Long-term potentiation increases tyrosine phosphorylation of the N-methyl-d-aspartate receptor subunit 2B in rat dentate gyrus in vivo

Kobi Rosenblum*, Yadin Dudai*†, and Gal Richter-Levin‡

*Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel; and ‡Department of Psychology, Haifa University, Haifa 31905, Israel

Communicated by Eric R. Kandel, Columbia University, New York, NY, June 4, 1996 (received for review January 26, 1996)

ABSTRACT Long-term potentiation (LTP) is a form of synaptic memory that may subserve developmental and behavioral plasticity. An intensively investigated form of LTP is dependent upon N-methyl-d-aspartate (NMDA) receptors and can be elicited in the dentate gyrus and hippocampal CA1. Induction of this type of LTP is triggered by influx of Ca²⁺ through activated NMDA receptors, but the downstream mechanisms of induction, and even more so of LTP maintenance, remain controversial. It has been reported that the function of NMDA receptor channel can be regulated by protein tyrosine kinases and protein phosphatases and that inhibition of protein tyrosine kinases impairs induction of LTP. Herein we report that LTP in the dentate gyrus is specifically correlated with tyrosine phosphorylation of the NMDA receptor subunit 2B in an NMDA receptor-dependent manner. The effect is observed with a delay of several minutes after LTP induction and persists in vivo for several hours. The potential relevance of this post-translational modification to mechanisms of LTP and circuit plasticity is discussed.

Long-term potentiation (LTP) is regarded as a form of synaptic memory that may subserve experience-dependent plasticity at the circuit and behavioral level (1). Although the relevance of experimentally induced LTP to normal brain function remains uncertain (2, 3), there is widespread agreement that cellular cascades recruited in LTP play a role in brain development (4, 5) and in learning (6–8). An intensively studied form of LTP is dependent upon N-methyl-d-aspartate (NMDA) receptors and can be elicited in the dentate gyrus and hippocampal CA1. Induction of this type of LTP is triggered by influx of Ca²⁺ through activated NMDA receptors, but the downstream mechanisms of induction, and even more so of LTP maintenance, are controversial (1, 6). These mechanisms may involve modulation of protein kinases and phosphatases, long implicated in short- and intermediate-term neuronal plasticity (9–11). Major candidates are ubiquitous serine/threonine protein kinases (12). Activation of Ca²⁺/calmodulin-dependent protein kinase II (CAM kinase II) has been specifically implicated in induction, and CAM kinase II up-regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors in induction and possibly in maintenance of NMDA receptor-dependent LTP (6, 13).

Protein tyrosine kinase have also been implicated in LTP; thus, it has been reported that inhibitors of tyrosine kinases impair LTP induction in the dentate gyrus and CA1 (14, 15) and that knockout of the tyrosine kinase gene fyn impairs LTP in CA1 (16). However, protein tyrosine kinase substrates that may play a role in LTP have not yet been identified.

We have recently reported that sensory experience results in enhanced tyrosine phosphorylation of a set of synaptic proteins in the insular cortex of the behaving rat (17). One of these proteins, of 180 kDa, is concentrated in the post-synaptic density and displays properties of the NMDA receptor subunit 2B (NR2B) (18, 19). This finding led us to test whether an experimentally controlled form of experience-dependent synaptic modification (i.e., LTP) also results in enhanced tyrosine phosphorylation of the NMDA receptor. We now report that this indeed is the case and, furthermore, that the time course of the post-translational modification suggests that tyrosine phosphorylation of NR2B plays a role in short- and intermediate-term mechanisms of experience-dependent circuit plasticity in the mammalian brain.

MATERIALS AND METHODS

Animals. Male Wistar rats (~60 days old, 200–250 g) were used. They were individually caged at 22 ± 2°C in a 12-hr light/12-hr dark cycle.

Chemicals. Polyclonal anti-phosphotyrosine (αPY) and αPY-agarose (monoclonal PY20) were from Zymed. Horse-radish peroxidase-linked protein A and the Enhanced Chemiluminescence (ECL) kit were from Amersham. MK-801 was from Research Biochemicals. All other chemicals and reagents were of analytical grade or the highest grade available.

Preparation of Anti-NR2B (αNR2B). We have prepared a polyclonal antibody to NR2B using a specific peptide as an immunogen. We have selected the peptide DEIELAYRRPRSPD (residues 1103–1118 in NR2B) because this sequence is specific to NR2B (20). The peptide was synthesized (Biological Services, Weizmann Institute of Science) and the peptide-keyhole limpet hemocyanin conjugate was injected to rabbits in complete Freund’s adjuvant. One of the sera thus obtained, ab013, was used throughout this study. It reacted with a 180-kDa polypeptide in rat forebrain homogenate as well as in a tyrosine-phosphorylated protein fraction purified from rat forebrain homogenate by affinity chromatography on αPY-agarose resin (see below). This 180-kDa was concentrated in the postsynaptic density (PSD) fraction (data not shown). The reaction with the 180-kDa polypeptide was not detected in the preimmune serum and was completely blocked by the peptide containing NR2B residues 1103–1118 at 10 μM (data not shown). In addition, we have expressed the NR2A and NR2B proteins in vitro, using the NR2A cDNA cloned in pCDNA3 and the NR2B cDNA cloned in pBlueScript KS− (provided by S. Nakani, Kyoto University, Kyoto) in the TNT-coupled reticulate lysate systems (Promega); ab013 precipitated the NR2B protein (a 162-kDa protein in the reticulate lysate transcription and translation system) but not the NR2A protein (data not shown).

Abbreviations: αPY, anti-phosphotyrosine antibodies; NR2B, NMDA receptor subunit 2B; αNR2B, anti-NR2B antibodies; LTP, long-term potentiation; NMDA, N-methyl-d-aspartate; PSD, postsynaptic density; EPSP, excitatory postsynaptic potential.

†To whom reprint requests should be addressed. e-mail: bndudai@weizmann.weizmann.ac.il.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Electrophysiological Procedures. Rats were prepared for acute stimulation of the perforant path and for recording of field potentials in the dentate gyrus under chloral hydrate anesthesia using standard procedures (21). Briefly, a bipolar 125-μm stimulating electrode was implanted in the perforant pathway [AP -8, L 2.5 relative to Bregma according to Paxinos and Watson (22), depth adjusted to yield maximal response of the dentate gyrus]. A recording electrode (glass filled with 2 M NaCl; tip diameter, 2.5-5 μm; 1-4 MΩ) was placed in the dentate gyrus [AP -4, L 2.5 relative to Bregma according to Paxinos and Watson (22), depth adjusted to yield the highest excitatory postsynaptic potential (EPSP) response to the stimulation of the perforant pathway]. The timing of the stimulus pulses was controlled by a computer, the output of which was fed to a constant-current stimulator (Master 8, AMPI). Stimuli were 100 μsec in duration, with intensity adjusted manually (range 0.5-1.0 V). Evoked responses were amplified, filtered (1-10 Hz), and stored for off-line analysis. Off-line measurements were made of amplitude of the population spike and the slope of the population EPSP using averages of five successive responses. LTP was induced unilaterally (10 × 10 pulses at 400 Hz; interburst interval, 5 sec) and the contralateral nonstimulated dentate was used as control. In part of the experiments, only low frequency test stimuli (0.07 Hz) were delivered throughout the recording session. Field potentials were recorded at various time points up to 24 hr after LTP induction.

Homogenization, Fractionation, and Sample Preparation. Rats were sacrificed by decapitation at various times after the induction of LTP, and their dentate gyri were removed and homogenized. Two homogenization and processing protocols were used. In protocol A, the dentate was homogenized in a glass-Teflon homogenizer in SDS sample buffer, containing 10% glycerol, 5% mercaptoethanol, 2.5% SDS, in 62.5 mM Tris-HCl (pH 6.8). This type of homogenate was then subjected to SDS/PAGE and immunoblot analysis with αPY, as detailed below, for the determination of the level of protein tyrosine phosphorylation. Protocol B was used in experiments in which the level of phosphotyrosine on NR2B was determined and included affinity purification of the tyrosine-phosphorylated protein fraction prior to SDS/PAGE and immunoblot analysis with αNR2B, as detailed below. In this protocol the dentate gyri hemispheres from two to four rats from the same experimental group were combined and homogenized in 0.35 M sucrose/0.5 mM EGTA/2 mM EDTA/2 mM Na3VO4/1 mM phenylmethylsulfonyl fluoride in 10 mM Tris-HCl (pH 7.4). The samples were centrifuged for 1 min at 2000 × g, the supernatant was collected, the pellet was resuspended as above, and the supernatants were combined and diluted 1:1 in 2% SDS. The sample was then sonicated for 10 min and boiled for 5 min, followed by dilution of 1:10 in 100 mM NaCl/5 mM EDTA/50 mM NaF/0.1% SDS/0.1% Triton X-100/1 mM Na3VO4, in 50 mM Tris-HCl (pH 7.5). The resulting aliquots were subjected to affinity chromatography on αPY-agarose.

After application and equilibration for 2 hr at room temperature, the resin was washed with 100-fold packed resin volume of the application buffer and the tyrosine phosphorylated protein fraction was then eluted with 40 mM p-nitrophenyl phosphate.

Western Blot Analysis. Aliquots in SDS sample buffer (equal amounts of protein) were subjected to SDS/PAGE (23) (7.5% polyacrylamide in the presence of SDS and 2-mercaptoethanol) and Western blot analysis (24). The amount of protein in each sample was always determined before loading (25), and the same amount of protein (range 25-50 μg) was loaded in each lane. After the run the lanes were also compared by Ponceau staining. After blocking with 1% BSA, the blot was reacted either overnight at 4°C or for 1 hr at room temperature with either αPY or αNR2B. The presence of the αPY or αNR2B was determined with horseradish peroxidase-linked protein A and the ECL kit. Quantification was carried out using a computerized densitometer and image analyzer (Molecular Dynamics).

RESULTS

LTP specifically induces an NMDA receptor-dependent increase in protein tyrosine phosphorylation. High frequency stimulation of the perforant path induced potentiation of both the EPSP slope (156 ± 11%, n = 9) and of the population spike (270 ± 28%, n = 9) in the dentate gyrus as measured 1 hr after tetanic stimulation. A similar stimulation protocol resulted in a significant potentiation also at 24 hr after tetanic stimulation [EPSP = 126 ± 6%, population spike = 250 ± 51%, n = 5]. As can be seen in Fig. 1, high-frequency stimulation led to a significant increase in the tyrosine phosphorylation of a set of proteins, including polypeptides of 97 kDa, 115 kDa, and 180 kDa. The tyrosine phosphorylation of some other polypeptides, among them polypeptides of 60 kDa and 40 kDa, was not affected (Fig. 1B). The effect on tyrosine phosphorylation was transient, peaking at about 3 hr and subsiding after 24 hr (Fig. 1C). At 200 min after tetanic stimulation, the ratio of tyrosine phosphorylation in the stimulated hemisphere over that in the nonstimulated hemisphere was 1.75 ± 0.17 for the 97-kDa polypeptide, 1.77 ± 0.22 for the 115-kDa polypeptide, and 1.68 ± 0.20 for the 180-kDa polypeptide (n = 3-5 each).

Insertion of the electrode with no stimulation was ineffective (Fig. 1C). Low-frequency stimulation had only a small short-lived effect (Fig. 1C). The NMDA receptor antagonist MK-801 (2 mg/kg, i.p., 1 hr before tetanic stimulation) abolished both LTP (20 min after tetanic stimulation, [EPSP = 103 ± 2%, population spike = 121 ± 11% of pretetanic values, n = 4) and the increase in protein tyrosine phosphorylation (Fig. 1C).

A 180-kDa species whose tyrosine phosphorylation is increased by LTP is NR2B. Using the αNR2B antibodies and the tyrosine-phosphorylated protein fraction affinity-purified from the dentate gyrus, we have identified a 180-kDa protein whose tyrosine phosphorylation was significantly increased by LTP as NR2B (Fig. 2). The ratio of tyrosine phosphorylated NR2B in the stimulated to the nonstimulated hemispheres (S/NS) was 1.84 ± 0.24 (n = 4 experiments, P < 0.04). When aliquots of dentate homogenates were examined by immunoblot analysis with αNR2B antibodies, no difference was detected in the overall level of NR2B between the stimulated and the nonstimulated hemispheres (S/NS = 0.93 ± 0.13, n = 4 experiments, see also Fig. 1A), demonstrating that the modulation observed in LTP is due to increased tyrosine phosphorylation rather than increased amount of receptor protein.

DISCUSSION

It has been reported that induction of LTP in the rat hippocampal CA1 region (14) and dentate gyrus (15) is blocked by tyrosine kinase inhibitors and that mice with mutation in the tyrosine kinase gene fyn display impaired LTP in CA1 (16). The present data directly demonstrate, to our knowledge for the first time, that an NMDA receptor is phosphorylated on tyrosine in vivo in response to persistent synaptic activation and identifies the modulated subunit as NR2B. Assuming that under the conditions used the αPY affinity resin retains tyrosine-phosphorylated NR2B whether it is phosphorylated on one or more tyrosine residues, one may infer from our data (i.e., that the number of NR2B molecules retained on the αPY resin increases after LTP with no change in the total number of NR2B molecules) that the increase in tyrosine phosphorylation is due to an increase in the number of phosphorylated receptors rather than merely an increase of phosphorylation on already phosphorylated receptors. An LTP-associated increase in the tyrosine phosphorylation of a 180-kDa glycopro-
gyrus phosphorylation of NR2B possibilities involved subserved (i.e., tyrosine phosphorylation induced at animals recently independently stimulation, resulting from the antagonist MK-801 of [Penate](identical amount < Control)).

Increased protein tyrosine phosphorylation in the dentate gyrus in response to synaptic activation in vivo. (A) Representative recording of LTP at 200 min (each trace is an average of five successive responses collected at 0.07 Hz). (B Left) Representative blot, with αPY, of aliquots of homogenates prepared 200 min after induction of LTP from the dentate gyrus of the experimental (LTP) and the contralateral nonstimulated (Control) hemisphere of the same rat. (Right) Similar to the Left but probed with αNR2B. This blot illustrates that the total amount of NR2B is unaffected by LTP (C) The tyrosine phosphorylation of the 180-kDa species as a function of time after stimulation. Solid circles, high-frequency stimulation, resulting in LTP; open circles, low-frequency stimulation; square, electrode insertion only; solid diamond, +MK801, tyrosine phosphorylation of the 180-kDa species at 200 min in rats in which LTP induction was blocked by an injection of the NMDA receptor noncompetitive antagonist MK-801 (2 mg/kg) 60 min prior to the high-frequency stimulation. The ratio PY (potentiated/control) in the LTP-induced animals at 200 min significantly differs from 1.0 and from the ratio PY (potentiated/control) in the low stimulation and MK-801-treated animals (P < 0.02).

What might be the functional significance of the LTP-induced tyrosine phosphorylation of NR2B? Based on its kinetics (i.e., a delay of a few minutes after induction of LTP and persistence of several hours), this post-translational modification may be considered as a candidate component of a mechanism involved in short- and intermediate-term mechanisms of LTP. With regard to potential mechanisms, several possibilities may be entertained. The first is that tyrosine phosphorylation of NR2B enhances NMDA currents in the dentate gyrus granule cells. Although a prevalent notion is that the increased synaptic efficacy in NMDA-dependent LTP is subserved mainly by upregulation of non-NMDA glutamatergic receptor channels (6), evidence exists for the contribution of potentiated NMDA channel currents as well (27–29). Furthermore, in rats hippocampal slice, NMDA-channel-mediated currents can be potentiated by a cholinergic-mediated increase in postsynaptic Ca2+ (30). Interestingly, we have recently found that carbamylcholine enhances the tyrosine phosphorylation of a set of synaptic proteins in rat cortex in vivo, including that of NR2B (31). Direct evidence for the functional modulation of the NMDA receptor channel was obtained in spinal dorsal horn neurons in culture, where NMDA receptor-mediated currents were reported to be potentiated by protein tyrosine kinase and by inhibitors of protein phosphatase and depressed by inhibitors of protein tyrosine kinase (32).

However, even if tyrosine phosphorylation of NR2B results in persistent potentiation of NMDA currents in the granule cells in vivo, the contribution of this process to the maintenance of LTP is expected to be small (33–35). Additional possibilities should, therefore, be considered. One is that the major effect of tyrosine phosphorylation of NR2B on the C-terminal cytoplasmic side of the receptor (36) is not necessarily reflected in the modification of channel properties but rather in altered function of an intracellular signal transduction machinery interfacing with the receptor. No experimental evidence as yet exists for this proposal. Another, more appealing possibility is that LTP-induced tyrosine phosphorylation of NR2B takes place in interneurons and, hence, results in modulation of local circuit activity in the dentate gyrus. Indeed, using αNR2B for immunocytochemistry, we find a substantial proportion of NR2B on dentate gyrus interneurons (K.R. and Y.D., unpublished results). It has been reported that in the dentate gyrus, application of the GABAergic agonist baclofen produces a concentration-dependent long-lasting potentiation and facilitates the induction of β-adrenergic receptor-mediated long-lasting potentiation (37). Furthermore, in CA1, maintenance of LTP was reported to be associated with

![Figure 1](https://example.com/fig1.png)

![Figure 2](https://example.com/fig2.png)
an overall impairment of GABAergic inhibition, potentially contributing to the pyramidal cell LTP (38). It is thus possible, for example, that the persistent tyrosine phosphorylation of NR2B observed in LTP alters excitability of interneurons, reducing feed-forward inhibition and hence modifying the computational properties of the circuit.

As noted above, there were reports of the involvement of some tyrosine kinases in LTP (14–16), and NMDA receptor blocking prevents not only induction of LTP but also tyrosine phosphorylation (Fig. 1C); nevertheless, we do not yet have evidence that selective inhibition of the LTP-induced tyrosine phosphorylation of the NR2B disrupts the outcome of LTP induction. Such a proof, as well as further elaboration of the mechanisms involved, would benefit from having selective inhibitors of the kinase or phosphatase reaction(s) that modulate(s) the tyrosine phosphate(s) on NR2B. At this point in time, all we can conclude is that the tyrosine phosphorylation of NR2B is specifically correlated with LTP induction and persists for many hours after induction.

Which protein tyrosine kinases and phosphatases might regulate the level of tyrosine phosphorylation of NR2B in LTP? Both types of enzyme are present in the postsynaptic density along with NR2B; among them the protein tyrosine kinase fyn, which has been suggested to phosphorylate NR2B (39). This is especially intriguing since a mutation in fyn impairs LTP (14). Another appealing candidate is PYK2, a protein tyrosine kinase highly expressed in the central nervous system and rapidly phosphorylated on tyrosine residues and activated in response to stimuli that elevate Ca2+ concentrations (40).

The potential relevance to the experience-dependent tyrosine phosphorylation of NR2B of other proteins that were reported to colocalize with NR2B in the PSD has also yet to be determined. One such protein is PSD-95, shown to interact with NR2B (41). We do know that PSD-95 is not identical with the 100-kDa protein (K.R. and Y.D., unpublished results), whose tyrosine phosphorylation is also enhanced with LTP [Fig. 1B, see also ref. (17)]. The identification of the 100-kDa and 115-kDa proteins (17) may cast additional light on intracellular molecular cascades involved in LTP and also on the experience-dependent regulation of the tyrosine phosphorylation of NR2B.

We have recently reported that when behaving rats sample an unfamiliar taste, either incidentally or in the context of conditioned taste aversion training, the tyrosine phosphorylation of several synaptic proteins is markedly enhanced in the insular cortex (17). The major experience-dependent modulated protein is a 180-kDa polypeptide abundant in the PSD that displays molecular properties of NR2B (18, 19). We propose that the increased tyrosine phosphorylation of NR2B in the insular cortex of a behaving rat in response to the sampling of an unfamiliar taste and the increased tyrosine phosphorylation in vivo of NR2B in the dentate gyrus in response to LTP are two manifestations of a similar experience-dependent neuronal mechanism.

We thank S. Nakanshi (Kyoto) for the NR2A and NR2B cDNA clones and Diego E. Berman for assisting us with the in vitro transcription and translation assay. We are grateful to S. Hazvi for technical assistance; M. Segal and V. Teichberg for valuable comments on the manuscript; and J. A. P. Rostas, V. A. Brent, K. Voss, M. L. Errington, T. V. P. Bliss, and J. W. Gurd for making the results of their work available prior to publication. The support of the United States-Israel Binational Science Foundation, Jerusalem (to Y.D.), the Saul Silverman Family Foundation, Toronto (to Y.D.), and The Alon Fellowship (to G.R.-L.) is gratefully acknowledged.