sitional refinement algorithm of X-PLOR (*26*). Between refinement cycles, the structure was inspected on a graphics display with the FRODO program (*27*). The Glu^{95} residue was exchanged with Ser, and segments from amino acids 180 to 190 and 224 to 231 in domain III were manually rebuilt. In later refinement cycles, solvent molecules, calcium, and sulfate were included. The structure was refined with data to 2.52 Å to a final *R* factor of 19.5% ($R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where F_o and F_c are the observed and the calculated structure factors, respectively). The refinement statistics were as follows: resolution = 8.0 to 2.52 Å; number of reflections used for refinement = 10,750; total number of non-hydrogen atoms = 2630; protein atoms = 2472; solvent = 151; calcium = 2; sulfate = 1; root-mean-square deviation from target values for bonds = 0.013 Å and for angles = 2.811°; mean B factors for protein atoms = 29.29 $Å^2$ and for solvent = 41.58 $Å^2$.

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Characterization of a Presynaptic Glutamate Receptor

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Glutamate receptors mediate excitatory neurotransmission in the brain and are important in the formation of memory and in some neurodegenerative disorders. A complementary DNA clone that encoded a 33-kilodalton protein (GR33) was obtained by screening a library with an antibody generated against glutamate binding proteins. The sequence of GR33 is identical to that of the recently reported presynaptic protein syntaxin. When GR33 was expressed in *Xenopus* oocytes, it formed glutamate-activated ion channels that are pharmacologically similar to those of *N*-methyl-D-aspartate receptors but with different electrophysiological properties. Mutation of the leucine 278 residue in the single putative transmembrane segment of GR33 affects the properties of the channel. Thus, in vivo GR33 may be a presynaptic glutamate receptor.

The family of glutamate-gated ion channels includes receptors activated by *N*-methyl-D-aspartate (NMDA), kainic acid, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (*1*). The NMDA receptors have been shown to participate in neuronal plasticity (*1*), long-term potentiation (*2*), and excitotoxicity (*3*) as well as in degenerative disorders (*4*). A large body of evidence indicates that these glutamate receptors are localized postsynaptically. However, recent findings demonstrate that regulation of glutamate release from presynaptic membrane involves one or more presynaptic glutamate autoreceptors (*5*, *6*). Here, we describe the cloning and functional characterization of a new glutamate receptor, which is located presynaptically.

A 1.7-kb fragment of complementary DNA (cDNA), GR33, was obtained from a rat striatal cDNA library with the use of a cDNA clone of 480 base pairs (bp) isolated by immunoscreening of a human cortex library with an antibody generated against glutamate binding proteins (*7*). The amino acid sequence of GR33, which consists of 288 residues, was deduced from the longest open reading frame. A search of the GenBank database revealed that the sequence of the GR33 protein is identical to that of the presynaptic protein syntaxin (p35B) (8) and is similar to a protein of unknown function called HPC-1 (9). Syntaxin has been suggested to dock synaptic vesicles near calcium channels at presynaptic active zones (*8*). At its COOH-terminus, this protein has a single hydrophobic region that may be a transmembrane segment (TM).

We tested whether GR33 could function as a glutamate receptor by injecting GR33 complementary RNA (cRNA) into *Xenopus* oocytes (*10*). The GR33 protein formed glutamate-activated channels with a pharmacological profile characteristic of NMDA receptors in several ways (Fig. 1). (i) Application of 100 μM glutamate or 200 μM NMDA evoked inward currents at negative potentials (Fig. 1, A and B) ($n = 32$). The effective dose for half-maximal response (ED_{50}) for NMDA was 10 μM (Fig. 1D), which is similar to data obtained in oocytes injected with either rat brain polyadenylated (*11*) or synthetic NMDA receptor subunit (NMDAR1) mRNAs (*12*). (ii) Omission of glycine from the medium significantly reduced the response to glutamate and to NMDA (Fig. 1, A and B). (iii) NMDA-evoked currents were reduced to 40% in the presence of the competitive NMDA receptor antagonist D-2-amino-7-phosphonoheptanoic acid (10 μM AP7) ($n = 5$) (Fig. 1B), and the responses to glutamate (100 μM) and to NMDA (200 μM) were completely blocked when the concentration of AP7 was increased to 100 μM (Fig. 1, A and B) ($n = 6$). Interestingly, in contrast to the "classic" NMDA receptor, another competitive antagonist, D-2-amino-4-phosphopentanoic acid (AP5; 100 μM), reduced NMDA (200 μM) currents only 40% ($n = 2$); however, AP5 completely blocked responses to glutamate (100

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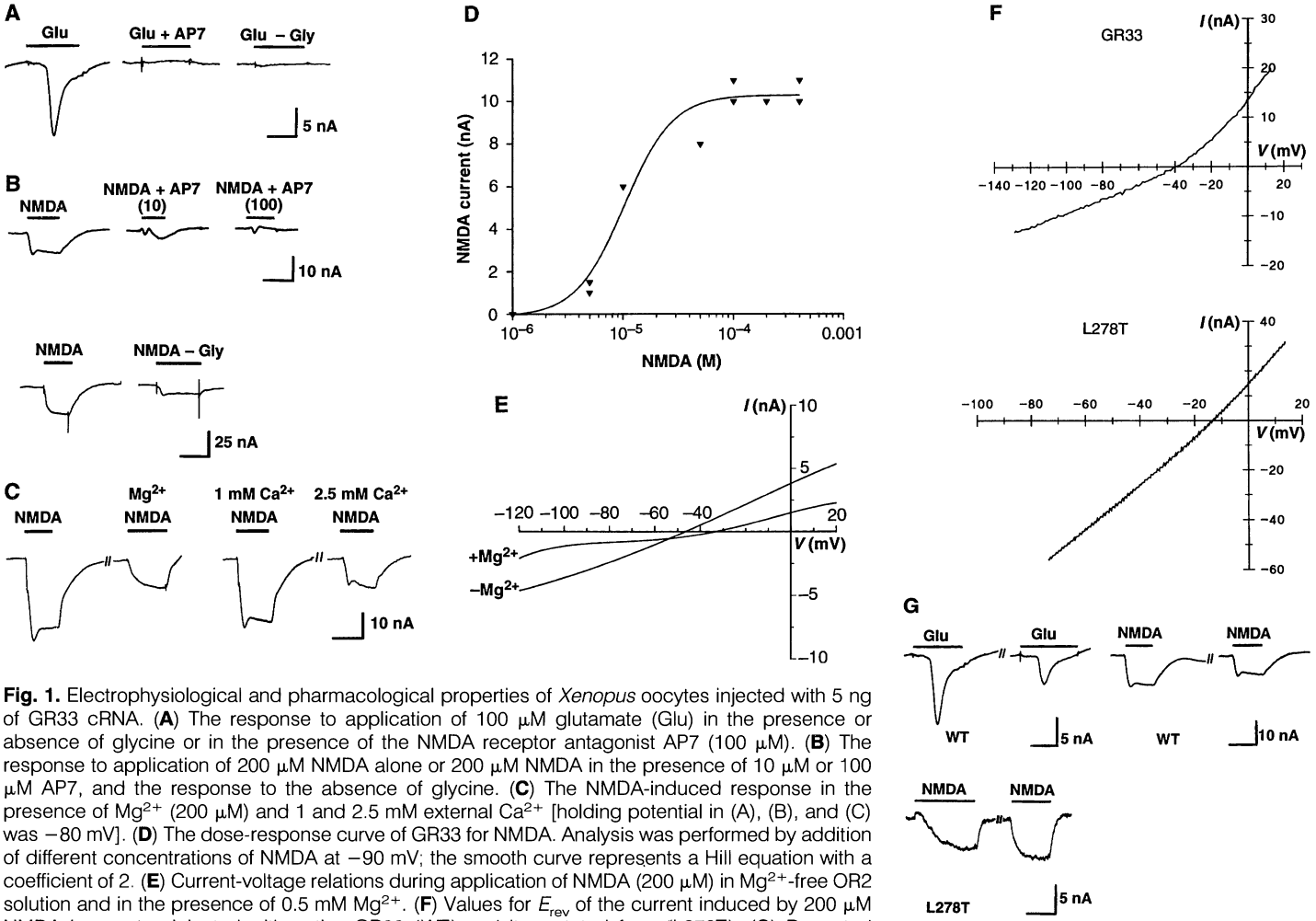

Fig. 1. Electrophysiological and pharmacological properties of *Xenopus* oocytes injected with 5 ng of GR33 cRNA. (**A**) The response to application of 100 μM glutamate (Glu) in the presence or absence of glycine or in the presence of the NMDA receptor antagonist AP7 (100 μM). (**B**) The response to application of 200 μM NMDA alone or 200 μM NMDA in the presence of 10 μM or 100 μM AP7, and the response to the absence of glycine. (**C**) The NMDA-induced response in the presence of Mg^{2+} (200 μM) and 1 and 2.5 mM external Ca^{2+} [holding potential in (A), (B), and (C) was −80 mV]. (**D**) The dose-response curve of GR33 for NMDA. Analysis was performed by addition of different concentrations of NMDA at −90 mV; the smooth curve represents a Hill equation with a coefficient of 2. (**E**) Current-voltage relations during application of NMDA (200 μM) in Mg^{2+}-free OR2 solution and in the presence of 0.5 mM Mg^{2+}. (**F**) Values for E_{rev} of the current induced by 200 μM NMDA in oocytes injected with native GR33 (WT) and its mutated form (L278T). (**G**) Repeated applications of glutamate (Glu) (100 μM) or of NMDA (200 μM) onto oocytes injected with GR33 (WT) or its mutated form (L278T). The series of applications was performed with intervals ranging from 30 s to 1 min. The electrophysiology of oocytes and the generation of the L278T mutation was performed as described (*22*). The horizontal bars show a 10-s interval.

μM; n = 2). (iv) Among other agonists of the ionotropic glutamate receptors (kainate, AMPA, and L-homocysteate) and of the metabotropic glutamate receptor (quisqualate), only L-homocysteate induced consistent responses. No responses were induced by glutamate agonists in control oocytes injected with the GR33 antisense mRNA or with water.

In addition to its pharmacological similarities with NMDA receptors, GR33 displayed distinctive electrophysiological properties. First, increasing the external Ca^{2+} concentration from 1 mM to 2.5 mM reduced to 50% the response of GR33 to NMDA (Fig. 1C), whereas the response of the NMDAR1 subunit was enhanced two- to fourfold under the same experimental conditions. Therefore, the GR33 receptor in oocytes might have a low permeability to Ca^{2+}, and calcium-dependent chloride channels would make little or no contribution to the NMDA-induced current. Indeed, substitution of 90% of the external Cl^- with methane sulfonate did not change the NMDA-evoked response. Second, the inhibition of the NMDA-induced response of GR33 by Mg^{2+} at −80 mV was incomplete (Fig. 1C), whereas the response of NMDAR1 was completely blocked. Moreover, the inhibition by Mg^{2+} of the NMDA-induced response of GR33 was not voltage-dependent (*12*) because Mg^{2+} reduced NMDA responses by 30 to 70% uniformly in a range of potentials from −100 mV to +20 mV (n = 10) (Fig. 1E). This and a shift in the reversal potential of NMDA current in the presence of Mg^{2+} (Fig. 1E) suggest a permeability of the GR33 channel to Mg^{2+} that could have a physiological role in the regulation of transmitter release by competing with Ca^{2+} influx in the presynaptic membrane.

Another important difference between the GR33 receptor and postsynaptic glutamate receptors is the reversal potential (E_{rev}). The E_{rev} of the GR33-gated NMDA currents was −46.9 mV (SE = 2.43 mV, n = 7) (Fig. 1F). Interestingly, an NMDA receptor located on the visual interneurons of the crayfish optic lobe was recently shown to elicit currents that reversed at −60 mV (*13*). This E_{rev} is unusual for NMDA-activated conductance because NMDA receptors generally gate a nonspecific cation current with a value for E_{rev} of about −10 mV. It might be that the contribution of K^+ ions to the NMDA-evoked current of the GR33 channel is more significant than it is for postsynaptic NMDA receptors. Alternatively, NMDA could elicit a mixture of inward and outward currents.

To eliminate the possibility that GR33 in the oocytes induces activation of an endogenous ion channel, we performed a mutagenesis study. The GR33 protein contains a single TM segment. Ligand-gated ion channels identified to date have four putative membrane-spanning segments that are hydrophobic, with the TM segment II (TMII) forming a channel pore together with other subunits (*14*). When the α helix structure of GR33-TM is compared with that of the TMII of NMDA receptor subunits (*15*) and the nicotinic acetylcholine

(ACh) receptor α subunit (*16*), three amino acids (Leu, Ser, and Thr) are placed in the same relative positions. In the ACh receptor α subunit, one of these amino acids, Leu, was shown to face the ion channel and to play a critical role in its function (*16*).

Accordingly, we replaced Leu^{278} of GR33 with Thr. This mutation [Leu^{278}→ Thr (L278T)] did not disturb expression of the glutamate-activated channel in the oocytes. However, the L278T mutation was found to induce a shift of about 30 mV of the E_{rev} of the NMDA current toward positive values (Fig. 1F). Furthermore, the mutation L278T modified the desensitization of the responses. In a series of NMDA applications onto oocytes injected with GR33-L278T, the first application had a "priming" effect: the amplitude of the subsequent responses was increased by about 50% (Fig. 1G). In contrast, there was an inactivation of glutamate and NMDA responses when oocytes were injected with native GR33 mRNA and subjected to a similar series of applications (Fig. 1G). These findings suggest that (i) the GR33 TM segment forms an ion channel; (ii) Leu^{278} is important for ion permeability as well as for the desensitization of responses; and (iii) as in other receptors, the NH_2-terminus of GR33-TM is oriented toward the extracellular side. Therefore, the appearance of the NMDA-evoked currents in the oocytes is a result of the formation of a receptor-channel complex by GR33-encoded protein. It is of interest that a recently cloned voltage-gated K^+ channel of 15 kD that contains a single membrane-spanning segment also forms a functional ion channel in oocytes (*17*).

Previous results (*8*) show that GR33 (syntaxin) contains a cytoplasmic domain that might play a role in the docking of synaptic vesicles at presynaptic active zones. Our electrophysiological results and the presumed orientation of GR33-TM in the membrane suggest that GR33 has an extracellular segment. Consistent with this, specific immunofluorescent labeling with GR33 antibodies was observed in living neuronal cultures from both the cortex and hippocampus of rat embryos (Fig. 2A). GR33 antibodies labeled varicosities as well as cell bodies of all neurons. No specific labeling was revealed with preimmune serum (Fig. 2B). In agreement with our results, Inoue and Akagawa (*18*) have stated that most of HPC-1 immunoreactivity is localized on the plasma membrane of neuronal cell somas and axons. Localization of GR33 on the cell bodies of embryonic neurons but not of the neurons of postnatal rats (*8*) is in agreement with changes in the distribution of NMDA receptors during development. Furthermore, antibodies against synaptotagmin, a vesicular protein that may be associated with GR33 (*8*), labeled cell bodies of cerebral cortex neurons (*19*). Thus, at least a part of the GR33 protein NH_2-terminus is extracellular and may contain the glutamate binding site.

The expression pattern of the GR33 gene was studied by RNA blotting and in situ hybridization. Northern (RNA) blot analysis identified a single 4.6-kb transcript and revealed that the GR33 gene is expressed in the hippocampus, cortex, striatum, and cerebellum. With the use of in situ hybridization on brain sections, the pattern of GR33 mRNA distribution was found to be similar to that of NMDAR1 (*12*). Hybridization signals were observed in the hippocampal CA1 and CA3 regions; in the granular cells of dentate gyrus; throughout the layers of the cortex, piriform cortex, and amygdala (Fig. 3); in the cells of the granular layer; and in the Purkinje cells of cerebellum.

These results and previous data (*8*) demonstrate that GR33 may be a type of presynaptic glutamate receptor and may likely be involved in the autoregulation of transmitter release. Moreover, syntaxin (GR33) is believed to be linked to the presynaptic N-type Ca^{2+} channel that is voltage-activated (*8*); the channel is blocked by ω-conotoxin and is involved in neurotransmitter release from presynaptic sites. Interestingly, transmitter release, mediated by this Ca^{2+} channel, has been demonstrated to be regulated by adenosine (*20*) and by NMDA (*21*). Therefore, there might be a direct connection between the GR33 receptor, the N-type Ca^{2+} channel, and the regulation of neurotransmitter release from nerve terminals. As in the case of presynaptic metabotropic glutamate receptors (*5*), GR33 might modulate the release of glutamate or other neurotransmitters from presynaptic terminals during enhanced synaptic activity, like long-term potentiation.

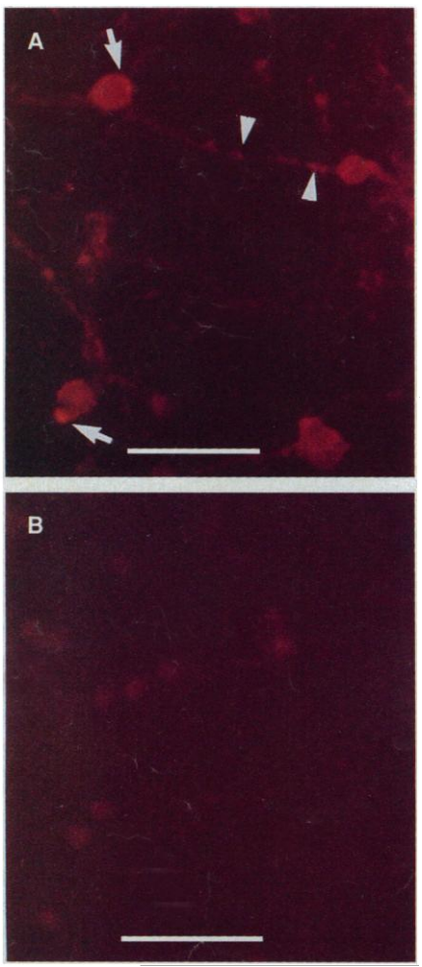

Fig. 2. Localization of the GR33-specific epitopes on the surface of living cortical neurons from rat E17 embryos. (**A**) Indirect immunofluorescence staining of cortex neurons in monolayer culture with GR33 antiserum without fixing or permeabilizing the cell membrane. (**B**) Preimmune serum. Arrowheads indicate localization of GR33 along neurites in multiple varicosities; arrows show the localization of GR33 on cell bodies. Cell culture preparation and immunostaining were performed as described (*23*). Antiserum was generated as described (*24*). Scale bars = 45 μM.

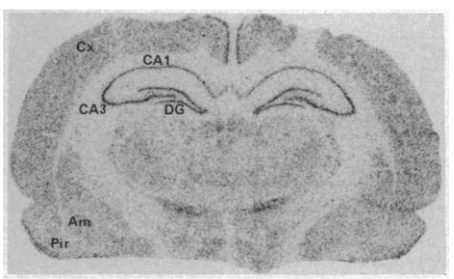

Fig. 3. Rat brain distribution of GR33 receptor mRNA. Positive film image of in situ hybridization of a rat brain frontal section with a GR33-specific, 45-mer oligonucleotide. CA1 and CA3, hippocampal CA1 and CA3 pyramidal cells; DG, granule cells of dentate gyrus; Cx, cortex; Am, amygdala; Pir, piriform cortex. Hybridization with tissue sections was performed as described (*25*).

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injected into BALB/c mice to generate antibodies. Injections were repeated as necessary. Titers of antibodies were estimated by enzyme-linked immunosorbent assay. The antisera were used to screen a human cortex cDNA library (Clontech, Palo Alto, CA). Out of 1 million recombinants, one positive clone that contained a cDNA insert of 480 bp was found, purified, and used to screen a rat striatum cDNA library generated in the laboratory in a lambda ZAP vector (Stratagene). One million clones were screened at high stringency with the human insert. One full-length cDNA clone (GR33) was obtained and sequenced in both strands.

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23. The dissociated cells from the cortex of fetal Wistar rats (E17) were cultured during 4 to 5 days in the absence of serum as described [S. Kure *et al.*, *Biochem. Biophys. Res. Commun.* **179**, 39 (1991)]. Non-neuronal cells were found to represent less than 0.5% of the population. Neurons were incubated with the primary GR33-specific antibodies (1:100) in the presence of 10% fetal calf serum during 2 hours at 37°C. After six washes with phosphate-buffered saline (PBS), the cells were exposed to secondary antibodies (1:50 dilution of rhodamine-conjugated goat antibody to rabbit immunoglobulins in PBS) for 1 hour at 37°C. Cells were washed twice with PBS and were observed with a fluorescence Nikon Diaphot TMD microscope.
24. A fragment of the GR33 cDNA that carried the coding region was generated by PCR amplification with GR33-specific oligonucleotides as primers. The product was subcloned into an *Escherichia coli* pGEX-2 expression vector [D. B. Smith and K. S. Johnston, *Gene* **67**, 31 (1988)] so that a fusion protein [glutathione-S-transferase–GR33] was obtained. The fusion protein was purified and used to produce rabbit polyclonal antibodies.
25. In situ hybridization was performed on 15-μm brain slices, fixed in 4% paraformaldehyde, and dried through ascending ethanol baths, with a 45-mer antisense oligonucleotide (specific to GR33 and labeled with ^{35}S-labeled adenosine triphosphate) at 42°C overnight in 4× saline sodium citrate (SSC) (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate) solution containing 50% formamide, 10% dextran sulfate, 10 mM dithiothreitol (DTT), 1× Denhardt's solution, and 250 μM transfer RNA. After a final washing at 55°C in 0.5× SSC and 10 mM DTT, the slices were dried and exposed to βmax film (Amersham) for 3 to 4 days.
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Transsynaptic Expression of a Presynaptic Glutamate Receptor During Hippocampal Long-Term Potentiation

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Repetitive activation of excitatory synapses in the hippocampus produces a persistent enhancement of synaptic efficiency known as long-term potentiation (LTP). In anesthetized and in freely moving rats, the induction of LTP in the perforant path led to a transient increase in the amount of messenger RNA (mRNA) coding for a presynaptic glutamate receptor (GR33) in dentate granule cells. The amount of GR33 mRNA was increased for at least 5 hours after the induction of LTP but was indistinguishable from control values 1 day after induction. The *N*-methyl-D-aspartate receptor antagonist 2-aminophosphonovalerate prevented the induction of both LTP and the increase in GR33 mRNA. The amount of GR33 protein was increased in the mossy fiber terminal zone of dentate granule cells 5 hours after the induction of LTP. These results suggest that the induction of LTP in synapses at one stage in a neural network may lead to modification in synaptic function at the next stage in the network.

Long-term potentiation (LTP) in the dentate gyrus and hippocampus is an extensively studied form of activity-dependent synaptic plasticity in the vertebrate nervous system (*1*) and is considered to be a cellular model for the changes that underlie learning and memory (*2*). Induction of LTP in the CA1 region of the hippocampus and in the dentate gyrus requires simultaneous presynaptic release of neurotransmitter and sufficient postsynaptic depolarization to cause activation of the *N*-methyl-D-aspartate (NMDA) receptor channel complex, which results in an increased calcium influx into the postsynaptic cell. This initial triggering event activates both pre- and postsynaptic mechanisms to generate a persistent increase in synaptic strength (*3*). In the CA3 region, however, one form of LTP, mossy fiber LTP, is independent of NMDA receptor activation (*4*). Mossy fibers are granule cell axons that make synaptic connections with spines located on the proximal dendrites of CA3 pyramidal cells (*5*). Although the mechanism of induction of LTP at the mossy fiber–CA3 synapse remains controversial (*6*), it is possible that maintenance of LTP is entirely presynaptically mediated (*4*) and involves presynaptic receptors that modulate the release of excitatory neurotransmitters (*7*). Recent studies that demonstrate that the presynaptic glutamate autoreceptor of the metabotropic (mGluR) type regulates transmitter release (*8*) suggest that presynaptic glutamate receptors have an important role in the maintenance of LTP.

One of the candidates for such a role is GR33, a presynaptic glutamate receptor with a pharmacological profile similar to that of the postsynaptic NMDA receptor (*9*). We studied the effect of LTP, which was induced in the perforant path–dentate gyrus synapses, on the expression of the GR33 receptor gene in the rat hippocampus. In situ hybridization (*10*) was performed on brain slices taken 2 or 5 hours after induction of LTP in anesthetized rats (*11*) ($n = 2$ for each time point) and 2 hours, 1 day, 2 days, or 5 days after induction of LTP in freely moving rats (*12*) ($n = 3$ for each time point). Freely

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