

Role of EGR1 in Hippocampal Synaptic Enhancement Induced by Tetanic Stimulation and Amputation

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Abstract. Hippocampal neurons fire spikes when an animal is at a particular location or performs certain behaviors in a particular place, providing a cellular basis for hippocampal involvement in spatial learning and memory. In a natural environment, spatial memory is often associated with potentially dangerous sensory experiences such as noxious or painful stimuli. The central sites for such pain-associated memory or plasticity have not been identified. Here we present evidence that excitatory glutamatergic synapses within the CA1 region of the hippocampus may play a role in storing pain-related information. Peripheral noxious stimulation induced excitatory postsynaptic potentials (EPSPs) in CA1 py-

ramidal cells in anesthetized animals. Tissue/nerve injury caused a rapid increase in the level of the immediate-early gene product Egr1 (also called NGFI-A, Krox24, or zif/268) in hippocampal CA1 neurons. In parallel, synaptic potentiation induced by a single tetanic stimulation (100 Hz for 1 s) was enhanced after the injury. This enhancement of synaptic potentiation was absent in mice lacking Egr1. Our data suggest that Egr1 may act as an important regulator of pain-related synaptic plasticity within the hippocampus.

Key words: Egr1 • NMDA • LTP • pain • hippocampus

Introduction

The hippocampus and related structures are important for certain types of learning and memory in both rodents and humans (Squire and Zola-Morgan, 1991; Squire, 1992; Eichenbaum, 1999). One special feature of hippocampal neurons is that they can fire spikes when an animal is at a particular location or performs certain behaviors in a particular location, providing a cellular basis for hippocampal involvement in spatial learning and memory (O'Keefe and Nadel, 1978; Muller et al., 1987; Nadel, 1991; Stevens, 1996; Eichenbaum et al., 1999). In addition to spatial information, hippocampal neurons have been reported to be responsive to different sensory stimuli including noxious somatosensory stimulation (Berger et al., 1976, 1980, 1983; Brankack and Buzsaki, 1986; Jirsa et al., 1992; Luntz-Leybman et al., 1992; Tamura et al., 1992; Sakurai, 1994; Stevens, 1996; Weiss et al., 1996). We are interested in the properties of hippocampal neuronal responses to somatosensory stimuli, especially high intensity stimuli triggering escape behavior in animals and pain in humans (Brankack and Buzsaki, 1986; Sinclair and Lo, 1986; Heale and Vanderwolf, 1994). One possible physiological function of these responses is that the hippocampus may be in-

involved in the formation of spatial memory associated specifically with potentially dangerous sensory experiences such as noxious or painful stimulation.

Excitatory synaptic transmission within the CA1 region of the hippocampus is mediated by glutamate. Glutamatergic synapses exhibit divergent synaptic plasticity, including long-term potentiation (LTP)¹ and long-term depression (LTD), depending on synaptic activity as well as postsynaptic membrane excitability (Bliss and Collingridge, 1993; Bear and Malenka, 1994; Malenka and Nicoll, 1999). High-frequency stimulation of excitatory synapses within the hippocampus activates various types of immediate-early genes (IEGs) in postsynaptic neurons (Morgan and Curran, 1991; Bliss and Collingridge, 1993; Ginty et al., 1993; Kandel, 1997), including Egr1 (also called NGFI-A, Krox24, or zif/268). The IEG Egr1 is critical for coupling extracellular signals to changes in cellular gene expression (Milbrandt, 1987; Deisseroth et al., 1996). In the hippocampus, Egr1 is upregulated by tetanic stimulation, which is known to induce LTP (Cole et al., 1989; Gashler and Sukhatme, 1995). The function of Egr1, however, is not fully understood.

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¹Abbreviations used in this paper: ACSF, artificial cerebrospinal fluid; DG, dentate gyrus; EPSPs, excitatory postsynaptic potentials; IEGs, immediate-early genes; LTD, long-term depression; LTP, long-term potentiation.

In this study, we tested the hypothesis that activity-dependent expression of *Egr1* in hippocampal neurons may contribute to plastic changes in excitatory synaptic transmission. Electrophysiological, immunocytochemical, and genetic approaches were used to test this hypothesis. First, we recorded from CA1 pyramidal neurons in anesthetized adult rats and showed that some of these neurons responded to peripheral noxious stimulation. Second, using immunocytochemical staining, the IEG *Egr1* was activated in hippocampal CA1 neurons after tissue injury in both rats and mice. Third, we demonstrated that in CA1 neurons, synaptic plasticity of excitatory glutamatergic transmission was altered after tissue injury. Finally, using mice lacking *Egr1*, we found that *Egr1* was required for plastic changes in the hippocampus caused by tissue injury, in addition to contributing to long-lasting synaptic enhancement in the normal hippocampus.

Materials and Methods

Animals and Treatment

Adult male rats (Sprague-Dawley rats, 220–400 g; Harlan) and mice (wild-type and mutant *Egr1* mice generated by Dr. J. Milbrandt) were used. As reported previously, we used two different amputation procedures under halothane anesthesia: in adult rats, the central digit of a rat hindpaw was removed (Wei et al., 1999); in mice, the tip of a mouse tail was removed (Zhuo, 1998). We did not perform rat tail amputation in part due to surgical complications (e.g., excessive bleeding). Wild-type and homozygous mutant *Egr1* mice were obtained by crossing heterozygous mutant mice bearing a targeted mutation of the *Egr1* gene. Genotypes were determined by PCR analysis (Lee et al., 1995) of genomic DNA extracted from mouse ear tissue. Mice were maintained in a C57BL/6 strain background and were age matched in each experiment.

In Vivo Electrophysiology

Intracellular recording and injection of dye were performed on adult rats under halothane anesthesia. A bipolar stimulating electrode was placed into one hindpaw. Recording electrodes had a tip resistance of 50–70 M Ω when filled with a solution of 4% neurobiotin (Vector) in 2 M potassium acetate. After placement of a microelectrode in the cortex above the hippocampus (AP 3.0–6.5 mm, ML 1.0–4.0 mm), the exposed surface of the brain was covered with soft paraffin wax. After impalement, neurons with stable membrane potentials of -60 mV or greater were selected for further study. After each successful recording, neurobiotin was iontophoresed into the cell by passing a positive current pulse (2 Hz, 300 ms, 0.5–1 nA) for 10 min. At the end of the experiment, the rat was deeply anesthetized and perfused transcardially with 0.01 M phosphate-buffered saline followed by 4% paraformaldehyde. The brain was removed and stored in fixative overnight. Coronal sections were cut at a 50- μ m thickness using a vibratome and incubated in 0.1% horseradish peroxidase-conjugated avidin-D (Vector) in 0.01 M potassium phosphate-buffered saline (KPBS, pH 7.4) with 0.5% Triton X-100 at room temperature for 6–8 h. After detection of peroxidase activity with 3',3'-DAB, sections were examined in KBPS. Sections containing labeled neurons were mounted on gelatin-coated slides for light microscopy.

In Vitro Electrophysiology

Mice were anesthetized with halothane and the tail tip (2.5 cm) was removed (Zhuo, 1998). Animals recovered from anesthesia within 2–3 min. In the sham-operated group, the same procedure was performed without tail amputation. Both amputated and sham-operated mice were put back in their cages with the access to food and water. 45 min later, mice were anesthetized with halothane and transverse slices of hippocampus 400 μ m thick were prepared rapidly and maintained in an interface chamber at 28°C, where they were subfused with saline (artificial cerebrospinal fluid; ACSF) consisting of 124 mM NaCl, 4.4 mM CaCl₂, 2.0 mM MgSO₄, 25 mM NaHCO₃, 1.0 mM Na₂HPO₄, and 10 mM glucose and bubbled with 95% O₂ and 5% CO₂. In some experiments, slices were harvested at different peri-

ods of time after amputation (\sim 0, 20, and 120 min). In a second group of experiments, mice were maintained deeply anesthetized by halothane (2–3%) for the 45 min between amputation and preparation of hippocampal slices.

In all experiments, slices recovered in the chamber for at least 2 h before recording. A bipolar tungsten stimulating electrode was placed in the stratum radiatum of the CA1 region, and extracellular field potentials were recorded using a glass microelectrode (3–12 M Ω , filled with ACSF), also in the stratum radiatum. Test responses were elicited at 0.02 Hz. In some experiments, picrotoxin (100 μ M) was included in bath solution to block inhibitory transmission. LTD was induced by low frequency stimulation (1 Hz for 15 min; Dudek and Bear, 1992). LTP was induced by a single tetanic stimulus (100 Hz for 1 s). Paired-pulse facilitation using various interpulse intervals (25–400 ms) was also measured. In experiments with wild-type and mutant mice, NMDA receptor-mediated field excitatory postsynaptic potentials (EPSPs) were measured in the presence of AMPA/kainate receptor antagonist CNQX (10 μ M). Stimulation at different intensities was tested and the fiber volley-EPSP slope curves were generated. Two-pathway experiments were performed in some cases to exclude the possibility that synaptic potentiation caused after amputation may be due to nonselective gradual increase in baseline responses. Independence of the two pathways was tested by paired-pulse facilitation (see Zhuo et al., 1993). While one train tetanic stimulation was delivered to the first pathway, the second, independent pathway received no training and served as a control. One-way analysis of variance (with Duncan's multiple range test for post hoc comparison) and Student's *t* test were used for statistical analysis.

Immunocytochemistry

After different treatments, rats or mice were deeply anesthetized with halothane and perfused transcardially with 50–100 ml saline followed by 150–500 ml of cold 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. The brain block, including hippocampus and lower lumbar and sacral spinal cord were removed, post-fixed for 4 h, and then cryoprotected by storing in a 30% sucrose, 0.1 M PB solution for 2 d at 4°C. Coronal brain and spinal cord sections (30- μ m thickness) were cut using a cryostat. Sections from sham-operated and experimental animals were simultaneously processed for immunostaining. Primary rabbit antibodies used included anti-*Egr1* (A310, 1:5,000; Day et al., 1990) and anti-c-Fos (1:20,000; Oncogene). Incubation with biotinylated goat anti-rabbit immunoglobulin (1:400, Vector) for 1 h was followed by incubation with avidin-biotin-peroxidase complexes (1:100, Vector) for 1 h. DAB with nickel was used as the final chromagen. Double-label immunostaining was done with anti-*Egr1* and combination of a monoclonal mouse anti-CaMKII α antiserum (1:1,000, Oncogene). Secondary antibodies conjugated to fluorescent markers FITC (1:50, used with *Egr1*) and Cy-3 (1:600, used with CaMKII α ; Jackson ImmunoResearch Laboratories) were used. Images of the CA1 areas of hippocampus sections at 0.7- μ m intervals with 20 \times lens were obtained with Bio-Rad Laboratories MRC 1000 laser-scanning confocal fluorescent imaging system.

Immunoprecipitation of *Egr1*

For two mice under brief anesthesia with halothane, amputation of 2.5-cm long tail segments was performed. Two control mice received only the same brief anesthesia. After 1 h, mice were killed and hippocampi were rapidly dissected and extracted in 1.2 ml ice-cold lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM pepstatin). Samples were sonicated three times and extracts were centrifuged (10,000 *g* for 15 min) to remove insoluble materials. Supernatants were incubated with the *Egr1*-specific monoclonal antibody 6H10 (Day et al., 1990) and protein A-Sepharose at 4°C overnight. The immunoprecipitates were washed three times with lysis buffer, separated on a SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with polyclonal anti-*Egr1* antisera (A310). This experiment was repeated twice with similar results.

Results

Hippocampal Pyramidal Cells Respond to Peripheral Noxious Stimuli in Adult Rats In Vivo

Previous studies using extracellular field recording or

spike-recording techniques revealed that neurons in the hippocampus show spike responses or mixed field potentials to peripheral noxious stimuli (Brankack and Buzsaki, 1986; Heale and Vanderwolf, 1994; Sinclair and Lo, 1986). However, due to technical limitations, several questions remain to be addressed: (a) it is unknown whether neuronal responses originate from CA1 pyramidal neurons or local inhibitory interneurons; and (b) spike recordings fail to reveal any subthreshold (below action potential firing threshold) EPSPs. Thus, it remains unclear whether peripheral noxious stimuli could induce EPSPs in hippocampal CA1 neurons. Although subthreshold EPSPs may not elicit action potentials, they could significantly affect the electrophysiological properties as well as plasticity of hippocampal neurons.

Intracellular recordings were performed from identified hippocampal CA1 pyramidal neurons in anesthetized adult rats ($n = 30$; Fig. 1 A). All neurons were identified with neurobiotin staining as CA1 pyramidal cells (Fig. 1 C). In >35% of recorded neurons (11/30), peripheral electrical stimulation of one hindpaw elicited EPSPs. The EPSPs were intensity-related and polysynaptic in nature (Fig. 1, B and D). These results provide the first direct evidence that hippocampal CA1 pyramidal neurons receive sensory, including nociceptive, inputs from the periphery.

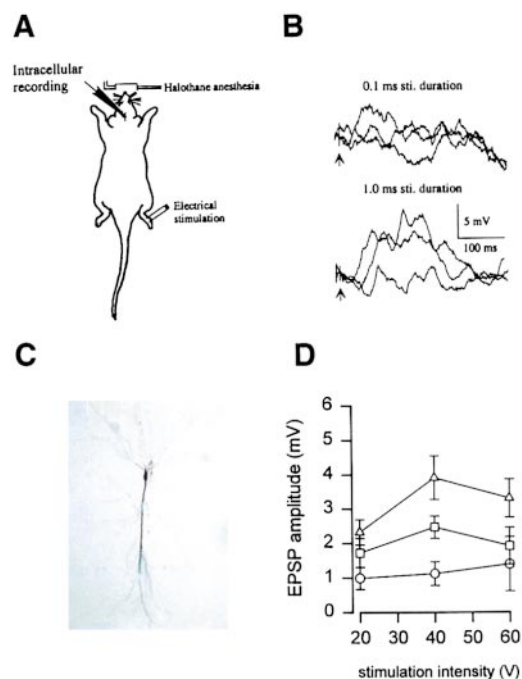


Figure 1. Peripheral noxious stimuli induced EPSPs from CA1 pyramidal neurons. (A) Diagram of an in vivo intracellular recording in an anesthetized rat. (C) An example of intracellularly stained CA1 pyramidal neuron (C). Representative traces (B) show the evoked responses of a CA1 neuron to stimuli of different durations. Each is the average of four traces. (Arrow) The stimulus artifact. (D) Plot of EPSP amplitude versus intensity of peripheral stimulation with different stimulus durations (triangles: 1.0 ms; squares: 0.5 ms; circles: 0.1 ms). Each point is the mean \pm SEM.

Amputation Causes Rapid Expression of *Egr1* in Hippocampus

Repetitive activation of excitatory glutamatergic synapses within the hippocampus activates various types of IEGs in postsynaptic neurons (Morgan and Curran, 1991; Bliss and Collingridge, 1993; Ginty et al., 1993; Deisseroth et al., 1996). In the hippocampus, *Egr1* is upregulated by strong synaptic activity, such as tetanic stimulation, which is known to induce LTP (Cole et al., 1989; Wisden et al., 1990). Because peripheral noxious shocks elicited EPSPs in hippocampal CA1 neurons, it is conceivable that prolonged noxious stimuli may activate *Egr1*. We tested whether tissue/nerve injury activated *Egr1* expression in hippocampal CA1 neurons. The central digit of a hindpaw in adult rats was removed. At 15 min after amputation, an increased level of *Egr1* protein was detected in the CA1 region of the hippocampus ($n = 5$, data not shown). The level of *Egr1* peaked at 45 min after amputation and remained high for at least 2 h ($n = 4-6$). This change in *Egr1* expression was regionally selective within the CA1 region of the hippocampus, as only minor changes were detected in the CA3 region or dentate gyrus (DG).

To use genetically manipulated mice, we tested whether similar changes could be observed in mice after tissue/nerve injury. Removal of the tip of a mouse tail induced an NMDA-dependent, long-lasting hyperalgesia in mice (Zhuo, 1998). Accordingly, the removal of a distal tail segment caused a significant increase of the expression of *Egr1* in the hippocampus, as revealed by both immunoprecipitation and immunocytochemistry (Fig. 2, A and B). Similar to our observations in rats, increased levels of *Egr1* were most dramatic in the CA1 region of the hippocampus (Fig. 2 B). To better demonstrate that *Egr1* activation occurred within CA1 pyramidal cells, we carried out double stainings with both *Egr1* and calcium/calmodulin-dependent protein kinase II (CaMKII). We found strong nuclear *Egr1* signal expression in CA1 pyramidal neurons visualized by CaMKII immunofluorescence ($n = 2$ mice, see Fig. 2 C for an example).

Only minor changes were observed in CA3 or DG (Table I). *Egr1* expression is selective for noxious stimuli; in experiments using non-noxious mechanical brush (with paintbrush for 12 s, $n = 3$) or non-noxious heating (at 40°C for 12 s, $n = 4$), we did not see any significant increase of *Egr1* expression in the hippocampus.

To determine whether *Egr1* was upregulated in neurons of the spinal dorsal horn or brainstem in response to injury, we examined its expression and that of *c-Fos* after amputation of the distal tail. While significant increases in *c-Fos* expression were observed in dorsal horn neurons, little or no change in *Egr1* levels was observed in the spinal cord (Fig. 2 F and Table I) or rostral ventromedial medulla (RVM; data not shown).

Peripheral Sensory Inputs and Activation of NMDA Receptors

Heightened *Egr1* expression caused by amputation was NMDA receptor dependent. Pretreatment with i.p. injected MK-801 (1 mg/kg, 30 min before amputation) attenuated amputation-induced increases in *Egr1* expression in the hippocampus (Fig. 2 D and Table I). In mice receiv-

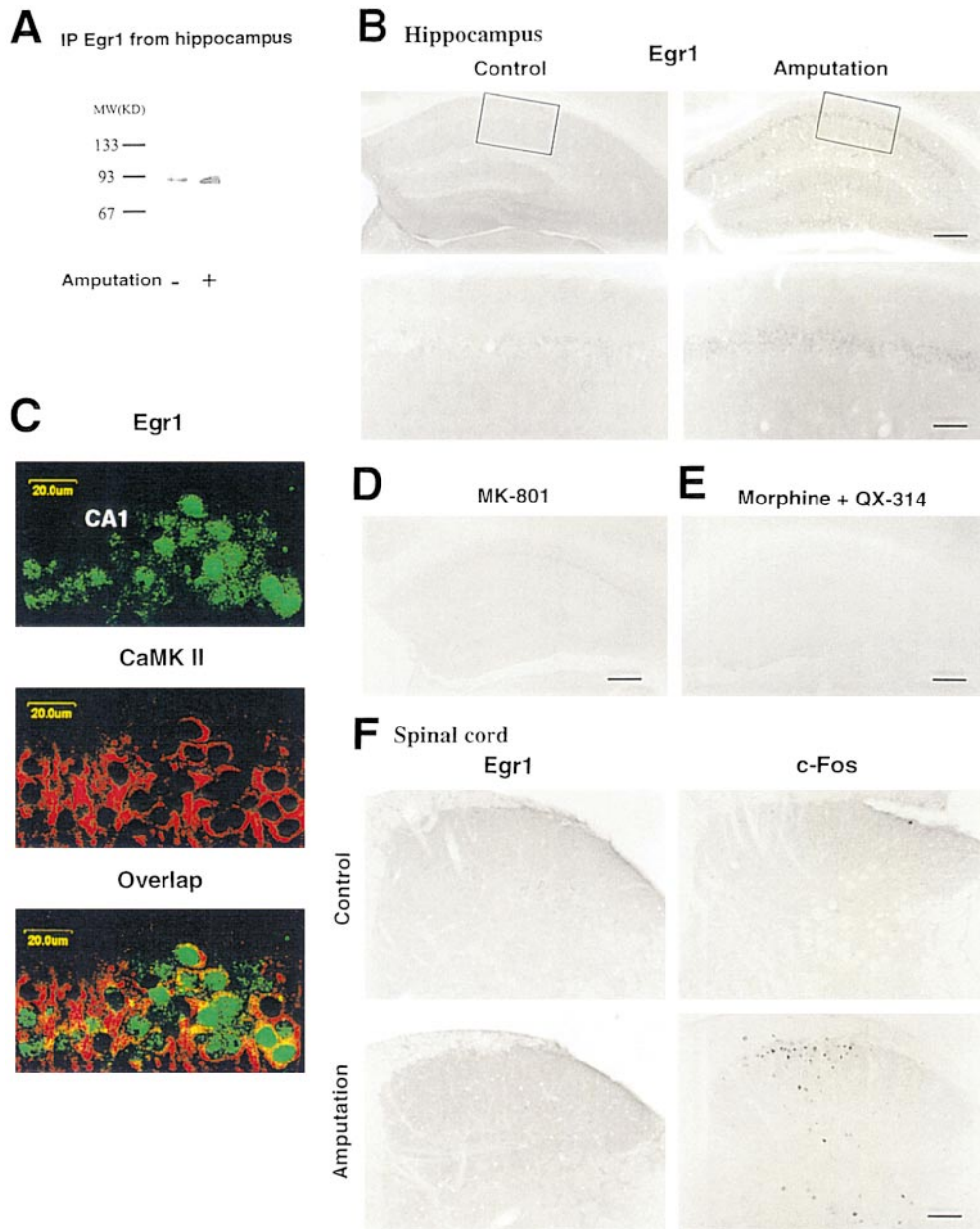


Figure 2. Amputation of a mouse distal tail segment increased hippocampal Egr1. Egr1 was isolated by immunoprecipitation from hippocampus and detected by Western blot in control mice (indicated by -) and mice 1 h after amputation (+). Increases in hippocampal Egr1 immunoreactivity at 45 min after amputation are compared with the hippocampus of normal mice (the bottom set of photos are high-magnification details of the indicated areas). Confocal images of double-labeled CA1 pyramidal neurons in the hippocampus of amputated mice for FITC-labeled Egr1 (green, top), Cy-3 labeled CaMKII (red, middle), and merged image (bottom) showing a strong nuclear Egr1 signal expression in many pyramidal neurons visualized by CaMKII immunofluorescence. (D) Pretreatment with MK-801 (1 mg/kg) almost completely blocked Egr1 activation. (E) Intraperitoneal morphine (10 mg/kg) and subcutaneous QX-314 (5%, 10 μ l) significantly decreased amputation-induced Egr1 activation. (F) Amputation increased c-Fos but not Egr1 immunoreactivity in the spinal dorsal horn. Bars: (B, top and E) 400 μ m; (B, bottom) 150 μ m; (F) 100 μ m.

ing i.p. morphine (10 mg/kg; 30 min before amputation) and local anesthetic blockade with subcutaneously applied QX-314 (5%; 10 μ l, 10 min before), the amputation induced significantly less Egr1 expression in the hippocampus (Fig. 2 E and Table I). These findings indicate that sensory inputs during tissue/nerve injury were critical for the induction of Egr1.

Plasticity within the Hippocampus after Amputation

Could changes in Egr1 expression also be accompanied by plastic changes in excitatory transmission in the CA1 region? Two major forms of synaptic plasticity have been reported in the hippocampus: LTP and LTD (Bear and Malenka, 1994). First, we measured LTP induced by a single tetanic stimulation (100 Hz, 1 s). In slices from sham-operated mice, a single tetanic stimulation induced only

a small synaptic potentiation that lasted \sim 1 h ($n = 6$, $141.2 \pm 15.4\%$ of control at 1 h after stimulation; Fig. 3 A). In contrast, a significantly greater potentiation was induced in slices prepared from mice 45 min after tail tip amputation ($n = 6$, $259.3 \pm 40.4\%$, $t(10) = 2.73$, $P < 0.05$ compared with sham mice; Fig. 3 A). This potentiation lasted for at least 2–4 h ($n = 3$; see Fig. 3 B for an example). To demonstrate that synaptic enhancement was input specific, we also performed two-pathway experiments. While one train tetanic stimulation caused prolonged enhancement in one pathway ($n = 4$, $189.4 \pm 3.4\%$), synaptic responses recorded from the second, independent pathway remained unchanged ($n = 4$, $91.5 \pm 9.8\%$; Fig. 3 C). The effect of amputation on synaptic potentiation was time related. We also carried out experiments using mice killed at three different time points after amputation (\sim 0, 20, and 120 min after amputation). As shown in Fig. 3 D,

Table 1. Tail Amputation-induced Enhancement of Egr1 and c-Fos Immunoreactivities in the Hippocampus and Spinal Dorsal Horn of Mice and Effects of Systemically Morphine or MK-801 and Local Anesthesia on Both Expressions

IEGs	Hippocampus			Spinal dorsal horn
	CA1	CA3	DG	
Egr1				
15 min post amputation (n = 4)	++	+	-	-
45 min post amputation (n = 4)	+++	+	-	-
2 h post amputation (n = 4)	+++	-	-	-
24 h post amputation (n = 4)	++	-	-	-
45 min post-MK-801 (n = 2)	+	-	-	-
45 min post-morphine + QX-314 (n = 3)	+	-	-	-
45 min post-MK-801 + morphine + QX-314 (n = 3)	-	-	-	-
c-Fos				
15 min post amputation	-	-	-	+
45 min post amputation	+	-	+	+++
2 h post amputation	+	+	-	+++
24 h post amputation	-	-	-	+
45 min post-MK-801	+	+	+	++
45 min post-morphine + QX-314	-	-	-	+
45 min post-MK-801 + morphine + QX-314	-	-	+	+

The change in level is rated high (+++), moderate (++), weak (+), or undetectable (-) compared to that in control animals.

we found no significant enhancement of synaptic potentiation induced by one train tetanic stimulation at either 0 min or 20 min after amputation. A similar amount of enhancement was observed in slices prepared at 120 min after amputation (n = 5, 270.3 ± 37.8%) relative to that at 45 min after amputation (n = 6, 258.2 ± 37.9%).

To test if the conscious experience of pain during the 45 min between the tail amputation and decapitation contribute to the observed alterations in hippocampal synaptic potentiation, we kept mice anesthetized throughout the 45 min between amputation and slice preparation in some experiments. Interestingly, we found that no significant enhancement of synaptic potentiation (n = 4, 131.5 ± 3.7%; no significant difference from slices of sham-treated animals). In addition, we measured Egr1 activation in hippocampus from mice receiving continuous halothane anesthesia after amputation and found that activation of Egr1 was completely blocked in these mice (n = 2).

LTD induced by low frequency stimulation (1 Hz, 15 min) was not affected by amputation (sham: n = 7, 66.8 ± 13.4% of control at 30 min after stimulation; amputated: n = 5, 50.3 ± 11.8%, no significant difference between the two groups; Fig. 3 E). To detect possible frequency-dependent changes, we applied the same number of pulses (n = 900) at two additional frequencies, 5 and 10 Hz. No significant difference was found between sham-operated and amputated mice (Fig. 3, F and G). Basal synaptic responses were not significantly different between sham (n = 25 slices/10 mice) and amputated animals (45 min after the amputation; n = 30 slices/15 mice, data not shown). Furthermore, paired-pulse facilitation, an indication of

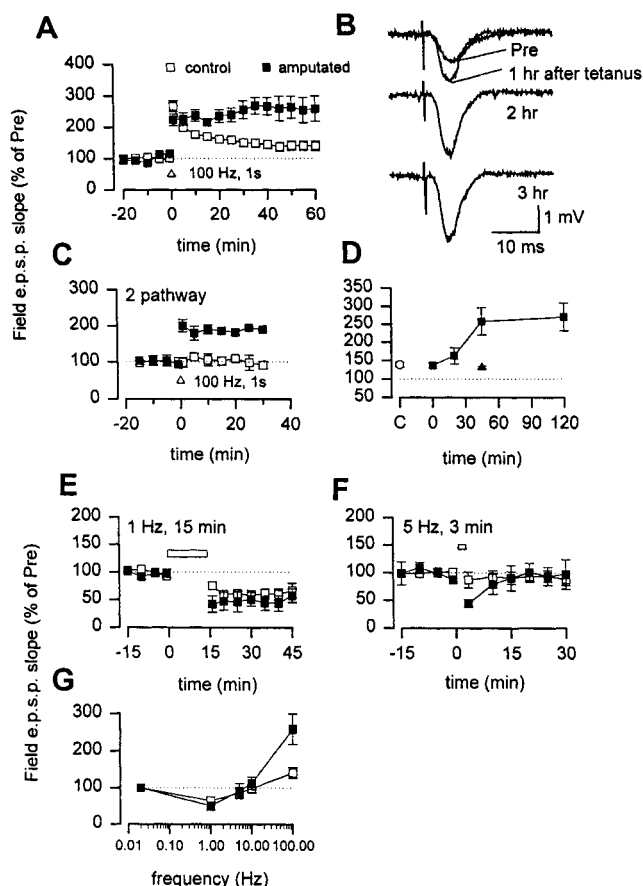


Figure 3. Amputation affected hippocampal LTP but not LTD. A single tetanic stimulation (100 Hz, 1 s) produced short-term potentiation in normal mice (n = 6, open squares). But 45 min after amputation, tetanic stimulation caused enhanced synaptic potentiation lasting for at least 60 min (n = 6, filled squares). An example illustrates that synaptic potentiation in slices prepared from amputated mice persisted for at least 3 h. Synaptic potentiation is input specific. As in A, a single tetanic stimulation induced enhanced potentiation (n = 4, filled squares) but synaptic responses at the second, independent pathway remained unaffected (open squares). Summarized time course curve of the effect of amputation on synaptic potentiation induced by one train tetanic stimulation (filled squares; open circle indicates sham-animals). Keeping mice under general anesthesia during the 45 min between amputation and decapitation prevented synaptic potentiation caused by amputation (filled triangles). LTD was not affected by amputation (control, n = 7, open squares; amputated, n = 5, filled squares). Synaptic responses to 5 Hz stimulation (for 3 min) also revealed no difference (control: n = 5, 80.0 ± 14.5%, open squares; amputated: n = 4, 94.2 ± 20.5%, filled squares). Summary of frequency-dependent responses.

possible presynaptic changes, was also not affected (sham: n = 9; amputated: n = 16, data not shown).

Activation of Egr1 in Hippocampal Slices

What is the molecular mechanism contributing to the enhancement of synaptic potentiation after amputation? We hypothesized that NMDA receptor-dependent Egr1 activation may play an important role in the synaptic enhancement caused by amputation. To test this, we first deter-

mined whether hippocampal slices could be used to detect possible changes in gene expression caused by amputation. Studies from different regions of the central nervous system have shown that some physiological changes can be detected using an in vitro brain slice technique (Shors et al., 1989; Kirkwood et al., 1996; Malenka and Nicoll, 1997; Wei et al., 1999). In hippocampal slices incubated in a recording chamber for at least 2 h, we found that levels of Egr1 were not upregulated ($n = 6$, data not shown). Bath application of glutamate (100 μ M) increased Egr1 in CA1 neurons ($n = 7$). Moreover, activation of NMDA receptors was critical for this upregulation, because pretreatment with 50 μ M AP-5 blocked Egr1 activation ($n = 6$). Thus, activation of Egr1 in vitro was also NMDA receptor dependent, as shown in vivo.

Hippocampal Synaptic Potentiation and Depression in Mice Lacking Egr1

Second, we wanted to determine if Egr1 contributes to hippocampal LTD and LTP using mice lacking Egr1 (Lee et al., 1995). LTD induced by a prolonged, 1 Hz stimulation (for 15 min) was not affected in mutant mice (wild-type: $n = 8$, $60.0 \pm 10.5\%$; mutant: $n = 6$, $75.2 \pm 7.5\%$, no significant difference between two groups; Fig. 4 A). Synaptic responses to repetitive stimulation at two other frequencies (5 and 10 Hz) were also not affected (Fig. 4 B; data with 10 Hz not shown). Moreover, as shown in Fig. 4 C, no significant difference in LTP induced by a single tetanic stimulation was found between the two groups (wild-type: $n = 8$, $121.8 \pm 10.2\%$; mutant: $n = 5$, $127.5 \pm 8.2\%$). Next, we sought to determine whether Egr1 is important for the enhancement of LTP caused by amputation (see Fig. 3 A). In both wild-type and mutant mice, we used alternative method for genotyping (i.e., by PCR analysis of genomic DNA extracted from mouse ear tissue). We performed experiments on Egr1-deficient and wild-type mice after amputation of the distal tail. In contrast to wild-type mice ($n = 5$, $238.3 \pm 36.1\%$ of control at 45 min after tetanic stimulation), amputation in mutant mice failed to cause enhancement of LTP ($n = 6$, $125.8 \pm 8.8\%$, $t(9) = 3.02$, $P < 0.01$ compared with wild-type mice; Fig. 4, D and E). This result suggests that Egr1 upregulation induced by amputation is critical for amputation-induced enhancement of LTP. Pharmacological inhibition of Egr1 activation with i.p. MK-801 (1 mg/kg, 30 min before amputation) revealed similar results ($n = 4$).

Egr1 Contributes to Late-Phase LTP

What could be the possible physiological functions of Egr1 in hippocampal plasticity? One possible function of Egr1 is to contribute to LTP. We performed several additional electrophysiological experiments in wild-type and mutant mice. First, we measured paired-pulse facilitation at different interpulse intervals. No significant difference was found between wild-type ($n = 6$ mice) and mutant mice ($n = 8$ mice; Fig. 5 A). Basal synaptic responses to stimulation were also not significantly different between wild-type ($n = 18$ slices/11 mice) and mutant animals ($n = 19$ slices/12 mice). However, late-phase LTP induced by a four train tetanic stimulation (39–40) was significantly decreased in mutant mice (wild-type: $n = 9$, $210.7 \pm 21.3\%$;

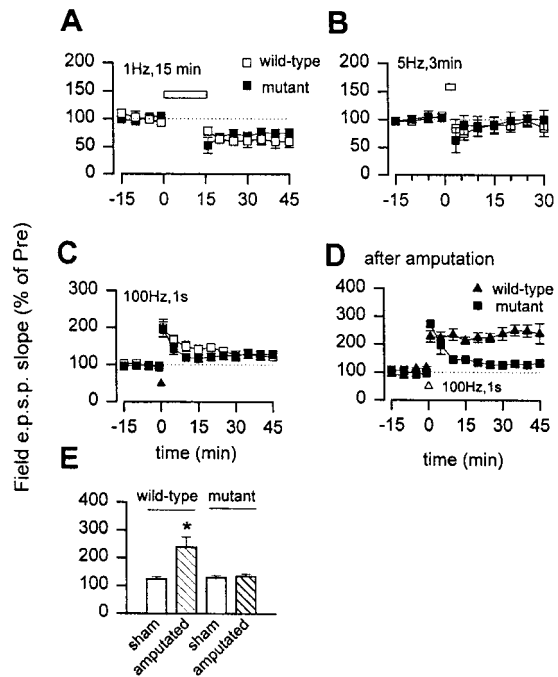


Figure 4. Hippocampal LTP and LTD in mice lacking Egr1. LTD was normal in mutant mice (wild-type, $n = 8$, open squares; mutant, $n = 6$, filled squares). Synaptic responses to 5 Hz stimulation was also normal (wild-type, $n = 4$, open squares; mutant, $n = 5$, filled squares). Synaptic potentiation induced by a single tetanic stimulation was similar (wild-type, $n = 8$, open squares; mutant, $n = 5$, filled squares). Amputation caused no synaptic enhancement of LTP in mutant mice (wild-type, $n = 5$, open squares; mutant, $n = 6$, filled squares). Summarized data of different treatments on the enhancement of LTP caused by amputation.

mutant: $n = 5$, $132.6 \pm 29.0\%$, $t(12) = 2.18$, $P < 0.05$; Fig. 5 D). Pharmacological experiments using 100 μ M AP-5 demonstrated that the NMDA receptor is essential for induction and expression of late-phase LTP ($n = 4$, $92.2 \pm 13.2\%$, Fig. 5 C). To detect if NMDA receptor-mediated responses may be affected in mutant mice, we measured NMDA receptor-mediated EPSPs in the presence of the AMPA/kainate receptor antagonist CNQX (10 μ M). We found no significant difference between wild-type ($n = 9$ slices/6 mice) and mutant animals ($n = 6$ slices/5 mice; Fig. 5 B), indicating that NMDA receptor function is not significantly affected. Finally, we also measured late-phase LTP of mutant slices in the presence of picrotoxin (100 μ M) and found a similar defect in late-phase LTP ($n = 4$, $138.9 \pm 8.3\%$). These results suggest the observed defect in late-phase LTP in mutant mice is not due to changes in NMDA receptor functions or inhibitory tone.

Discussion

In this study, we show that noxious somatosensory stimuli elicit both EPSPs and elevations in Egr1 expression in hippocampal CA1 neurons. The same maneuver, tail tip amputation, that led to the latter result also led to an enhancement of synaptic potentiation in the CA1 region. Moreover, this enhancement of plasticity was not present

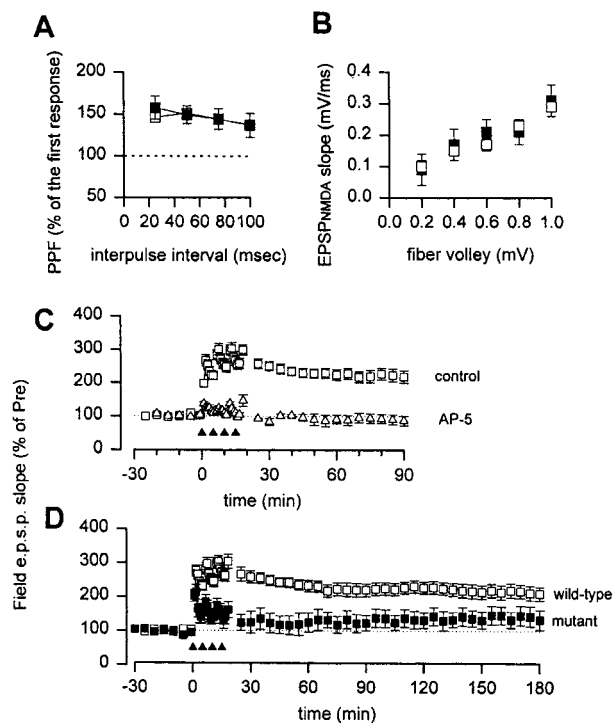


Figure 5. *Egr1* contributes to NMDA receptor-dependent late-phase LTP. (A) Wild-type ($n = 6$, open squares) and mutant slices ($n = 8$, filled squares) showed no significant difference in paired-pulse facilitation of the field EPSP at different interpulse intervals. (B) Wild-type ($n = 9$, open squares) and mutant slices ($n = 6$, filled squares) showed no significant difference in NMDA receptor-mediated EPSPs. (C) The induction of late-phase LTP in wild-type mice was completely blocked by 100 μ M AP-5 in bath solution ($n = 4$). (D) Late-phase LTP was significantly decreased in mutant mice (wild-type, $n = 9$, open squares; mutant, $n = 5$, filled squares).

in mice lacking *Egr1*. Finally, late-phase LTP but not early-phase LTP or LTD was abolished in *Egr1* knockout mice.

It may not be readily evident why tail tip amputation should induce changes in hippocampal physiology different from decapitation, which is performed during hippocampal slice preparation. We hypothesized that after an extremely painful stimulus, such as tail tip amputation, a mouse, awake and alert after recovering from the brief, light anesthesia, would react in many ways, in particular, that changes may occur in susceptibility to synaptic plasticity in the hippocampus. According to this model, decapitation would induce none of these effects, the animal never experiences that stimulation consciously. Supporting this hypothesis, we found that keeping mice under halothane anesthesia between tail amputation and decapitation blocked the enhancement of synaptic potentiation. Furthermore, experiments using slices harvested at different time points after amputation consistently demonstrated that a certain period of consciousness is required for synaptic enhancement to occur. Slices were allowed to recover in the recording chamber for at least 2 h (often more time elapsed before the beginning of a given recording). Since 45 min between amputation and slice preparation

was sufficient time to achieve a maximal effect on hippocampal synaptic potentiation, more than sufficient time elapsed after slice preparation to allow any similar effect of that maneuver to take hold. Taken together, these observations lend strong support to our conclusion that the difference observed between sham-operated and amputated animals was specifically a result of the physiological responses of a conscious animal during the minutes following severe tissue/nerve injury.

Electrical and Biochemical Responses of Hippocampal Neurons to Somatosensory Stimuli

We demonstrate in this study that >35% of hippocampal CA1 pyramidal cells respond to peripheral noxious shocks, providing the first intracellular *in vivo* recordings to support previous observations implicating the hippocampus in pain-related physiological functions. Previous electrophysiological evidence using extracellular recording techniques failed to determine which neurons were the source of the signal recorded. In addition, peripheral stimulation generates many subthreshold depolarizations in CA1 neurons which would not generate spikes but may nonetheless influence the summated output.

Interestingly, these EPSPs showed intensity-related responses, that is, larger EPSPs were observed with higher-intensity peripheral electrical shocks. These findings suggest that these neurons may also encode the intensity of stimulation. Gentle touch, however, did not cause any significant responses. We should point out that not all CA1 neurons responded to peripheral noxious shocks, suggesting that only a subpopulation of CA1 neurons are responsive to noxious stimuli. Hippocampal neurons receive inputs from many areas of the CNS, including the rostro-ventral medulla (Vertes and Kocsis, 1997), suggesting that there are multiple potential pathways through which these hippocampal CA1 neurons can be activated.

Hippocampal neurons also respond to somatosensory stimuli by changes in gene expression. Mixed changes in the expression of *c-Fos* in the hippocampus after tissue injury has been reported previously, including increases after subcutaneous formalin injection (Aloisi et al., 1997) or decreases after noxious tooth pulp stimulation (Funahashi et al., 1999). However, *c-Fos* seems not to play an important role in hippocampal synaptic transmission and plasticity. For example, mice lacking *c-Fos* showed normally early- and late-phase LTP (unpublished observation). In this study, using a different animal model, we showed that tissue injury (amputation of a single digit in rats or distal tail in mice) causes rapid expression of *Egr1* in the hippocampus, including CA1 neurons. In awake animals, activation of *Egr1* requires noxious stimulation. Non-noxious stimuli, such as mechanical brush and warm thermal heating, failed to induce significant *Egr1* expression in the hippocampus. Most of the expression was seen in the CA1 area but not DG of the hippocampus.

Activation of NMDA receptors was important for the expression of *Egr1* caused by tissue injury *in vivo* or bath application of glutamate to hippocampal slices *in vitro*. These results are consistent with previous studies in rats showing that *Egr1* expression was induced by synaptic activity through NMDA receptors (see Introduction). In the

spinal cord, we found that Egr1 was not significantly activated in dorsal horn sensory neurons. These results differ from a previous report using heat-induced tissue injury (Wisden et al., 1990), suggesting that amputation may activate central pathways distinct from those activated by some other types of tissue injury (see Zhuo, 1998, for discussion).

Egr1 Contributes to Hippocampal Synaptic Potentiation

Despite the well-documented, activity-dependent stimulation of Egr1 expression in hippocampal neurons, no report is available concerning a possible contribution of Egr1 to NMDA receptor-dependent synaptic plasticity within the hippocampus. In this study, using mice lacking Egr1 gene, we present the first evidence that Egr1 is important for late-phase LTP. This effect of Egr1 deletion is relatively selective. LTP induced by a single tetanic stimulation or LTD were normal in mice lacking Egr1. Furthermore, other basic electrophysiological properties of these synapses seem to be normal (e.g., paired-pulse facilitation, basal field EPSPs and NMDA receptor-mediated EPSPs). It is obviously important to identify, in future studies, further cellular target proteins downstream from Egr1 which may contribute to an enhancement of synaptic potentiation. Although Egr1 is also upregulated by seizure (Mack et al., 1990), genetic deletion of Egr1 in mice seems to have no effect on the rate of kindling and associated mossy fiber sprouting in the hippocampus (Zhang et al., 1998).

Synaptic Enhancement after Amputation

One typical question for gene-related pathways is whether they are activated under physiological/pathological conditions and, upon activation, how they may affect the properties of central synapses. This study provides a possible answer for Egr1 in the hippocampus. We found that activation of Egr1 within the hippocampus occurs after tissue injury. Parallel with the activation of Egr1 by tissue injury, changes in synaptic plasticity obtained from hippocampal slices from animals with tissue injury were observed. A single tetanic stimulation, which normally induced moderate synaptic potentiation, induced a larger and longer-lasting potentiation in the CA1 area of the hippocampus after amputation. Interestingly, we found that keeping amputated animals anesthetized throughout the 45 min between amputation and hippocampal slice preparation prevented synaptic enhancement. Consistent with electrophysiological observations, the activation of Egr1 by amputation was also blocked. Halothane is known to decrease central neuronal excitability by inhibiting excitatory glutamatergic transmission and enhancing inhibitory transmission in rats and mice (Jones et al., 1992; Jones and Harrison, 1993; Perousansky et al., 1995; Kirson et al., 1998). It is likely that halothane inhibits glutamatergic transmission (such as the activation of NMDA receptors) and prevents the activation of Egr1, which is required for synaptic enhancement caused by amputation.

The other forms of synaptic plasticity tested were not affected, such as paired-pulse facilitation and LTD. These changes are not likely due to general stress during or after the amputation. Hippocampal synaptic plasticity is differentially affected in animals under behavioral stress com-

pared with animals after tail amputation. After behavioral stress, hippocampal LTP was inhibited (Shors et al., 1989; Diamond et al., 1994) and LTD was facilitated (Xu et al., 1997), suggesting that distinct mechanisms are involved.

We believe that the synaptic enhancement after amputation did not reflect a generally elevated neuronal excitability. First, in experiments using two-pathway stimulation in the same slice, we showed that synaptic enhancement was selectively observed in the pathway receiving a single tetanic stimulation. Synaptic responses were not affected at the second pathway. Second, we have showed that basal responses to stimulation as well as paired-pulse facilitation were not affected after amputation. Finally, if neuronal excitability were uniformly enhanced, we might be expected to see less LTP (due to an occlusive, or saturating, effect); instead, we observed the opposite effect (enhanced LTP).

We also further test the possible relationship between activated Egr1 and changes in synaptic plasticity using mice lacking Egr1. Interestingly, the enhancement of potentiation caused by a single tetanic stimulation was blocked in mice lacking Egr1. These results suggest that Egr1 or Egr1-related signaling pathways could serve as a temporary marker within neurons for peripheral tissue injury.

Physiological Significance

Although the evidence we have so far does not allow us to assign confidently any physiological role to Egr1 *in vivo*, we suggest that Egr1 might serve as an important molecule for nociception or pain-related plasticity within the hippocampus. Egr1 may not only serve as a signaling molecule downstream from the NMDA receptor, thereby contributing to late-phase LTP, but it may also associate sensory nociceptive, non-spatial information with spatial memory. The hippocampus and related structures are known to play a critical role in spatial as well as non-spatial memory formation (Squire and Zola-Morgan, 1991; Squire, 1992; Eichenbaum, 1999). Such an association may explain why a patient retains a vivid memory of the place where an injury was sustained. Second, the hippocampus may also affect the perception of pain or pain-related unpleasantness. Lesion of the hippocampus or reversible blockade of hippocampal neuronal activity are reported to affect behavioral nociceptive responses in animals (Jackson and Regestein, 1979; Plaznik et al., 1983; McKenna and Melzack, 1992) and humans (Gol and Faibisch, 1966, 1967). Our results provide evidence at molecular and cellular levels that the hippocampus may play a role in pain memory.

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References

Aloisi, A.M., M. Zimmermann, and T. Herdegen. 1997. Sex-dependent effects of formalin and restraint on c-Fos expression in the septum and hippocam-

- pus of the rat. *Neuroscience*. 81:951–958.
- Bear, M.F., and R.C. Malenka. 1994. Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.* 4:389–399.
- Berger, T.W., B. Alger, and R.F. Thompson. 1976. Neuronal substrate of classical conditioning in the hippocampus. *Science*. 192:483–485.
- Berger, T.W., R.I. Laham, and R.F. Thompson. 1980. Hippocampal unit-behavior correlations during classical conditioning. *Brain Res.* 193:229–248.
- Berger, T.W., P.C. Rinaldi, D.J. Weisz, and R.F. Thompson. 1983. Single-unit analysis of different hippocampal cell types during classical conditioning of rabbit nictitating membrane response. *J. Neurophysiol.* 50:1197–1219.
- Bliss, T.V., and G.L. Collingridge. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*. 361:31–39.
- Brankack, J., and G. Buzsaki. 1986. Hippocampal response evoked by tooth pulp and acoustic stimulation: depth profiles and effect of behavior. *Brain Res.* 378:303–314.
- Cole, A.J., D.W. Saffen, J.M. Baraban, and P.F. Worley. 1989. Rapid increase of an immediate-early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature*. 340:474–476.
- Day, M.L., T.J. Fahrner, S. Aykent, and J. Milbrandt. 1990. The zinc finger protein NGFI-A exists in both nuclear and cytoplasmic forms in nerve growth factor-stimulated PC 12 cells. *J. Biol. Chem.* 265:15253–15260.
- Deisseroth, K., H. Bitto, and R.W. Tsien. 1996. Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron*. 16:89–101.
- Diamond, D.M., M. Fleshner, and G.M. Rose. 1994. Psychological stress repeatedly blocks hippocampal primed burst potentiation behaving rats. *Behav. Brain Res.* 62:1–9.
- Dudek, S.M., and M.F. Bear. 1992. Homosynaptic long-term depression in area CA1 of hippocampus and effects of *N*-methyl-D-aspartate receptor blockade. *Proc. Natl. Acad. Sci. USA*. 89:4363–4367.
- Eichenbaum, H. 1999. Conscious awareness, memory and the hippocampus. *Nat. Neurosci.* 2:775–776.
- Eichenbaum, H., P. Dudchenko, E. Wood, M. Shapiro, and H. Tanila. 1999. The hippocampus, memory, and place cells: is it spatial memory or a memory space? *Neuron*. 23:209–226.
- Funahashi, M., Y.F. He, T. Sugimoto, and R. Matsuo. 1999. Noxious tooth pulp stimulation suppresses *c-fos* expression in the rat hippocampal formation. *Brain Res.* 827:215–220.
- Gashler, A., and V.P. Sukhatme. 1995. Early growth response protein 1 (*Egr1*): prototype of a zinc-finger family of transcription factors. *Prog. Nucleic Acid Res. Mol. Biol.* 50:191–224.
- Ginty, D.D., J.M. Kornhauser, M.A. Thompson, H. Bading, K.E. Mayo, J.S. Takahashi, and M.E. Greenberg. 1993. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science*. 260:238–241.
- Gol, A., and G.M. Faibisch. 1966. Hippocampectomy for relief of intractable pain. *Tex. Med.* 62:76–79.
- Gol, A., and G.M. Faibisch. 1967. Effects of human hippocampal ablation. *J. Neurosurg.* 26:390–398.
- Heale, V.R., and C.H. Vanderwolf. 1994. Dentate gyrus and olfactory bulb responses to olfactory and noxious stimulation in urethane anaesthetized rats. *Brain Res.* 652:235–242.
- Jackson, W.J., and Q.R. Regestein. 1979. Hippocampal lesions impair prolonged titrated avoidance by rhesus monkeys. *Exp. Neurol.* 63:28–34.
- Jirsa, R., P. Poc, and T. Radil. 1992. Hippocampal auditory evoked response threshold in the rat: behavioral modulation. *Brain Res. Bull.* 28:149–153.
- Jones, M.V., and N.L. Harrison. 1993. Effects of volatile anesthetics on the kinetics of inhibitory postsynaptic currents in cultured rat hippocampal neurons. *J. Neurophysiol.* 70:1339–1349.
- Jones, M.V., P.A. Brooks, and N.L. Harrison. 1992. Enhancement of gamma-aminobutyric acid-activated Cl-currents in cultured rat hippocampal neurons by three volatile anesthetics. *J. Physiol.* 449:279–293.
- Kandel, E.R. 1997. Genes, synapses, and long-term memory. *J. Cell. Physiol.* 173:124–125.
- Kirkwood, A., M.G. Rioult, and M.F. Bear. 1996. Experience-dependent modification of synaptic plasticity in visual cortex. *Nature*. 381:526–528.
- Kirson, E.D., Y. Yaari, and M. Perouansky. 1998. Presynaptic and postsynaptic actions of halothane at glutamatergic synapses in the mouse hippocampus. *Brit. J. Pharmacol.* 124:1607–1614.
- Lee, S.L., L.C. Tourtellotte, R.L. Wesselschmidt, and J. Milbrandt. 1995. Growth and differentiation proceeds normally in cells deficient in the immediate-early gene NGFI-A. *J. Biol. Chem.* 270:9971–9977.
- Luntz-Leybman, V., P.C. Bickford, and R. Freedman. 1992. Cholinergic gating of response to auditory stimuli in rat hippocampus. *Brain Res.* 587:130–136.
- Mack, K., M. Day, J. Milbrandt, and D.I. Gottlieb. 1990. Localization of the NGFI-A protein in the rat brain. *Mol. Brain Res.* 8:177–180.
- Malenka, R.C., and R.A. Nicoll. 1997. Learning and memory: never fear, LTP is hear. *Nature*. 390:552–553.
- Malenka, R.C., and R.A. Nicoll. 1999. Long-term potentiation—a decade of progress? *Science*. 285:1870–1874.
- McKenna, J.E., and R. Melzack. 1992. Analgesia produced by lidocaine microinjection into the dentate gyrus. *Pain*. 49:105–112.
- Milbrandt, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science*. 238:797–798.
- Morgan, J.L., and T. Curran. 1991. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu. Rev. Neurosci.* 14:421–451.
- Muller, R.U., J.L. Kubie, and J.B. Ranck, Jr. 1987. Spatial firing patterns of hippocampal complex-spike cells in a fixed environment. *J. Neurosci.* 7:1935–1950.
- Nadel, L. 1991. The hippocampus and space revisited. *Hippocampus*. 1:221–229.
- O'Keefe, J., and L. Nadel. 1978. The hippocampus as a cognitive map. Oxford University Press, London.
- Perousansky, M., D. Baranov, M. Salman, and Y. Yaari. 1995. Effects of halothane on glutamate receptor-mediated excitatory postsynaptic currents. A patch-clamp study in adult mouse hippocampal slices. *Anesthesiology*. 83:109–119.
- Plaznik, A., W. Danysz, and W. Kostowski. 1983. Some behavioral effects of microinjections of noradrenaline and serotonin into the hippocampus of the rat. *Physiol. Behav.* 31:625–631.
- Sakurai, Y. 1994. Involvement of auditory cortical and hippocampal neurons in auditory working memory and reference memory in the rat. *J. Neurosci.* 14:2606–2623.
- Shors, T.J., T.B. Seib, S. Levine, and R.F. Thompson. 1989. Inescapable versus escapable shock modulates long-term potentiation in the rat hippocampus. *Science*. 244:224–226.
- Sinclair, J.G., and G.F. Lo. 1986. Morphine, but not atropine, blocks nociceptor-driven activity in rat dorsal hippocampal neurons. *Neurosci. Lett.* 68:47–50.
- Squire, L.R. 1992. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychol. Rev.* 99:195–231.
- Squire, L.R., and S. Zola-Morgan. 1991. The medial temporal lobe memory system. *Science*. 253:1380–1386.
- Stevens, C.F. 1996. Spatial learning and memory: the beginning of a dream. *Cell*. 87:1147–1148.
- Tamura, R., T. Ono, M. Fukuda, and K. Nakamura. 1992. Spatial responsiveness of monkey hippocampal neurons to various visual and auditory stimuli. *Hippocampus*. 2:307–322.
- Vertes, R.P., and B. Kocsis. 1997. Brainstem-diencephalo-septohippocampal systems controlling the theta rhythm of the hippocampus. *Neuroscience*. 81:893–926.
- Wei, F., P. Li, and M. Zhuo. 1999. Loss of synaptic depression in mammalian anterior cingulate cortex following amputation. *J. Neuroscience*. 19:9346–9354.
- Weiss, C., M.A. Kronforst-Collins, and J.F. Disterhoft. 1996. Activity of hippocampal pyramidal neurons during trace eyeblink conditioning. *Hippocampus*. 6:192–209.
- Wisden, W., M.L. Errington, S. Williams, S.B. Dunnett, C. Waters, D. Hitchcock, G. Evan, T.V.P. Bliss, and S.P. Hunt. 1990. Differential expression of immediate-early genes in the hippocampus and spinal cord. *Neuron*. 4:603–614.
- Xu, L., R. Anwyl, and M.J. Rowan. 1997. Behavioral stress facilitates the induction of long-term depression in the hippocampus. *Nature*. 387:497–500.
- Zhang, D., L.S. Butler, and J.O. McNamara. 1998. Kindling and associated mossy fibre sprouting are not affected in mice deficient of NGFI-A/NGFI-B genes. *Neuroscience*. 83:251–258.
- Zhuo, M., S.A. Small, E.R. Kandel, and R.D. Hawkins. 1993. Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. *Science*. 260:1946–1950.
- Zhuo, M. 1998. NMDA receptor-dependent long term hyperalgesia after tail amputation in mice. *Eur. J. Pharmacol.* 349:211–220.