices, in which both the induction and expression are presynaptic (Nicoll and Malenka, 1995). It also demonstrates unambiguously that this form of LTP is a single-cell phenomenon that does not require any cooperative interaction among neighboring neurons. Finally, we present evidence that mossy fiber LTP is associated with the recruitment of release sites that prior to a tetanus remained silent, and that the mechanism responsible for the enhanced release of transmitter occurs, at least in part, at some step subsequent to the entry of $Ca^{2+}$ into the presynaptic terminal. Determining the ex-

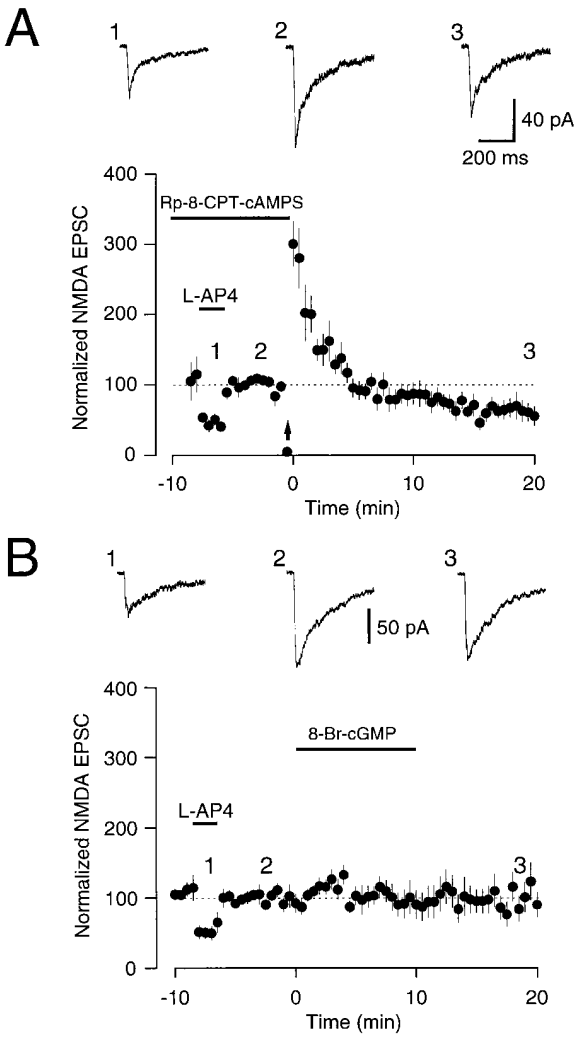


Figure 7. The PKA Inhibitor, Rp-8-CPT-cAMPS, Blocks LTP

(A) Effect of 20 min application of Rp-8-CPT-cAMPS (100 μM) on LTP in L−AP4-sensitive cells (n = 9). Top traces: averaged NMDAR EPSCs in L−AP4 (30 μM) (1), control (2), and 20 min after tetanic stimulation (3). The neurons were preincubated with Rp-8-CPT-cAMPS for 10 min before recording and for an additional 10 min before tetanic stimulation.

(B) Summary graphs of effects of 8-Br-cGMP (100 μM, 10 min) in L−AP4-sensitive cells (n = 7). Top traces: averaged synaptic responses in L−AP4 (30 μM) (1), control (2), and 25 min after application of 8-Br-cGMP (3).

act presynaptic protein(s) that are modified by PKA and are responsible for the lasting changes in transmitter release remains a challenging problem, the solution to which should be greatly facilitated by the development of this culture system.

## Experimental Procedures

### Tissue Culture

Microdot cultures were prepared from hippocampal neurons of the dentate region from prenatal day 35–55 guinea pig fetuses (Charles River). Extreme care was taken to isolate the dentate region prior to dissociation, so that cultures contained neurons predominantly from this part of the hippocampus. Autaptic recordings were obtained from isolated neurons grown on collagen/poly-D-lysine microdots, as described previously (Tong and Jahr, 1994). Multicell cultures were made on collagen/poly-D-lysine coated cover slips. Multicell cultures were used only for the results presented in Figure 3. Tetrodotoxin (100 nM) and D-AP5 (10 μM) were added to the culture medium 5–8 days after plating. Recordings were made at room temperature (22°C–25°C) from 9–28 day-old neurons.

### Whole-Cell Experiments

Whole-cell recordings of autaptic excitatory postsynaptic currents and mEPSCs were made (Axopatch-1D) with low resistance patch pipettes (1−3 MΩ) containing 110 mM K-gluconate, 10 mM NaCl, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, 0.2 mM GTP, 20 mM $K_2$-creatine phosphate and 50 U/ml phosphocreatine kinase, adjusted to pH 7.3 with KOH. During the course of this study, we found that there was a variable run down of the NMDAR-mediated responses in both granule and nongranule cells. Similar to previous findings (Rosenmund and Westbrook, 1993a, 1993b) we found that the run down could largely be prevented by the addition of phalloidin (1 mM) in the pipette solution. Therefore, the summary graphs in this paper combine data from phalloidin-treated and untreated cells. Control extracellular medium contained 140 mM NaCl, 3.5 mM KCl, 10 mM HEPES, 20 mM glucose, 2–3 mM $CaCl_2$, and 50 μM picrotoxin, adjusted to pH 7.4 with NaOH. To isolate NMDAR EPSCs, CNQX (10 μM) was added to solutions containing 20 μM glycine. To isolate AMPAR EPSCs, 1 mM $Mg^{2+}$ and D−AP5 (50 μM) were added to solutions nominally free of glycine. Autaptic EPSCs were evoked every 10 s or 15 s with 0.5 ms–2 ms voltage jumps to −20 or 0 mV from a holding potential of −60 to −90 mV. In multicell cultures, focal extracellular stimulation with a patch pipette filled with the normal extracellular medium was used to activate presynaptic inputs. Tetanus-induced LTP was obtained by using a 50 Hz stimulus for 1 s in voltage clamp. This procedure was performed four times at 2 s intervals. All tetani were given in the presence of 1 mM $Mg^{2+}$ and 50 μM D-AP5. Currents were low pass filtered at 0.5–2 kHz and digitally sampled at 2.5 kHz–10 kHz. Series resistance compensation (80%–90%) was used in all experiments. The series and input resistances were monitored throughout each experiment with a −1 to −4 mV calibration pulse given 30 ms before each stimulation. For most illustrations, the partially clamped action potential and associated capacitative currents evoked by the stimulating voltage jump were isolated with CNQX and D−AP5 and subtracted from the averaged EPSCs. The remaining artifact was blanked. Illustrated traces are averages of 8–16 responses, except where indicated.

Solution changes were made with gravity-fed flow tubes, as described previously (Lester et al., 1993; Tong and Jahr, 1994). High purity salts (Aldrich, Gold Label) and HPLC grade water (Fisher Scientific, Fair Lawn, NJ) were used. Other chemicals were obtained from Sigma, except for CNQX (Tocris Neuramin), D-AP5 (Tocris Neuramin), and $K_2$-creatine phosphate (Calbiochem).

### Data Analysis

Evoked EPSCs were stored on a 486 computer equipped with a Labmaster A/D board and analyzed offline. To measure synaptic strength of the NMDAR EPSC, the charge transfer was determined by measuring the area from the peak of the EPSC to 150 ms after the peak. When AMPAR EPSCs were recorded, their peak amplitude was used. AMPAR mEPSCs were stored on videotape and analyzed offline, as previously described (Wyllie et al., 1994). Results are expressed as mean ± SEM.

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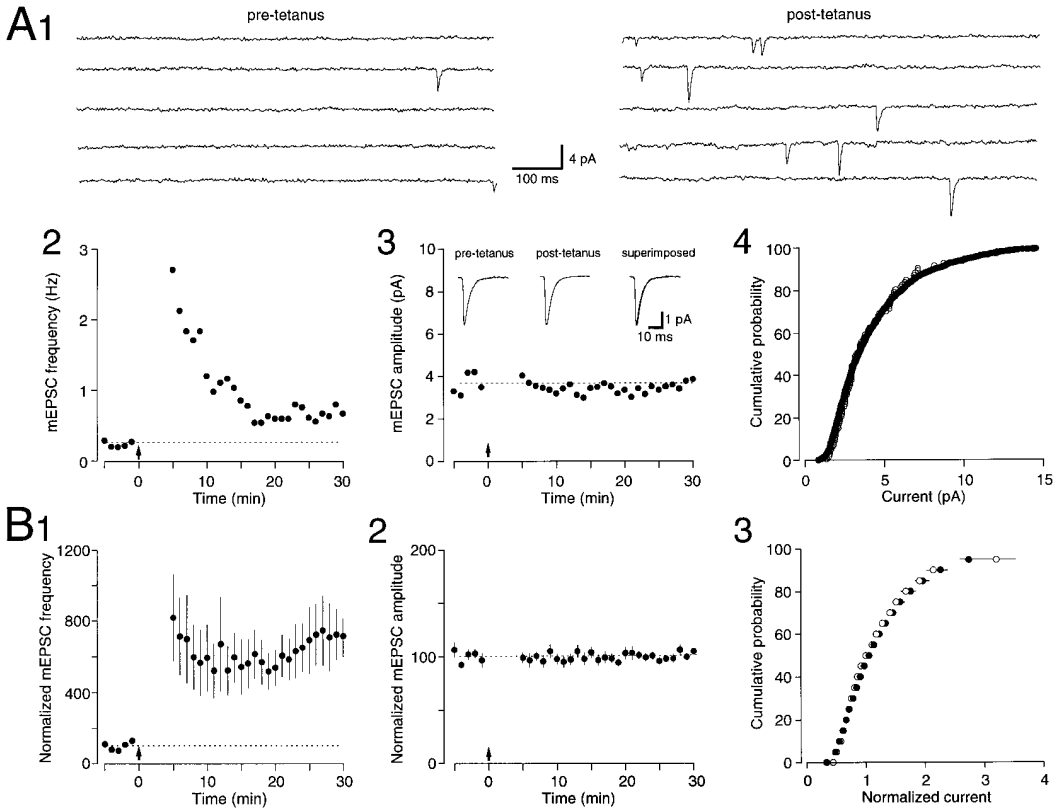


Figure 8. Tetanic Stimulation Causes a Long-Lasting Enhancement of the Frequency but Not Amplitude of mEPSCs

(A1) Examples of mEPSCs before (pretetanus), and 30 min after tetanic stimulation (posttetanus).

(A2) The time course of the change in mEPSC frequency from the same cell shown in (A1).

(A3) The time course of mEPSC amplitude. Inset shows the averages of 59 mEPSCs before and 2667 mEPSCs after the tetanic stimulation.

(A4) Cumulative probability plot of the amplitudes of the mEPSCs before (open circles) and after tetanic stimulation (closed circles).

(B) Summary graphs of the effects of tetanic stimulation on mEPSC frequency (B1) mean mEPSC amplitude (B2) and cumulative probability plots of mEPSC amplitude (B3) in L−AP4-sensitive cells (n = 9). The average cumulative probability plot was obtained by normalizing each distribution to the pretetanus median value. For clarity, the last point of each plot is not illustrated. Open circles are pretetanus and closed circles are posttetanus. Tetrodotoxin was present for 5 of the 9 experiments. Since no difference was observed between these two conditions, the results from the nine experiments were averaged together.

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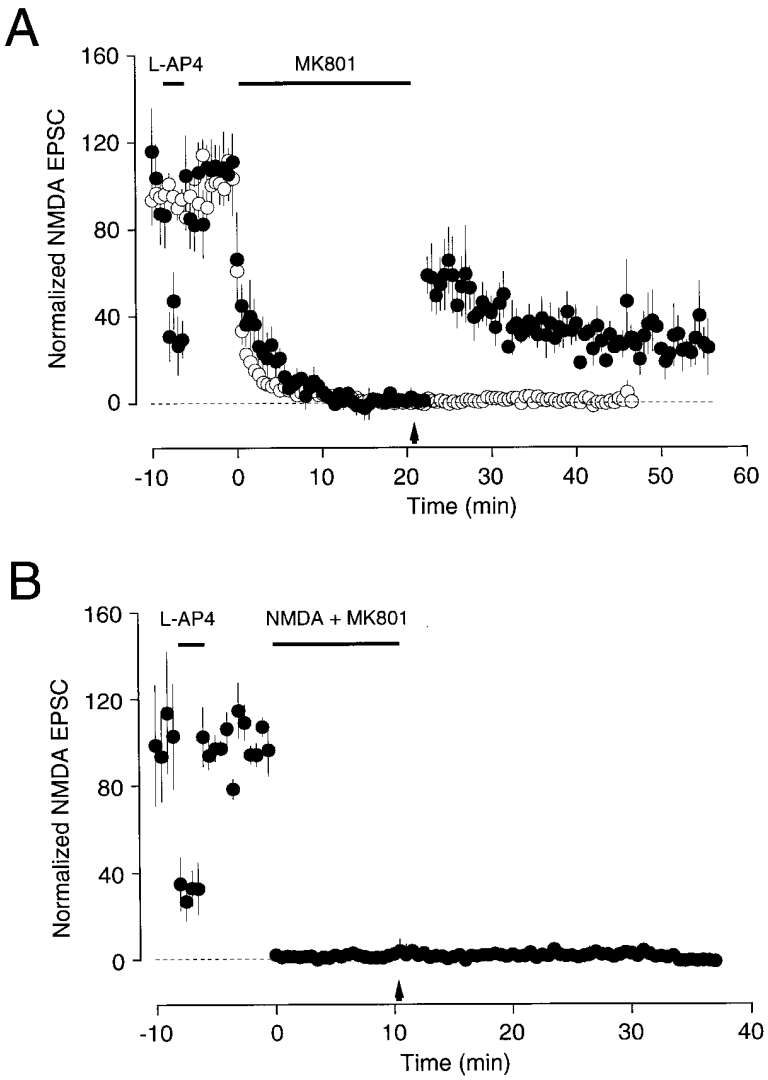


Figure 9. Tetanic Stimulation of Granule Cell Synapses Turns on Previously Silent Release Sites

(A) In L−AP4-sensitive cells (closed circles) (n = 5) synaptic NMDARs were blocked by low-frequency stimulation in the presence of MK-801 (5 μM). Following complete blockade of the synaptic response, a tetanic stimulation was given in the presence of D-AP5. D-AP5 and MK-801 were then washed out. Tetanic stimulation resulted in the reappearance of synaptic responses that remained for the rest of the experiment. At the end of the experiment, D-AP5 was applied, which completely blocked the resurrected responses (data not shown). The exact same experiment was performed on a series of L−AP4-insensitive cells (open circles) (n = 6), but in this case tetanic stimulation failed to restore synaptic responses.

(B) After establishing that the autapses were sensitive to L−AP4, NMDA (50 μM) and MK-801 (5 μM–10 μM) were coapplied. This resulted in the rapid and complete blockade of the NMDA EPSC, as well as the inward current generated by the applied NMDA. Tetanic stimulation in this condition failed to restore any synaptic response (n = 6).

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