Volume 300, number 1, 39–45 FEBS 10843 March 1992

Cloning, expression and modulation of a mouse NMDA receptor subunit

Makoto Yamazaki[a,b], Hisashi Mori[a], Kazuaki Araki[a], Kazuhiro J. Mori[b] and Masayoshi Mishina[a]

[a]*Department of Neuropharmacology, Brain Research Institute* and [b]*Department of Biology, Faculty of Science, Niigata University, Niigata 951, Japan.*

Received 10 January 1992; revised version received 1 February 1992

The primary structure and presence of two forms of the mouse *N*-methyl-D-aspartate (NMDA) receptor channel subunit ζ1 have been disclosed by cloning and sequencing the cDNAs. The ζ1 subunit shows ~20% amino acid sequence identities with the rodent α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)- or kainate-selective GluR subunits and has structural features common to neurotransmitter-gated ion channels. Functional homomeric ζ1 channels expressed in *Xenopus* oocytes by injection of the subunit-specific mRNA exhibit current responses characteristic for the NMDA receptor channel such as activation by glycine, Ca^{2+} permeability, blocking by Mg^{2+} and activation by polyamine. It has been found that the ζ1 channel activity is positively modulated by treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA).

Glutamate receptor; Glycine; *N*-methyl-D-aspartate receptor channel; Mg^{2+} block; 12-*O*-Tetradecanoylphorbol 13-acetate

1. INTRODUCTION

Glutamate receptor (GluR) channels mediate most of the fast excitatory synaptic transmission in the central nervous system and are classified into three major subtypes, that is, receptors for kainate, AMPA and NMDA based on the pharmacological and electrophysiological properties [1,2]. The NMDA receptor channel highly permeable to Ca^{2+} plays a key role in synaptic plasticity, thought to underlie memory and learning as well as development of the nervous system [3,4]. Furthermore, abnormal activation of NMDA receptor channels has been suggested to lead to neuronal cell death observed in various acute and chronic disorders such as ischemia, stroke, Alzheimer's dementia and Huntington's disease [5,6].

A family of rodent GluR channel subunits has been characterized by cloning and expression of the cDNAs [7–17]. The rat GluR1-GluR4 (or GluRA-GluRD) subunits [7–9,11] and mouse α1 (GluR1) and α2 (GluR2) subunits [10] are highly homologous with each other (~70% amino acid sequence identities), thus classified as the α subfamily of the GluR channel. The members of the α subfamily form homomeric and heteromeric channels responsive to L-glutamate, quisqualate, AMPA and kainate. The apparent affinities of these channels are higher for quisqualate and AMPA than for kainate, indicating that the α subfamily represents the AMPA-selective GluR channels. Additional members of the GluR channel family have been identified by screening under low stringency conditions using cDNAs of the α subfamily subunits as probes. The rat GluR5 and GluR6 subunits [12,14] and the mouse β2 (GluR6) subunit [16] are highly homologous with each other and are classified as the β subfamily of the GluR channels. The β2 (GluR6) subunit forms homomeric channels responsive to L-glutamate and kainate, but not to AMPA, indicating that the β subfamily represents the kainate-selective GluR channel. The other subfamily γ includes the rat KA-1 subunit [13] and the mouse γ2 subunit [17]. The KA-1 subunit expressed in mammalian cells exhibits high affinity binding for kainate. The γ2 subunit when expressed together with the β2 subunit yields functional GluR channels selective for kainate. Thus the γ subfamily also represents the kainate-selective GluR channels.

Further screening under low stringency conditions using these GluR subunit cDNAs as probes has revealed the presence of additional GluR channel subfamilies. Here, we report that one of these subunits, designated as ζ1, is found in two forms and constitutes functional GluR channels with characteristics of the NMDA receptor channel; the larger form corresponds to the rat NMDA receptor subunit NMDAR1 [15]. We have found that TPA treatment positively modulates the ζ1 NMDA receptor channel activity.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APV, D-2-amino-5-phosphonovalerate; GluR, glutamate receptor; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

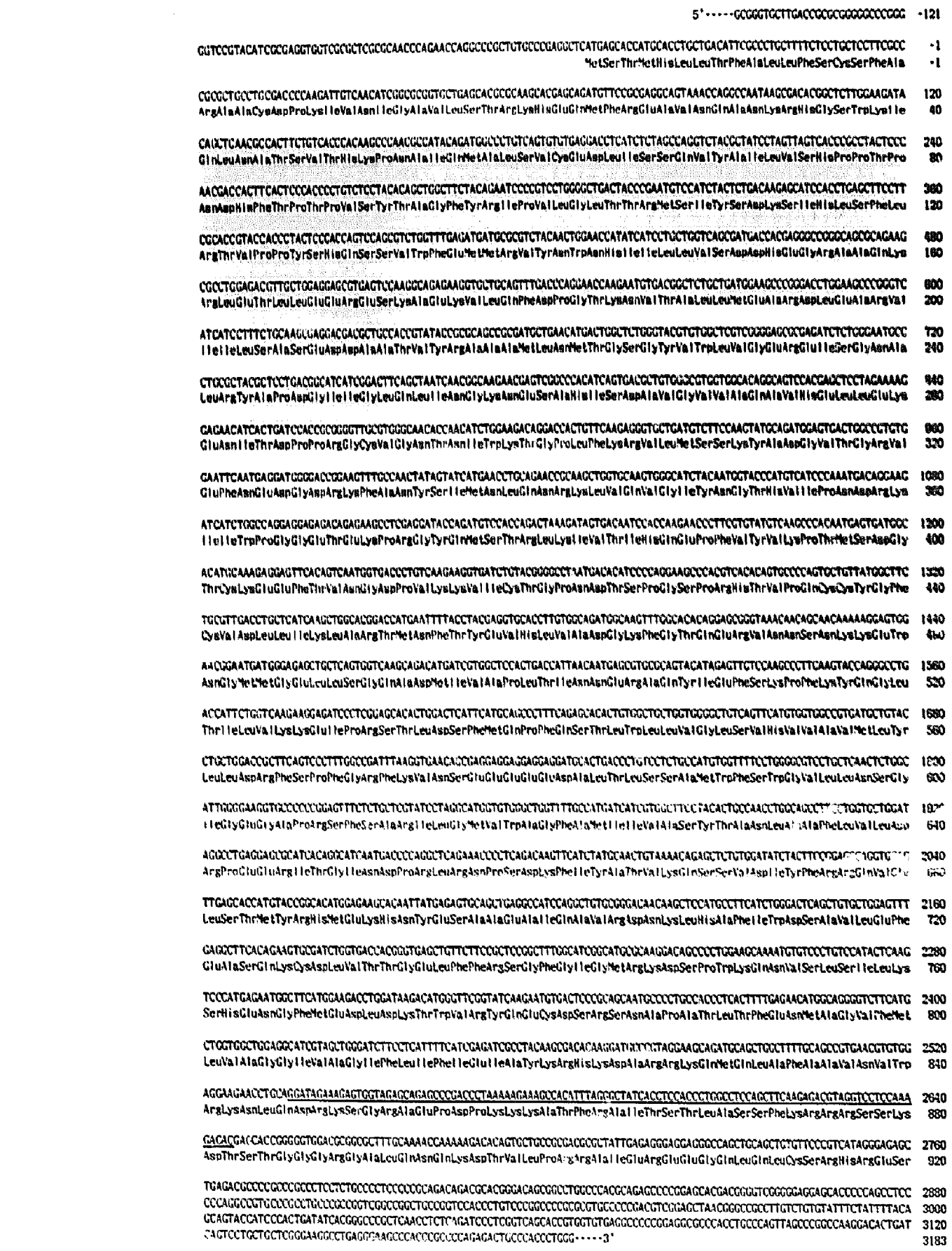
Correspondence address: M. Mishina, Department of Neuropharmacology, Brain Research Institute, Niigata University, Asahimachi, Niigata 951, Japan. Fax: (81) (25) 225 6458.

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing of cDNAs*

Screening of mouse forebrain and cerebellar cDNA libraries constructed in λgt10 under low stringency conditions with mouse α1 and

Published by Elsevier Science Publishers B.V.



5'-----GCGGGTGCTTGACCGCGCGGGGCCCCGG -121

GGTCCGTACATCGCGAGGTGGTCGCGCTCGCGCAACCCAGAACCAGGCCCGCTGTGCCCGAGGCTCATGAGCACCATGCACCTGCTGACATTCGCCCTGCTTTTCTCCTGCTCCTTCGCC -1
MetSerThrMetHisLeuLeuThrPheAlaLeuLeuPheSerCysSerPheAla -1

CGCGCTGCCTGCGACCCCAAGATTGTCAACATCGGCGCGGTGCTGAGCACGCCCAAGCACGAGCAGATGTTCCGCGAGGCAGTAAACCAGGCCAATAAGCGACACGGCTCTTGGAAGATA 120
ArgAlaAlaCysAspProLysIleValAsnIleGlyAlaValLeuSerThrArgLysHisGluGlnMetPheArgGluAlaValAsnGlnAlaAsnLysArgHisGlySerTrpLysIle 40

CAGCTCAACGCCACTTCTGTCACCCACAAGCCCAACGCCATACAGATGGCCCTGTCAGTGTGTGAGGACCTCATCTCTACGCCAGGTCTACGCTATCCTAGTTAGTCACCCGCCTACTCCC 240
GlnLeuAsnAlaThrSerValThrHisLysProAsnAlaIleGlnMetAlaLeuSerValCysGluAspLeuIleSerGlnValTyrAlaIleLeuValSerHisProProThrPro 80

AACGACCACTTCACTCCCACCCCTGTCTCCTACACAGCTGGCTTCTACAGAATCCCCGTCCTGGGGCTGACTACCCGAATGTCCATCTACTCTGACAAGAGCATCCACCTGAGCTTCCTT 360
AsnAspHisPheThrProThrProValSerTyrThrAlaGlyPheTyrArgIleProValLeuGlyLeuThrThrArgMetSerIleTyrSerAspLysSerIleHisLeuSerPheLeu 120

CGCACCGTACCACCCTACTCCCACCAGTCCAGCGTCTGGTTTGAGATGATGCCGCGTCTACAACTGGAACCATATCATCCTGCTGGTCAGCGATGACCACGAGGGCCGGGCAGCGCAGAAG 480
ArgThrValProProTyrSerHisGlnSerSerValTrpPheGluMetMetArgValTyrAsnTrpAsnHisIleIleLeuLeuValSerAspAspHisGluGlyArgAlaAlaGlnLys 160

CGCCTGGAGACGTTGCTGGAGGAGCGTGAGTCCAAGGCAGAGAAGGTGCTGCAGTTTGACCCAGGAACCAAGAATGTGACGGCTCTGCTGATGGAAGCCCGGGACCTGGAAGCCCGGGTC 600
ArgLeuGluThrLeuLeuGluGluArgGluSerLysAlaGluLysValLeuGlnPheAspProGlyThrLysAsnValThrAlaLeuLeuMetGluAlaArgAspLeuGluAlaArgVal 200

ATCATCCTTTCTGCAAGCGAGGACGACGCTGCCACCGTATACCGCGCAGCCGCGATGCTGAACATGACTGGCTCTGGGTACGTGTGGCTCGTCGGGGAGCCGCAGATCTCTCGGAATGCC 720
IleIleLeuSerAlaSerGluAspAspAlaAlaThrValTyrArgAlaAlaMetLeuAsnMetThrGlySerGlyTyrValTrpLeuValGlyGluArgGlnIleSerGlyAsnAla 240

CTGCGCTACGCTCCTGACGGCATCATCGGACTTCAGCTAATCAACCGCCAAGAACGAGTCGGCCCCACATCAGTGACGCTGTGGGCGTGGTGGCACAGGCAGTCCACCAGCTCCTACAAAAG 840
LeuArgTyrAlaProAspGlyIleIleGlyLeuGlnLeuIleAsnGlyLysAsnGluSerAlaHisIleSerAspAlaValGlyValValAlaGlnAlaValHisGluLeuGluLys 280

GAGAACATCACTGATCCACCGCGGGTTGCGTGGGCAACACCAACATCTGGAAGACAGGACCACTGTTCAAGAGGGTGCTGATGTCTTCCAACTATGCAGATGGAGTCACTGCCCCTCTG 960
GluAsnIleThrAspProProArgGlyCysValGlyAsnThrAsnIleTrpLysThrGlyProLeuPheLysArgValLeuMetSerSerLysTyrAlaAspGlyValThrGlyArgVal 320

GAATTCAATGAGGATGGGGACCGGAAGTTTGCCAACTATAGTATCATGAACCTGCAGAACCCGCAAGCTGGTGCAAGTGGGCATCTACAATGCTACCCATGTCATCCCAAATGACACGCAAG 1080
GluPheAsnGluAspGlyAspArgLysPheAlaAsnTyrSerIleMetAsnLeuGlnAsnArgLysLeuValGlnValGlyIleTyrAsnGlyThrHisValIleProAsnAspArgLys 360

ATCATCTGGCCAGGAGGAGAGACAGAGAAGCCTCGAGGATACCAGATGTCCACCAGACTAAAGATAGTGACAATCCACCAAGAACCCTTCCTGTATGTCAAGCCCACAATGAGTGATGCC 1200
IleIleTrpProGlyGlyGluThrGluLysProArgGlyTyrGlnMetSerThrArgLeuLysIleValThrIleHisGlnGluProPheValTyrValLysProThrMetSerAspGly 400

ACATTCAAAGAGGAGTTCACAGTCAATGGTGACCCTGTCAAGAAGGTGATCTGTACGGGCCCTAATGACACATCCCCAGGAAGCCCACGTCACACAGTGCCCCAGTGCTGTTATGGCTTC 1320
ThrCysLysGluGluPheThrValAsnGlyAspProValLysLysValIleCysThrGlyProAsnAspThrSerProGlySerProArgHisThrValProGlnCysCysTyrGlyPhe 440

TGCGTTGACCTGCTCATCAAGCTGGCACGGACCATGAATTTTACCTACGAGGTGCACCTTGTGGCAGATGGCAAGTTTGGCACACAGGAGCGGGTAAACAACAGCAACAAAAAGGAGTGG 1440
CysValAspLeuLeuIleLysLeuAlaArgThrMetAsnPheThrTyrGluValHisLeuValAlaAspGlyLysPheGlyThrGlnGluArgValAsnAsnSerAsnLysLysGluTrp 480

AACGGAATGATGGGAGAGCTGCTCAGTGGTCAAGCAGACATGATCGTGGCTCCCACTGACCATTAACAATGAGCGTGCGCAGTACATAGAGTTCTCCAAGCCCTTCAAGTACCAGGGCCTG 1560
AsnGlyMetMetGlyGluLeuLeuSerGlyGlnAlaAspMetIleValAlaProLeuThrIleAsnAsnGluArgAlaGlnTyrIleGluPheSerLysProPheLysTyrGlnGlyLeu 520

ACCATTCTGGTCAAGAAGGAGATCCCTCGGAGCACACTGGACTCATTCATGCAGCCCTTTCAGAGCACACTGTGGCTGCTGGTGGCGCTGTCAGTTCATGTGGTGGCCGTGATGCTGTAC 1680
ThrIleLeuValLysLysGluIleProArgSerThrLeuAspSerPheMetGlnProPheGlnSerThrLeuTrpLeuLeuValGlyLeuSerValHisValValAlaValMetLeuTyr 560

CTGCTGGACCGCTTCAGTCCCTTTGGCCGATTTAAGGTCAACACCGAGGAGGAGGAGGAGGATGCCACTGACCCTGTCCTCTGCCATGTGGTTTTCCTGGGGCGTCCTGCTCAACTCTGCC 1800
LeuLeuAspArgPheSerProPheGlyArgPheLysValAsnSerGluGluGluGluGluGluAspAlaLeuThrLeuSerSerAlaMetTrpPheSerTrpGlyValLeuLeuAsnSerGly 600

ATTGGGGAAGGTGCCCCCCGGAGTTTCTCTGCTCGTATCCTAGTCATGGTGTGGGCTGGTTTTGCCATGATCATCGTGGCTTCCTACACTGCCAACCTGGCAGCCTTCCTGGTGCTGGAT 1920
IleGlyGluGlyAlaProArgSerPheSerAlaArgIleLeuGlyMetValTrpAlaGlyPheAlaMetIleIleValAlaSerTyrThrAlaAsnLeuAlaAlaPheLeuValLeuAsp 640

AGGCCTGAGGAGCGCATCACAGGCATCAATGACCCCAGGCTCAGAAACCCCTCAGACAAGTTCATCTATGCAACTGTAAAACAGAGCTCTGTGGATATCTACTTTCGGCAGCAGGTGCTG 2040
ArgProGluGluArgIleThrGlyIleAsnAspProArgLeuArgAsnProSerAspLysPheIleTyrAlaThrValLysGlnSerSerValAspIleTyrPheArgArgGlnValLeu 680

TTGAGCACCATGTACCGGCACATGGAGAAGCACAATTATGAGAGTGCAGCTGAGGCCATCCAGGCTGTGCGGGACAACAAGCTCCATGCCTTCATCTGGGACTCAGCTGTGCTGGAGTTT 2160
LeuSerThrMetTyrArgHisMetGluLysHisAsnTyrGluSerAlaAlaGluAlaIleGlnAlaValArgAspAsnLysLeuHisAlaPheIleTrpAspSerAlaValLeuGluPhe 720

GAGGCTTCACAGAAGTGCGATCTGGTGACCACGGGTGAGCTGTTCTTCCGCTCCGGCTTTGGCATCGGCATGCGCAAGGACAGCCCCTGGAAGCAAAATGTGTCCCTGTCCATACTCAAG 2280
GluAlaSerGlnLysCysAspLeuValThrThrGlyGluLeuPhePheArgSerGlyPheGlyIleGlyMetArgLysAspSerProTrpLysGlnAsnValSerLeuSerIleLeuLys 760

TCCCATGAGAATGGCTTCATGGAAGACCTGGATAAGACATGGGTTCGGTATCAAGAATGTGACTCCCGCAGCAATGCCCCTGCCAACCTCACTTTTGAGAACATGGCAGGGGTCTTCATG 2400
SerHisGluAsnGlyPheMetGluAspLeuAspLysThrTrpValArgTyrGlnCysAspSerArgSerAsnAlaProAlaThrLeuThrPheGluAsnMetAlaGlyValPheMet 800

CTGGTGGCTGGAGGCATCGTAGCTGGGATCTTCCTCATTTTCATCGAGATCGGCTACAAGCGACACAAGGATGTCCTGTAGGAAGCAGATGCAGCTGGCTTTTGCAGCCGTGAACGTGTGG 2520
LeuValAlaGlyGlyIleValAlaGlyIlePheLeuIlePheIleGluIleAlaTyrLysArgHisLysAspAlaArgArgLysGlnMetGlnLeuAlaPheAlaAlaValAsnValTrp 840

AGGAAGAACCTGCAGGATAGAAAGAGTGGTAGAGCAGAGCCCGACCCTAAAAAGAAAGCCACATTTAGGGCTATCACCTCCACCCTGGCCTCCAGCTTCAAGAGACGTAGGTCCTCCAAA 2640
ArgLysAsnLeuGlnAspArgLysSerGlyArgAlaGluProAspProLysLysLysAlaThrPheArgAlaIleThrSerThrLeuAlaSerSerPheLysArgArgArgSerSerLys 880

GACACGACCACCGGGGGTGGACGCGGCGCTTTGCAAAACCAAAAAGACACAGTGCTGCCGCCACGCGCTATTGAGAGGGAGGAGGGCCAGCTGCAGCTGTGTTCCCGTCATAGGGAGAGC 2760
AspThrSerThrGlyGlyGlyArgGlyAlaLeuGlnAsnGlnLysAspThrValLeuProArgArgAlaIleGluArgGluGluGlyGlnLeuGlnLeuCysSerArgHisArgGluSer 920

TGAGACGCCCCCGCCCGCCCTCCTCTGCCCCTCCCCCGCAGACAGACGCACGGGACAGCGGCCTGGCCCACGCAGAGCCCCGGAGCACGACGGGGTCGGGGGAGGAGCACCCCCAGCCTCC 2880
CCCAGGCCGTGCCCGCCTGCCCGCCGGTCGGCCGGCTGCCGGTCCACCCTGTCCCGGCCCCCCGCGTGCCCCCGACGTCGGAGCTAACGGGCCGCCTTGTCTGTGTATTTCTATTTTACA 3000
GCAGTACCATCCCACTGATATCACGGGCCCGCTCAACCTCTCAGATCCCTCGGTCAGCACCGTGGTGTGAGGCCCCCGGAGGCGCCCACCTGCCCAGTTAGCCCGGCCAAGGACACTGAT 3120
CAGTCCTGCTGCTCGGGAAGGCCTGAGGGAAGCCCACCCGCCCCAGACACTGCCCACCCTGGG-----3' 3183

α2 subunit cDNAs [10] as probes yielded several cDNA clones encoding a novel GluR subunit [16,17]. Additional cDNA clones encoding a novel putative GluR subunit were obtained by series of screening of the same libraries with newly isolated cDNAs as probes. The cDNA insert of some of these recombinant phage carrying ζ1 subunit cDNAs were subcloned into the *Eco*RI site of the plasmid pBluescript II SK (−) (Stratagene) to yield the plasmids pHe9, pHe12, pHeF3, pHeF32, pHeF35, pFN4 and pFN12, respectively.

The nucleotide sequence (residues −149 to 3183) of the full-length cDNA clone He12 was determined on both strands by the dideoxy chain termination method [18]. Nucleotide sequence analysis revealed that the clone HeF3 carried parts of the He12 sequence corresponding to residues −28 to 2534 and 2646 to 3183 in succession, and the clone He9 these corresponding to residues 962 to 2534 and 2646 to 2813, indicating that 111 nucleotides (residues 2535 to 2645) were missing in these clones. We conclude that the HeF3 cDNA encodes a smaller form of the ζ1 subunit, designated as ζ1-2. Partial nucleotide sequence analysis showed that the 111 nucleotide segment was present in the clones HeF35, FN4 and FN12, and was absent in the clone HeF32. Analysis of nucleotide and amino acid sequences was carried out using GENETYX software (SDC) and Gene Works (IntelliGenetics).

2.2. *Expression of cDNAs*

Oligodeoxyribonucleotide 5′---CCA$_6$GGTGCA---3′
3′---ACCGTGGT$_6$CC---5′

was inserted into the *Pst*I site of the pBluescript II KS(−) (Stratagene) in the same orientation as the T3 promoter to yield the expression vector pBKSA. The 3.4 kb *Eco*RI fragment from λHe12 was subcloned into the *Eco*RI site of pBKSA in the same orientation as the T3 promoter to yield the plasmid pBKSAζ1. The 1.4 kb *Nhe*I fragment from pBKSAζ1, the 1.7 kb *Nhe*I *Eco*RI fragment from pHe9 and 3.0 kb *Nhe*I *Eco*RI fragment from pBKSA were ligated to yield the plasmid pBKSAζ1-2, carrying the coding sequence of the ζ1-2 subunit. The ζ1 subunit-specific and ζ1-2 subunit-specific mRNAs were synthesized in vitro with T3 RNA polymerase (BRL) using *Not*I-cleaved pBKSAζ1 and pBKSAζ1-2 as templates, respectively. Transcription was primed with cap dinucleotide, mGpppG (0.5 mM). *Xenopus laevis* oocytes were injected with the ζ1 subunit-specific or the ζ1-2 subunit-specific mRNA; the concentration of the mRNA was 1.0 μg/μl and the average volume injected was ~50 nl per oocyte. Whole cell currents were recorded at −70 mV membrane potential as described [10,19]. For TPA treatment, oocytes were perfused with normal Ringer's solution containing 1 μM TPA for 10 min.

3. RESULTS

3.1. *Two forms of the mouse NMDA receptor subunit ζ1*

A number of cDNAs encoding a putative GluR subunit have been isolated by series of screening under low stringency conditions of mouse forebrain and cerebellar cDNA libraries using mouse GluR α1 and α2 subunit cDNAs [10] and newly isolated putative GluR subunit cDNAs [16,17] as probes. Fig. 1 shows the nucleotide sequence and deduced amino acid sequence of one of these subunits, designated as the ζ1 subunit. The initial methionine is assigned to the first methionine residue found in a large open reading frame. The amino-terminal hydrophobic segment is assumed to represent a signal peptide as proposed for the GluR α1 and α2 subunits [10]. The proposed mature ζ1 subunit is composed of 920 amino acids with a calculated molecular weight of 103,477. The mouse ζ1 subunit shares 99.8% amino acid sequence identity with the rat NMDA receptor subunit NMDAR1 [15] and ~70% identities with the subunits of the AMPA- or kainate-selective GluR channel [7–14,16,17], thus representing the mouse counterpart of the rat NMDAR1 subunit. Analysis of several ζ1 subunit cDNA clones suggests the presence of a smaller form of the ζ1 subunit, designated as ζ1-2, whose sequence is identical to that of the ζ1 subunit except that 37 amino acids (residues 846–882) are deleted. Thus, the mature ζ1-2 subunit is composed of 883 amino acids with a calculated molecular weight of 99,313.

3.2. *Structural characteristics*

Analysis and comparison of the local hydropathicity profile of the ζ1 subunit suggest the presence of four putative transmembrane segments (M1–M4) in the carboxyl-terminal half of the molecule (Fig. 2). In accord with this model is the recent finding that the arginine 586 in the M2 segment of the α2 subunit determines the Ca^{2+} permeability of AMPA-selective GluR channels [19,20]. The ζ1 subunit contains an asparagine residue at the corresponding position in segment M2 (amino acid residue 598). In addition to the putative transmembrane regions, the region preceding segment M1 is also well conserved among GluR subunits, where the agonist binding site has been postulated [10,11]. A point mutation introduced into this region of the α1 subunit (mutation α1-K445E) strongly reduces the EC_{50} value for L-glutamate [19]. The putative cytoplasmic domain between segments M3 and M4 contains potential phosphorylation sites for Ca^{2+}-calmodulin dependent protein kinase type II [21] and protein kinase C [22]. Ten out of twelve potential *N*-glycosylation sites are found in the proposed extracellular domain.

3.3. *Functional expression*

The ζ1 and ζ1-2 subunits were expressed in *Xenopus* oocytes by injection of the respective mRNAs synthesized in vitro from cloned cDNAs. Oocytes injected with the ζ1 subunit-specific mRNA showed small but clear responses to 10 μM L-glutamate, 100 μM L-

←

Fig. 1. Nucleotide sequence of cDNA encoding the mouse NMDA receptor ζ1 subunit and the deduced amino acid sequence. Nucleotide residues are numbered in the 5′ to 3′-direction, beginning with the first residue of the codon specifying the amino-terminal residue of the mature subunit and the preceding residues are indicated by negative numbers. Amino acid residues are numbered beginning with the amino-terminal residue of the mature subunit and the preceding residues are indicated by negative numbers. Numbers of the nucleotide and amino acid residues at the right-hand end of the individual lines are given. The 111 nucleotides (residues 2535 to 2645) missing in the ζ1-2 subunit are underlined. The nucleotide sequence data will appear in the DDBJ, EMBL and GeneBank Nucleotide Sequence Database under the accession number D10028.

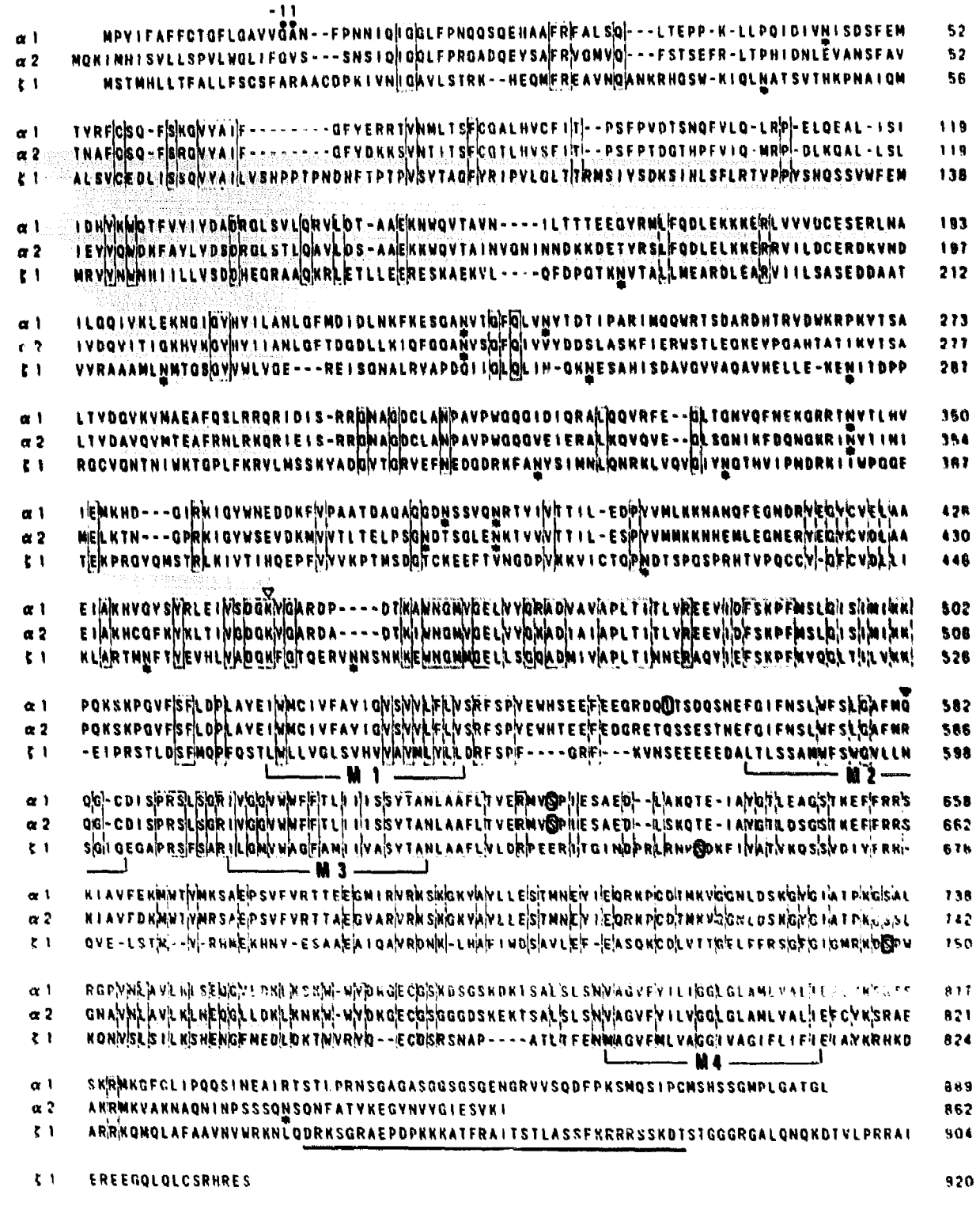


Fig. 2. Alignment of the deduced amino acid sequences of the mouse GluR α1, α2 and ζ1 subunits. Numbers of the amino acid residues at t right-hand end of the individual lines are given. Sets of identical amino acid residues in the homologous region are enclosed. The 37 amino a residues (residues 846–882) missing in the ζ1-2 subunit are underlined. The putative transmembrane segments (M1–M4) are indicated. T asparagine residues as potential *N*-glycosylation sites in the predicted extracellular domain are marked below with asterisks. Consensus phosp rylation sites for Ca^{2+}-calmodulin dependent protein kinase type II in the predicted intracellular domain are marked with open circles. The positi of the point mutations α1-K445E [19] and α2-R586Q [19,20,37] affecting the agonist binding and channel properties, respectively, are indicat by arrowheads.

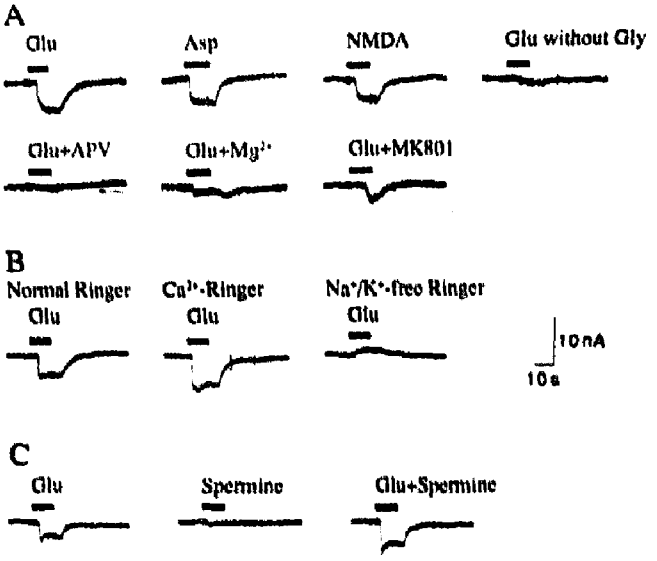


Fig. 3. Functional properties of the ζ1 subunit expressed in *Xenopus* oocytes. Agonist solutions contained 6 μM glycine unless otherwise indicated. Membrane potential, −70 mV. Inward current is downward. The duration of agonist application is indicated by bars without taking into account the dead-space time in the perfusion system (~2 s). (A) Whole-cell current activated by bath application of 10 μM L-glutamate, 100 μM L-aspartate, 100 μM NMDA, 10 μM L-glutamate without glycine, 10 μM L-glutamate with 100 μM APV, 10 μM L-glutamate with 100 μM $MgCl_2$, and 10 μM L-glutamate with 1 μM (+)-MK801. (B) Whole-cell current responses to 10 μM L-glutamate recorded from an oocyte in normal frog Ringer's solution (left), Ca^{2+}-Ringer's solution (middle) and Na^+- and K^+-free Ringer's solution (right). (C) Whole-cell currents activated by bath application of 10 μM L-glutamate (left), 250 μM spermine (middle) and 10 μM L-glutamate with 250 μM spermine (right).

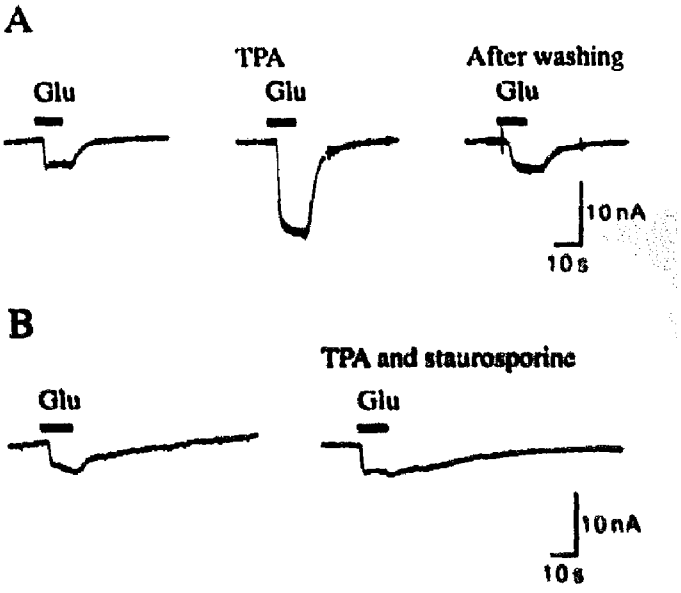


Fig. 4. Augmentation of ζ1 channel activity by TPA treatment. The experimental conditions were as in Fig. 3 unless otherwise specified. (A) Whole-cell currents activated by bath application of 10 μM L-glutamate before (left) and after treatment with 1 μM TPA for 10 min (middle), and after perfusion with normal frog Ringer's solution for a further 20 min (right). (B) Whole-cell currents activated by bath application of 10 μM L-glutamate before (left) and after treatment with 1 μM TPA in the presence of 5 μM staurosporine for 10 min (right).

aspartate, 100 μM NMDA (Fig. 3A) and 100 μM quisqualate (not shown) in the presence of 6 μM glycine at −70 mV membrane potential. No response was detected for 100 μM kainate and 100 μM AMPA even in the presence of glycine. The response to 10 μM L-glutamate was hardly detectable in the absence of glycine, an absolutely required activator of the NMDA receptor [23,24], and was diminished in the presence of 100 μM D-2-amino-5-phosphonovalerate (APV), a specific competitive antagonist of the NMDA receptor [25] (Fig. 3A). 100 μM Mg^{2+}, known to block the NMDA receptor channel in a voltage-dependent manner [26,27], and 1 μM (+)-MK-801, a channel blocker of the NMDA receptor [28], reduced the response to L-glutamate (Fig. 3A). The ζ1 channel activity was attenuated by the presence of 100 μM Zn^{2+}, reported to induce a non-competitive inhibition of the NMDA response in voltage-independent manner [29,30] (not shown). Similar results were obtained for the ζ1-2 subunit. The homomeric ζ1 channels exhibited a clear inward current in Na^+- and K^+-free Ringer's solution containing 20 mM Ca^{2+} (Ca^{2+}-Ringer's solution [19]), whereas a marginal outward current was observed in control Na^+- and K^+-free Ringer's solution (Fig. 3B), indicating that the ζ1 channels are permeable to Ca^{2+}. Spermine, reported to potentiate the NMDA receptor [31], enhanced the response of ζ1 homomeric channels by 1.4 ± 0.1 fold (n=6) (Fig. 3C). This effect was reversible. These pharmacological and electrophysiological properties are in good agreement with those of the NMDA-type GluR channel [1,2,31].

3.4. *Modulation of channel activity*

Treatment of the oocytes with 1μM TPA for 10 min enhanced the response of the ζ1 channels to 10 μM L-glutamate by 2.7 ± 0.4 fold (mean ± S.E.M., n=10) (Fig. 4A). The channel activity decreased gradually to the initial level by washing. This enhancing effect cannot be non-specific because the TPA treatment exerted no appreciable effect on the current amplitude of the heteromeric α1/α2 GluR channels expressed in oocytes (not shown). Furthermore, the effect of TPA was abolished by the simultaneous treatment with 5 μM staurosporine, a protein kinase inhibitor [32] (Fig. 4B). Enhancing effect of spermine was observed even after treatment with TPA. The ζ1-2 channels were similarly modulated by TPA treatment and polyamine.

4. DISCUSSION

The present investigation has shown the presence of two forms of a mouse NMDA receptor subunits, de-

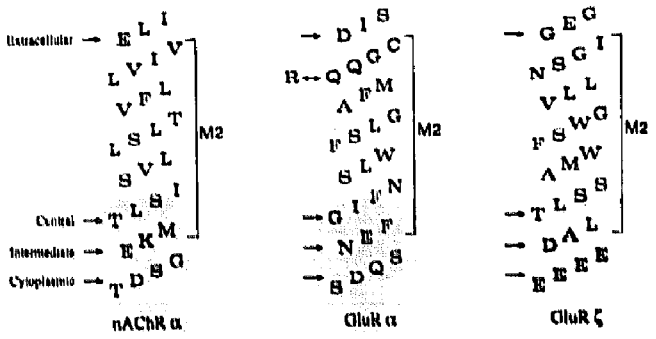


Fig. 5. Putative channel-forming regions of the GluR α1 and ζ1 subunits proposed by analogy of the nAChR α subunit. The one-letter amino acid notation is used. Arrows indicate the amino acid residues of the *Torpedo californica* nAChR α subunit constituting four rings as major determinants of ion flow through the channel [33,34] and the corresponding residues of the mouse GluR α1 and ζ1 subunits. The glutamine residue of the GluR α subunit corresponding to the arginine residue of the GluR α2 subunit as a critical determinant of the Ca^{2+} permeability [19,20] is also indicated.

signated as ζ1 and ζ1-2. The NMDA receptor subunit ζ1 as well as its smaller form ζ1-2 is endowed with most of the properties characteristic for the NMDA receptor, such as activation by glycine, Ca^{2+} permeability, blocking by Mg^{2+}, inhibition by Zn^{2+} and activation by polyamine [1,2,31], in accord with the rat counterpart of the ζ1 subunit [15]. In the absence of glycine, L-glutamate or NMDA alone failed to evoke the response of the ζ1 or ζ1-2 channels, in consistent with the absolute requirement of glycine for activation of rat NMDA receptor channels expressed in *Xenopus* oocytes by injection of rat brain poly(A)$^+$ RNA [24]. On the other hand, the rat counterpart of the ζ1 subunit has been reported to be activated by MNDA in the absence of glycine [15], although possible contamination of glycine in the assay system used can not be excluded. The lower channel activities of homomeric ζ1 or ζ1-2 channels as compared with NMDA channel activities observed in oocytes injected with mouse brain poly(A)$^+$RNA may imply the requirement of additions subunits for the NMDA receptor channel.

Since the GluR channels of NMDA-type and non-NMDA type are structurally similar but are functionally distinct, it is of interest to precisely inspect the primary structures of the glutamate-gated ion channels. Under the assumption that the basic structure is similar between the GluR channel and the nicotinic acetylcholine receptor (nAChR) channel, one can figure out the putative channel-forming region of the GluR channels (Fig. 5). Negatively-charged and polar residues are found in the ζ1 subunit at the positions corresponding to the four rings of the nAChR channel, major determinants of the ion flow through the channel [33,34]. Asparagine residue occupies the position corresponding to glutamine or arginine residue of the GluR α1 and α2 subunits, which determines the Ca^{2+} permeability of the GluR channel [19,20]. Unique to the ζ1 subunit is the presence of a contiguous stretch of negatively-charged aspartate and glutamate residues at the amino-terminal side of the segment M2. The corresponding region of the nAChR includes the intermediate ring constituting the narrowest constriction of the channel. In view of the fact that a point mutation introduced into the intermediate ring of the nAChR channel decreases not only the conductance but also the sensitivity to Mg^{2+} [33], the stretch of negatively charged aspartate and glutamate residues of the GluR ζ1 subunit may be responsible for the voltage-dependent channel block by Mg^{2+}. On the other hand, the carboxyl-terminal region of segment M3 is highly conserved among GluR subunits and thus may play an important role common to the GluR channel family.

Our finding that the ζ1 channel activity is enhanced by TPA treatment raises an intriguing possibility that the NMDA receptor channel can be regulated by protein kinases and such modulation might play a role in long-term potentiation, excitotoxity and developmental synaptic plasticity, in which NMDA receptors are thought to mediate key steps [1–4]. In this respect, it is to be noted that potentiation of the NMDA receptor by a μ opioid has been reported to be mediated by protein kinase C [35]. Furthermore, protein kinase C has been suggested to mediate the induction and maintenance of long-term potentiation [36].

Acknowledgements: We thank Drs. Kenji Sakimura, Tatsuya Kutsuwada, Michiaki Nagasawa and Katzutaka Ikeda for help with cDNA cloning and electrophysiological measurements, Misses Miyuki Nagao and Mami Ikarashi for experimental assistance, and Miss Mutsuko Motokoshi for help in the preparation of the manuscript. This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Institute of Physical and Chemical Research, the Ministry of Health and Welfare of Japan, the Naito Foundation, and the Yujin Memorial Grant.

REFERENCES

[1] Mayer, M.L. and Westbrook, G.L. (1987) Prog. Neurobiol. 28, 197–276.
[2] Monaghan, D.T., Bridges, R.J. and Cotman, C.W. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 365–402.
[3] Collingridge, G.L. and Bliss, T.V.P. (1987) Trends Neurosci. 10, 288–293.
[4] McDonald, J.W. and Johnston, M.V. (1990) Brain Res. Rev. 15, 41–70.
[5] Choi, D.W. (1988) Neuron 1, 623–634.
[6] Olney, J.W. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 47–71.
[7] Hollmann, M., O'Shea-Greenfield, A., Rogers, S.W. and Heinemann, S. (1989) Nature 342, 643–648.
[8] Keinänen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakmann, B. and Seeburg, P.H. (1990) Science 249, 556–560.
[9] Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C. and Heinemann, S. (1990) Science 249, 1033–1037.
[10] Sakimura, K., Bujo, H., Kushiya, E., Araki, K., Yamazaki, M.,

Yamazaki, M., Meguro, H., Warashina, A., Numa, S. and Mishina, M. (1990) FEBS Lett. 272, 73–80.
[11] Nakanishi, N., Shneider, N.A. and Axel, R. (1990) Neuron 5, 569–581.
[12] Bettler, B., Boulter, J., Hermans-Borgmeyer, I., O'Shea-Greenfield, A., Deneris, E.S., Moll, C., Borgmeyer, U., Hollmann, M. and Heinemann, S. (1990) Neuron 5, 583–595.
[13] Werner, P., Voigt, M., Keinänen, K., Wisden, W. and Seeburg, P.H. (1991) Nature 351, 742–744.
[14] Egebjerg, J., Bettler, B., Hermans-Borgmeyer, I. and Heinemann, S. (1991) Nature 351, 745–748.
[15] Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1991) Nature 354, 31–37.
[16] Morita, T., Sakimura, K., Kushiya, E., Yamazaki, M., Meguro, H., Araki, K., Abe, T., Mori, K.J. and Mishina, M. (1992) Mol. Brain. Res., in press.
[17] Sakimura, K., Morita, T., Kushiya, E. and Mishina, M. (1992) Neuron 8, in press.
[18] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[19] Mishina, M., Sakimura, K., Mori, H., Kushiya, E., Hurabayashi, M., Uchino, S. and Nagahari, K. (1991) Biochem. Biophys. Res. Commun. 180, 813–821.
[20] Hollmann, M., Hartley, M. and Heinemann, S. (1991) Science 252, 851–853.
[21] Pearson, R.B., Woodgett, J.R., Cohen, P. and Kemp, B.E. (1985) J. Biol. Chem. 260, 14471–14476.
[22] Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y. and Nishizuka, Y. (1985) J. Biol. Chem. 260, 12492–12499.
[23] Johnson, J.W. and Ascher, P. (1987) Nature 325, 529–531.
[24] Kleckner, N.W. and Dingledine, R. (1988) Science 241, 835–837.
[25] Davies, J., Francis, A.A., Jones, A.W. and Watkins, J.C. (1981) Neurosci. Lett. 21, 77–81.
[26] Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. and Prochiantz, A. (1984) Nature 307, 462–465.
[27] Mayer, M.L., Westbrook, G.L. and Guthrie, P.B. (1984) Nature 309, 261–263.
[28] Wong, E.H.F., Kemp, J.A., Priestley, T., Knight, A.R., Woodruff, G.N. and Iversen, L.L. (1986) Proc. Natl. Acad. Sci. USA 83, 7104–7108.
[29] Peters, S., Koh, J. and Choi, D.W. (1987) Science 236, 589–593.
[30] Westbrook, G.L. and Mayer, M.L. (1987) Nature 328, 640–643.
[31] Wong, E.H.F. and Kemp, J.A. (1991) Annu. Rev. Pharmacol. Toxicol. 31, 401–425.
[32] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[33] Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K. and Numa, S. (1988) Nature 335, 645–648.
[34] Imoto, K., Konno, T., Nakai, J., Wang, F., Mishina, M. and Numa, S. (1991) FEBS Lett. 289, 193–200.
[35] Chen, L. and Huang, L.Y.M. (1991) Neuron 7, 319–326.
[36] Hu, G.-Y., Hvalby, O., Walaas, S.I., Albert, K.A., Skjeflo, P., Andersen, P. and Greengard, P. (1987) Nature 328, 426–429.
[37] Verdoorn, T.A., Burnashev, N., Monyer, H., Seeburg, P.H. and Sakmann, B. (1991) Science 252, 1715–1718.