Instituto Juan March
de Estudios e Investigaciones

33 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Mechanisms of Synaptic Function

Organized by

J. Lerma and P. H. Seeburg

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M. V. L. Bennett
A. L. de Blas
J. Bockaert
G. L. Collingridge
M. G. Darlison
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PROGRAMME

MOLECULAR MECHANISMS OF SYNAPTIC FUNCTION

MONDAY, October 17th, 1994

I. Chairperson: R.S. Zukin

P.H. Seeburg	- Glutamate-gated ion channels in brain: Properties and genetic control.
M.G. Darlison	- Molecular characterization and functional expression of invertebrate ionotropic glutamate receptors.
M.V.L. Bennett	- Subunit composition determines spermine potentiation of NMDA receptors containing NR1 and NR2 isoforms.
J. Rossier	- Patch-clamp and RT-PCR techniques applied to single cells.

II. Chairperson: M.L. Mayer

R. A. Nicoll	- Postsynaptic mechanisms involved in Ca^{++} mediated synaptic plasticity.
G.L. Collingridge	- Roles of mGluRs in long-term potentiation.
C.E. Jahr	- Clearance of glutamate from excitatory synapses of cultured hippocampal neurons.
G.L. Westbrook	- Probing excitatory synaptic function with Mk-801.

Short Presentations:

D.M. Kullmann - Amplitude fluctuations of NMDA and AMPA/kainate receptor-mediated EPSCs in CA1 cells of the guinea pig hippocampus.

G. Maguire - The role of glutamate receptor desensitization and regulation in the formation of glutamatergic EPSCs in retinal neurons.

TUESDAY, October 18th, 1994

III. Chairperson: P. Ascher

K. Keinänen - Purification and characterization of AMPA receptors overexpressed in insect cells.

Y. Stern-Bach - Structure/function relationships of ionotropic glutamate receptors.

J. Lerma - Native kainate receptors in mammalian neurons.

M.L. Mayer - Allosteric modulation of native and recombinant AMPA and kainate receptors.

IV. Chairperson: G. L. Collingridge

J.P. Pin - The metabotropic glutamate receptors: Intracellular domains and G-protein coupling.

J. Bockaert - Modulation of voltage-sensitive calcium channels by metabotropic glutamate receptors (mGluR2/3 and mGluR1).

J. Sánchez-Prieto - Presynaptic glutamate receptors in the control of glutamate release.

K. Moriyoshi - Analysis of physiological functions of glutamate receptors at the molecular level.

Short Presentations:

J. Cudeiro - Nitric oxide modulates visual processing in the feline thalamus by regulation of NMDA-mediated activity.

R. Schneggenburger - Fractional calcium currents through AMPA- and NMDA-receptor channels in different types of central neurons.

WEDNESDAY, October 19th, 1994

V. Chairperson: P.H. Seeburg.

P. Ascher - Mechanosensitivity of NMDA receptors.

J. Garthwaite - Nitric oxide, synaptic plasticity and excitotoxicity.

P. Krogsgaard -Larsen - Design and molecular pharmacology of glutamate receptor agonists, partial agonists and antagonists.

A.L. de Blas - Study of the $GABA_A$ receptors with subunit-specific antibodies.

P.H. Seeburg - Concluding Remarks

INTRODUCTION

J. Lerma

Introduction

Most synapses at Central Nervous System use an amino acidic substance as the transmitter agent. Glutamatergic and GABAergic synapses are ubiquitously distributed throughout the brain, and they are involved in almost every physiological phenomenon from the spinal cord to the cerebral cortex. In the middle of this century, Hayashi found that the application of glutamate to the surface the cerebral cortex, an amino acid highly concentrated in every neuron of the brain, induced a strong electroconvulsive activity, constituting what probably was the first evidence for this amino acid as a neuroexcitatory substance. Since then, further studies showed that glutamate is the most widely used excitatory neurotransmitter in the Central Nervous System.

In more recent years we have learnt that the diverse effects of glutamate are mediated by a number of membrane receptor proteins. The two main classes of glutamate receptors have been characterized and they belong to one of these two families: the ionotropic receptors, which are ligand-gated channels, permeable to cations, and the metabotropic receptors, coupled to GTP-binding proteins. The activation of the first class results in an increase in membrane conductance, while activation of the second class produces a cascade in second messenger-mediated events. During the last few years, more than twenty different subunits of the glutamate ionotropic receptor and several metabotropic receptors have been identified. In addition, the study of mechanisms for neuron communication has been approached by new and refined techniques: E.g., the development of the patch-clamp technique and its application to a number of experimental situations. Cloning and expression of genes coding for different subunits of amino acid receptors is providing a detailed understanding of the fine structure and molecular determinants of membrane protein complexes responsible for fast synaptic transmission, synaptic plasticity and triggering mechanisms of neuronal death. Available information begins to be substantial and some surprising results in the particular field of excitatory amino acid receptors indicate the need for a reevaluation of our ideas of how receptor subtypes allow transfer of information among cells.

The aim of this workshop on the "Molecular Mechanisms of Synaptic Function" was not to revisit synaptic transmission, but to discuss several aspects of the molecular estructure and modulation of amino acid receptors. How the protein domains are involved in kinetics properties and ion selectivity; the regulation of expression of genes encoding for different receptor subtypes; the role of alternative splicing and the influence of subunit composition in functional receptor properties; the interaction of ligands with allosteric regulatory sites and its consequence on receptor function, and finally, to examine the role of amino acid receptors in synaptic plasticity and the mechanisms by which it is induced. In summary, this workshop tried to contribute to an interchange of ideas in this rapidly growing field.

Session I

Glutamate-Gated Ion channels in Brain: Properties and Genetic Control.

Peter H. Seeburg[1], Nail Burnashev[2], Johannes Mosbacher[2], Peter Jonas[2], Bert Sakmann[2], Georg Köhr[1], Hilda Lomeli[1], Miyoko Higuchi[1], Rolf Sprengel[1] and Hannah Monyer[1].
[1] Laboratory of Molecular Neuroendocrinology, University of Heidelberg, Center for Molecular Biology (ZMBH), Im Neuenheimer Feld 282 and [2] Max-Planck-Institut für Medizinische Forschung, Jahnstr. 29, D-69120 Heidelberg, Germany

L-glutamate, the principal excitatory neurotransmitter in CNS activates ionotropic receptors in postsynaptic membranes. These glutamate receptor channels (GluRs) are configured from different subunits of which 16 have been characterized by cloning, and can be grouped into different families based on sequences and functional properties [1]. Native GluRs are grouped into AMPA receptors, NMDA receptors and high-affinity kainate receptors. In contrast to AMPA and NMDA receptors, the physiological role of the high-affinity kainate receptors in central neurons is not resolved. All three receptor types can contribute to neuronal death in pathophysiological conditions.

AMPA receptors mediate the majority of the fast synaptic excitatory action of L-glutamate. These channels possess rapid kinetics, and are largely impermeable to Ca^{2+} ions. AMPA receptors assemble from subsets of four subunits, GluR-A to -D (alternatively termed GluR1 to 4) which share approximately 70% sequence identity. Each subunit can self-assemble to form homooligomeric channels that can be activated by L-glutamate and by excitatory amino acid analogs such as kainate domoate [1]. When several AMPA receptor subunits are co-expressed, heterooligomeric channels form with properties distinct from those of homooligomeric channels. One particular property that varies with subunit composition is the Ca^{2+} permeability of AMPA receptor channels. This Ca^{2+} permeability is very low for channels configured with participation of GluR-B but is high for AMPA channels formed in the absence of GluR-B [1]. Since GluR-B is highly expressed in most CNS structures, native AMPA receptor channels have generally low Ca^{2+} permeabilities. Thus neurons can regulate the Ca^{2+} influx through AMPA receptors by controlling the read-out of the GluR-B gene. Indeed, molecular and biophysical analysis of single live neurons in brain slices shows that cells with Ca^{2+} permeable AMPA receptors (generally GABA-ergic interneurons) express little GluR-B relative to other subunits whereas neurons with low Ca^{2+} permeability of AMPA receptors have high levels of GluR-B relative to other AMPA receptor subunits [2]. The determinant for the dominant behaviour of GluR-B regarding divalent ion permeability of AMPA receptor channels resides in the channel forming region M2. GluR-B carries a positively

charged residue (arginine, R) in M2 while all other AMPA receptor subunits carry an uncharged residue (glutamine, Q) in the homologous position. Thus, the particular residue in the Q/R site of M2 profoundly influences the ion conductance and gating properties of AMPA receptor channels. However, the arginine residue of GluR-B M2 is not encoded by the GluR-B gene whose sequence specifies that a glutamine residue should occupy the Q/R site. It appears that a single nucleotide in the exonic CAG codon for glutamine is changed at the transcript level to a CGG codon for the critical channel determinant in GluR-B. This process termed RNA editing is mediated by an intronic sequence in the GluR-B gene, which is unique to that gene and is not found in the genes for the other AMPA receptor subunits [3]. It has been demonstrated that RNA editing also affects the Q/R position in several subunits of high-affinity kainate receptors in central and peripheral neurons [1].

We have recently found another position in AMPA receptors where RNA editing changes a gene-specified codon, which is in an extracellular receptor domain, adjacent to the alternatively spliced modules Flip and Flop [4]. In this position, a gene-directed arginine (R) codon is converted into a glycine (G) codon in GluR-B, -C, -D mRNA but not in GluR-A. Interestingly, the extent of editing at this R/G site is developmentally controlled. Furthermore, AMPA receptor channels edited in this position have faster recovery rates from L-glutamate-induced desensitization. This and the developmental progression of R/G editing suggest that RNA editing may be involved in the fine-tuning of excitatory transmission [4].

Central excitatory synapses often operate with both, AMPA receptors and NMDA receptors. The latter possess high Ca^{2+} / Na^{+} permeability ratios, but are characterized by slower kinetics than AMPA receptor channels. Furthermore, NMDA receptor activity is subject to a voltage-dependent Mg^{2+} block of the ion pore. An additional difference to AMPA receptors is that NMDA receptor activation requires the presence of glycine as co-agonist to L-glutamate. In molecular terms, NMDA receptors are configured from two different subunit types termed NMDAR1 (NR1) and NMDAR2 (NR2). For the latter subunit there exist four variants (NR2A to NR2D) each encoded by its own gene. The NR1 subunit gene can be alternatively spliced to yield 8 different molecular variants. The NR2 subunit genes show strict developmental and regional expression patterns [5]. The participation of NR2 subunits in NMDA receptor formation endows NMDA receptors with subtype-specific properties, e.g., strength of Mg^{2+} block, Ca^{2+} block of Na^{+} current, desensitization time course, time course of deactivation, and glycine sensitivity. Thus, central neurons can carry NMDA receptors which differ in molecular, biophysical and pharmacological properties. The hallmark

properties of NMDA receptors, high Ca^{2+} permeability and voltage-dependent Mg^{2+} block were traced to an asparagine residue in the M2 of its subunits. The critical asparagine (N) occupies a position homologous to the Q/R site of AMPA receptor subunits. Indeed, introducing in AMPA receptors an N into the Q/R site endows them with high Ca^{2+} permeability.

NMDA receptors are key players in paradigms of neuronal plasticity and in ischemic cell death. The complex receptor structure possesses several allosteric sites via which receptor activation can be modulated. One such site is termed the redox site at which reducing agents can potentiate glutamate-activated NMDA currents [6]. This potentiation is thought to play a role in ischemic and traumatic injury and leads to excessive influx of Ca^{2+} into cells. It can be shown that the time course and extent of potentiation is subunit specific and is correlated with differences in kinetic parameters.

1. P.H.Seeburg, *Trends Neurosci.* **16**, 359-365 (1993).
2. P.Jonas et al. *Neuron* **12**, 1281-1289 (1994).
3. M.Higuchi et al., *Cell* **75**, 1361-1370 (1993).
4. H.Lomeli et al. *Science*, in press (1994).
5. H.Monyer et al. *Neuron* **12**, 529-540 (1994).
6. G.Köhr et al. *Neuron* **12**, 1031-1040 (1994).

MOLECULAR CHARACTERIZATION AND FUNCTIONAL EXPRESSION OF INVERTEBRATE IONOTROPIC GLUTAMATE RECEPTORS

Mark G. Darlison[1], Thorsten Stühmer[1], Muriel Amar[1,2], Robert J. Harvey[1], Hye-Chin Kim[1], Jan van Minnen[3] and Isabel Bermudez[2]

[1]Institut für Zellbiochemie und klinische Neurobiologie, Universitäts-Krankenhaus Eppendorf, Universität Hamburg, 20246 Hamburg, Germany, [2]School of Biological and Molecular Sciences, Oxford Brookes University, Oxford OX3 0BP, United Kingdom, and [3]Department of Organismic Zoology, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands

The existence of ion-channel receptors in invertebrate nervous tissue and muscle, that can be gated by the neurotransmitter L-glutamate, is well established. However, in contrast to the situation in mammals, relatively little information is available concerning the structures and functional properties of these. We have investigated the molecular nature of glutamate-gated ion channels (GluRs) in the fresh-water mollusc *Lymnaea stagnalis*, an animal with a simple nervous system (~15,000 neurons) which has been the subject of intensive neurophysiological investigation. Using the polymerase chain reaction, we have cloned full-length complementary DNAs (cDNAs) for three different *Lymnaea* GluR-like subunits; in addition, we have isolated partial cDNAs for two further GluR-like polypeptides. All of the three fully-characterized clones encode polypeptides with an M_r of ~100,000 daltons; however, their products display markedly different sequence similarities to mammalian GluRs. One *Lymnaea* subunit exhibits greatest similarity (43% to 46% identity) to the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-selective GluR1 to GluR4 subunits, a second displays greatest similarity (44% to 48% identity) to the kainate-selective GluR5 to GluR7 subunits, while the third displays no more than 32% identity to any mammalian GluR. The invertebrate GluR5 to GluR7-like polypeptide, which we have named InvGluR-K1, has been functionally expressed, as a homo-oligomer, in *Xenopus laevis* oocytes. Large, microampere, whole-cell currents are routinely observed upon the application of either L-glutamate (EC_{50} = 1.2 ± 0.3 μM) or several other glutamate receptor agonists (rank order of potency: L-glutamate » kainate > ibotenate > L-glutamic acid diethyl ester ≈ AMPA); these currents can be blocked by 6,7-dinitroquinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1-(4-chlorobenzoyl)-piperazine-2,3-dicarboxylic acid (CBPD). Ionic replacement experiments reveal that the agonist-induced current is carried almost entirely by sodium and potassium ions. We have also localized the transcripts for the various GluR-like polypeptides in the *Lymnaea* nervous system by *in situ* hybridization. The mRNA that encodes InvGluR-K1 is found in all of the central ganglia, including the buccal ganglia. Interestingly, the properties and deduced location of InvGluR-K1 are consistent with it being a component of the receptor, present on cells of the buccal ganglia, that mediates the excitatory effects of L-glutamate in the central pattern generator that controls the feeding behaviour of the snail.

SUBUNIT COMPOSITION DETERMINES SPERMINE POTENTIATION OF NMDA RECEPTORS CONTAINING NR1 AND NR2 ISOFORMS. M.V.L. Bennett, X. Zheng, L. Zhang and R.S. Zukin. Dept. of Neurosci., Albert Einstein College of Med., Bronx, NY 10461.

Alternative RNA splicing of the NR1 subunit confers differential structural, physiological and pharmacological properties to recombinant NMDA receptors. Insertion of a 21 amino acid cassette in the predicted amino terminal domain of the NR1 subunit reduces agonist affinity and potentiation by spermine andn zinc, and increases whole cell currents of expressed recombinant receptors. $NR1_{011}$, the most prominent NR1 splice variant in rat forebrain, and $NR1_{100}$, prominent in midbrain, were expressed in *Xenopus* oocytes singly and in combination with NR2A, NR2B and NR2C. To identify structural features of the N1 insert that control current amplitude and spermine and Zn^{2+} potentiation, we constructed mutant NR1 receptors with amino acid substitutions in N1. Neutralization of all six positively charged residues in N1 by replacement with alanines fully rescued spermine and Zn^{2+} potentiation. Positive charges in N1 did not appear to affect spermine or Zn^{2+} affinity. Neutralization of positive charges in N1 diminished the responses to the level of those of NR1 receptors lacking N1. These observations suggest that the positively charged N1 insert may increase agonist-induced currents by causing a conformational change similar to that produced by spermine and Zn^{2+} in NR1 receptors lacking N1.

Differential associatiion of the N41 subunit with NR2 also affects spermine potentiation of heteromeric recombinant NMDA receptors. $NR1_{011}$ homomers and $NR1_{011}$/NR2B receptors exhibited spermine potentiation by two mechanisms: by increasing glycine affinity and by increasing current through receptors with bound NMDA and glycine. $NR1_{011}$/NR2A receptors exhibited only the increase in glycine affinity and $NR1_{011}$/NR2C receptors exhibited neither. $NR1_{100}$ homomers and $NR1_{100}$/NR2B and $NR1_{100}$/NR2A receptors exhibited spermine potentiation only by increase in glycine affinity. Spermine produced no potentiation of $NR1_{100}$/NR2C receptors. Thus, the NR2B subunit "permits" both forms of spermine potentiation; the NR2A subunit permits spermine potentiation only by increase in glycine affinity and the NR2C subunit permits neither form of potentiation. These observations are of interest because the NR1 and NR2 subunits are differentially distributed and developmentally regulated. At early ages postnatal, NR2B subunit mRNA is more highly expressed than NR2A and NR2C mRNAs in hippocampus, neocortex and caudate putamen. These findings may account for observed differences among neurons in polyamine actions and suggest that these actions will vary in a cell-specific and age-related manner.

Jean Rossier
Institut Alfred Fessard
C N R S
91198 Gif-sur-Yvette CEDEX
F R A N C E

Patch-clamp and RT-PCR techniques applied to single cells

The diversity of known glutamate gated channels has been markedly increased by the molecular cloning of multiple subunits and their spliced and edited variants. These subunits can potentially form different oligomeric complexes with diverging properties. A crucial question is therefore to determine the actual subunit composition of naturally occurring glutamate receptors. We have coupled patch-clamp recordings and reverse transcription followed by PCR amplification to correlate the presence of messenger RNAs for each subunit and the functional properties of native glutamate receptors at the single cell level. We describe here results on the AMPA receptors of hippocampal neurones and on the N-methyl-D-aspartate (NMDA) receptors of cerebellar granule cells obtained with this technique.

The single cell RT PCR provides electrophysiologists using the patch clamp technique with a convenient method to link electrophysiological recordings to a molecular analysis of the mRNAs expressed in a single cell. This molecular analysis can be used either to correlate cell responses with their molecular basis or to identify cell types according to the expression of specific markers. The core of the molecular analysis is PCR which makes it fast, sensitive and simple.

The NMDA subtype of glutamate receptors is an oligomer formed of different subunits named NR1 and NR2A, B, C and D, the combination of which determines the channel functional properties. Rat cerebellar slice cultures were used to study the involvement of spontaneous activity and extracerebellar inputs on the changes of NR2A-C expression observed during the postnatal development of granule and Purkinje cells. The functional properties of the NMDA receptors and the expression of the NR2A-C mRNAs were correlated in single neurons by coupling patch-clamp recording and reverse transcription followed by PCR. Granule cells grown in standard culture conditions expressed mainly NR2A mRNAs when examined after 15 to 40 days *in vitro*. Consistent with this observation, their responses to NMDA were poorly reduced by 3 µM of ifenprodil, a non-competitive antagonist which discriminates NR2A and NR2B subunits in expression systems. The NR2C subunit, abundantly expressed *in vivo* by adult synapses, were formed between granule cells and mossy fibers originating from co-cultured brainstem explants. In cerebellar cultures which had been chronically exposed to tetrodotoxin for 15 to 35 days *in vitro* to reduce spontaneous activity, granule cells expressed predominantly NR2B subunits (as is the case only during the first two postnatal weeks *in vivo*) and their responses to NMDA were largely inhibited by 3 µM of ifenprodil. These results provide evidence that the expression of NR2A and B subunits is regulated through an activity-dependent mechanism leading to the formation of NMDA receptors with different pharmacological properties. Finally, in none of the experimental conditions described above, were detected any NR2 subunits in Purkinje cells, although NR1 was consistently expressed. This is in agreement with the absence of NMDA responses.

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Session II

POSTSYNAPTIC MECHANISMS INVOLVED IN Ca^{++} MEDIATED SYNAPTIC PLASTICITY.

R.A. Nicoll, T. Manabe, and D.J.A. Wyllie, Department of Pharmacology, University of California, San Francisco, San Francisco, CA 94143-0450. Supported by grants from the NIH and SERC.

It is universally agreed that the induction of NMDA receptor-dependent LTP is post-synaptic, requiring the entry of Ca^{++} through the NMDA receptor into the postsynaptic spine. There is considerable debate as to whether LTP is expressed as an increase in glutamate release, or an increase in sensitivity of AMPA receptors on the postsynaptic membrane, or both. We have taken two approaches to address this issue. First, we designed experiments that allowed us to determine if there is a change in postsynaptic sensitivity following manipulations that elevate Ca^{++} throughout the postsynaptic cell. Second, we designed experiments to monitor the probability of transmitter release (P_r) so that we could determine whether the release of glutamate increases during LTP.

In the first series of experiments we used the application of NMDA (Manabe et al, Nature 355:50, 1992) and the activation of postsynaptic voltage-sensitive Ca^{++} channels with depolarizing voltage pulses to potentiate synaptic transmission (Kullmann et al, Neuron 9:1175, 1992; Wyllie et al, Neuron, 12:127, 1994). Such experiments were designed so that all synapses on the cell would be exposed to the potentiating stimulus, thus maximizing our ability to detect postsynaptic changes. Following each manipulation we monitored the amplitude of miniature (m)EPSCs and responses to exogenously applied AMPA, two parameters generally accepted to be determined postsynaptically. We found that both manipulations resulted in a large increase in the amplitude of evoked EPSCs. Accompanying this potentiation was a large increase in the size of mEPSCs, as well as an increase in the size of AMPA responses. The selective CAM kinase II inhibitor KN-62 strongly antagonized the potentiation induced by voltage pulses. These results provide convincing evidence that during Ca^{++}-dependent potentiation there is a clear increase in the sensitivity of postsynaptic AMPA receptors. Unlike LTP, however, the voltage pulse-induced potentiation and NMDA-induced potentiation, although dependent on a rise in postsynaptic Ca^{++}, are transient. However, blockade of protein phosphatases by calyculin A converts the voltage pulse-induced potentiation into a long lasting form.

In the second series of experiments we used the use-dependent NMDA receptor antagonist MK-801 to monitor glutamate release (Manabe and Nicoll, Jap. J. Physiol., in press). Synaptic stimulation in the presence of MK-801 results in a progressive decay in the amplitude of the NMDA receptor-mediated component of the EPSC. The decay rate of the amplitude of the EPSC to repeated stimulation is very sensitive to manipulations that change P_r; high P_r results in a rapid decay. Despite the sensitivity of this assay for detecting changes in P_r, the P_r of synapses expressing LTP was no different from naive synapses. These results appear to rule out changes in P_r as contributing importantly to the expression of LTP. The only presynaptic mechanisms that would go undetected with this assay are the presynaptic recruitment of synapses that were entirely silent before LTP or presynaptic changes in quantal size.

In summary, two sets of experiments were designed to address the issue of whether the expression of LTP is pre- or postsynaptic. One set was designed to test for possible postsynaptic changes, and the other was designed to test for presynaptic changes. Both sets of experiments support a postsynaptic mechanism underlying the expression of NMDA receptor-dependent LTP, which may be mediated by activation of CAM kinase II.

Roles of mGluRs in long-term potentiation

Z. A. Bortolotto, C. H. Davies, Z. I. Bashir & G. L. Collingridge *Department of Pharmacology, The University of Birmingham, U.K.*

The induction of long-term potentiation (LTP) in area CA1 of the hippocampus normally requires the transient synaptic activation of N-methyl-D-aspartate (NMDA) receptors. We have started to use metabotropic glutamate receptor (mGluR) antagonists, in particular (+)-α-methyl-4-carboxyphenylglycine (MCPG), (Watkins & Collingridge, 1994; *Trends Pharmacol Sci* **15**, 333-342) to explore the possible roles of mGluRs in NMDA receptor-dependent hippocampal LTP.

Field EPSPs were recorded from stratum radiatum in rat hippocampal slices (Bashir et al., 1993; *Nature* **363**, 347-350). In experimentally naïve slices, (+)-MCPG (200 μM) blocked the induction of LTP, but not short-term potentiation (STP), in a reversible manner in most slices tested (n=33/39). In parallel experiments, we first induced LTP and reset the baseline and then tested the ability of MCPG to block the induction of further LTP. On each occasion tested, MCPG was ineffective even if the concentration was increased to 1 mM . Thus, a tetanus conditions a pathway such that the induction of LTP no longer requires the activation of mGluRs. The conditioning can be induced by delivering tetani in the presence of a specific NMDA antagonist (D-AP5; 50 μM) or by applying a specific mGluR agonist (1S,3R-ACPD; 10 μM). The conditioning can be blocked by delivering the tetanus in the presence of MCPG (200 μM) or a protein kinase inhibitor (K-252b; 0.1 μM). These results show that the activation of mGluRs during a tetanus negates the need to activate them during subsequent tetani because of a conditioning effect (i.e., there is an mGluR activated "molecular memory").

We have explored ways of erasing the kinase-dependent molecular memory. Following the induction of LTP, low frequency stimulation (900 shocks at 2 Hz) depotentiated the response to near pre-tetanus levels. Thereafter, MCPG invariably blocked the induction of LTP. Thus, low frequency stimulation re-instigates the need for mGluR activation for the induction of LTP, presumably by dephosphorylation of the substrate(s) phosphorylated by the K-252b-sensitive kinase(s). Both the induction of depotentiation and the erasing of the molecular memory is also prevented by MCPG, suggesting that mGluRs activate the phosphatase cascades.

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CRAIG E. JAHR

Vollum Institute for Advanced Biomedical Research
Oregon Health Sciences University L-474
PORTLAND, OR. 97201-3098 (USA)

Clearance of glutamate from excitatory synapses of cultured hippocampal neurons

Glutamate is released from presynaptic terminals of excitatory synapses by exocytosis of vesicles that have concentrated glutamate to about 100 mM. Because the postsynaptic membranes are only 20 nm from presynaptic release sites, a very high concentration of glutamate must exist at the postsynaptic receptors for a brief period after release. Clearance of glutamate from the cleft results from diffusion and possibly also from binding to glutamate transporters. We have investigated the rate of clearance as well as the role of glutamate transporters in clearance by measuring the percent inhibition of excitatory postsynaptic currents by very low affinity competitive antagonists of the AMPA receptor in the presence and absence of transporter blockers. In cultures of hippocampal neurons, we have found in both conditions that clearance is very fast, on the order of 100 μs, and that inhibiting binding of glutamate to transporters prolongs the presence of free glutamate in the cleft, but only very briefly. Block of glutamate transporters enhances AMPA receptor epsc amplitudes but not decay time courses, consistent with only a slight prolongation of free glutamate in the cleft. The rise time of miniature AMPA receptor epscs is slowed only slightly in the presence of low affinity competitive antagonists of the AMPA receptor. Based on a kinetic model of AMPA receptor activation (Jonas et al., J. Physiol., 472:615, 1993), we predict that binding of glutamate to transporters in or very near the cleft results in significant buffering of released glutamate that can decrease postsynaptic receptor occupancy.

Probing excitatory synaptic function with MK-801

Gary L. Westbrook
Vollum Institute, Portland OR, USA

Kinetic studies using rapid application of defined concentrations of agonist to whole-cell and outside-out patch preparations are useful in separating agonist binding steps from intrinsic gating of ligand-gated ion channels. For the NMDA channel, this approach has been used to examine the mechanisms of glycine regulation and desensitization, the number of agonist binding sites and the channel properties that shape the synaptic response (Mayer et al., 1989; Sather et al., 1990; Lester et al., 1990; Clements and Westbrook, 1991; Lester and Jahr, 1992; Hestrin 1992). Recently, the open channel blocker MK-801 has been employed to determine the open probability (Po) of NMDA channels. However this technique has resulted in a wide range of reported open probabilities (Huettner and Bean, 1988; Jahr, 1992; Rosenmund et al., 1993; Hessler et al., 1993), albeit using somewhat different analytical approaches and preparations. The discrepancy could be due to alteration of NMDA channels in outside-out patches. For example, desensitization and the calcium-sensitivity of NMDA channels are affected by patch recording (Benveniste et al., 1990; Sather et al., 1990; Sather et al., 1992;, Lester and Jahr, 1992; Rosenmund and Westbrook 1993a,b; Lester et al., 1993). Alternatively, synaptic NMDA channels may be different than extrasynaptic somatic channels, the usual source of outside-out patches.

To discriminate between these possibilities, we compared the Po of NMDA channels in whole-cell recording and outside-out patches from cultured hippocampal neurons. Currents were evoked by rapid application of saturating concentrations of NMDA in the presence or absence of MK-801. The reduction of the peak amplitude and the acceleration of the decay of the current in MK-801 were used to derive Po by fitting the current traces to a multistate kinetic model. The Po in whole-cell was low similar to that estimated for synaptically-activated NMDA channels. In contrast, ensemble average currents from outside-out patches were much more rapidly blocked in the presence of MK-801, indicative of a significantly higher Po. The Po in patches increased with the duration of recording reaching a value approximately 8 times that of the whole-cell Po. This time-dependence implies that channel gating is sensitive to mechanical alterations during pulling of the patch or to the loss of soluble cytoplasmic constituents during patch dialysis.

We also tested whether the disparity in the Po could be attributed to different gating of synaptic vs. extrasynaptic channels. Using microisland cultures containing a single excitatory neuron, the Po of whole-cell currents was measured before and after all synaptic NMDA channels ($\geq$90%) were blocked by evoking EPSCs in the presence of MK-801. The Po of synaptic- and extrasynaptic channels was equivalent, suggesting that Po recorded in the whole-cell configuration is the same as at the synapse. An interesting additional finding was that 81 $\pm$ 4% of NMDA channels on the membrane surface were synaptic. The low open probability for synaptic channels implies that with each presynaptic stimulus only 50% of channels that bind glutamate actually open, thus the NMDA receptor-mediated EPSC has a significant functional 'reserve'. This work was supported by NIH grants NS26494 and MH46613.

Amplitude fluctuations of NMDA and AMPA/kainate receptor-mediated EPSCs in CA1 cells of the guinea pig hippocampus.

Dimitri M Kullmann, Dept. of Clinical Neurology, Institute of Neurology, Queen Square, London WC1N 3BG, UK.

AMPA/kainate and NMDA receptors are thought to be colocalized at many glutamatergic synapses in the brain, accounting for the two components of evoked or spontaneous EPSCs which can be separated pharmacologically and electrophysiologically. It is however not known whether the ratio of the synaptic signals mediated by the two receptor types is constant between different synapses on the same cell. I have compared the trial-to-trial amplitude fluctuations of synaptic signals mediated by AMPA/kainate and NMDA receptors, by recording population EPSCs evoked in CA1 pyramidal cells by stratum radiatum stimulation. The coefficient of variation (CV) of the NMDA component was consistently smaller than that of the AMPA/kainate component. This is unlikely to arise from differences in the variability of quanta mediated by the two receptor types, but could be explained if AMPA/kainate receptors were absent or non-functional at a large proportion of synapses. The CV of the AMPA/kainate receptor mediated component was reduced by induction of long-term potentiation (LTP), a result which has previously been taken to imply an increase in the presynaptic release probability. LTP however had no effect on either the average amplitude or the CV of the NMDA component. An alternative explanation for the results is that LTP induction uncovers clusters of latent AMPA/kainate receptors. This would account for an increase in quantal content, but with no change in transmitter release and therefore no need for a retrograde messenger.

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THE ROLE OF GLUTAMATE RECEPTOR DESENSITIZATION AND REGULATION IN THE FORMATION OF GLUTAMATERGIC EPSCs IN RETINAL NEURONS. Greg Maguire, Sensory Sciences Center, University of Texas, Houston, Texas, USA.

Glutamate receptors in the vertebrate retina are can be comprised of at least 7 ionotropic and 6 metabotropic subunits. This diversity is recognized in natively expressed glutamate receptors by the wide variety of functional subtypes in the intact retina. Two important functional characteristics of the GluRs that I am currently studying, and which underlie specific retinal operational abilities are: 1) regulation by intracellular pathways activated by another neurotransmitter, dopamine, and 2) rapid versus slow desensitization. Whole cell and perforated patch techniques are used to record the GluR currents and GluR mediated EPSCs in retinal bipolar and amacrine neurons, using both isolated cells and cells in the semi-intact retinal slice preparation. Using these techniques neurons can be classified according to their natural stimulus, light, then examined for glutamate receptor responses and glutamatergic EPSCs, and then examined for morphological characteristics using Lucifer yellow filing of the recorded neuron. I have found that transient amacrine cells, which respond briefly to a step of light, express rapidly desensitizing GluRs and that this rapid desensitization underlies the transient amacrine cell's brief EPSCs, and forms the brief spiking character of this AC type. In contrast, sustained amacrine cells respond longer to a step of light, have glutamatergic EPSCs with much longer relaxation times, and express more of the slowly desensitizing GluRs. Cyclothiazide lengthens the relaxation time of the EPSC in transient amacrine but not sustained amacrine neurons. In bipolar cells of the retina, I have found that the glutamate elicited currents are enhanced by dopamine through a D1/G-protein/cAMP/PKA pathway, and that this enhancement underlies the ability of dopamine to enhance the cone photoreceptor driven synaptic inputs to bipolar cells. This does not happen in the GluRs associated with rod input. Thus glutamate receptor subtypes in bipolar cells, and their regulation by other transmitters, are important for controlling the relative contribution of rod and cone inputs to the visual system, one of the most important tasks performed by the visual system

Session III

PURIFICATION AND CHARACTERIZATION OF AMPA RECEPTORS OVEREXPRESSED IN INSECT CELLS

Kari Keinänen, VTT Biotechnology and Food Research
P.O.Box 1500, FIN-02044 VTT, Espoo, Finland

AMPA-selective glutamate receptors GluR-B and GluR-D (1) were expressed in recombinant baculovirus-infected Sf21 insect cells at levels of 15-30 pmol of high-affinity [^{3}H]AMPA binding sites per mg of protein (2). The GluR-B and GluR-D subunits formed functional homomeric and heteromeric GluR channels as shown by agonist-triggered inward currents in the infected cells. From a panel of nonionic and ionic detergents tested for solubilization of the high-affinity AMPA binding sites, Triton X-100, Triton X-114, n-dodecylmaltoside and n-octyl-glucoside proved most effective, solubilizing up to 90 % and 70 % of GluR-B and GluR-D, respectively. The detergent-solubilized receptors bound to immobilized wheat germ lectin and were subsequently eluted by N-acetylglucosamine, indicating the presence of N-linked glycosylation. For purification of the receptor, an polyhistidinyl affinity tag engineered at the C-terminus of GluR-D was employed. Affinity-tagged GluR-D bound to Ni^{2+}-charged Chelating Sepharose and was eluted by 100-200 mM imidazole. This single affinity step provided about 60-fold purification of the receptor to a specific activity exceeding 1000 pmol/mg of protein. The purified receptor preparation contains a major 105-kDa component which is recognized by polyclonal anti-GluR-D antiserum. Purified GluR-D binds [^{3}H]AMPA with a K_d of 40 nM and displays ligand binding pharmacology characteristic of AMPA-selective GluRs. Cross-linking and gel-filtration experiments indicate a multimeric structure for insect cell -expressed AMPA receptors. Further refinement of the purification procedure in combination with scaled-up cell culture should facilitate production of GluRs for detailed biochemical and structural studies.

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STRUCTURE/FUNCTION RELATIONSHIPS OF IONOTROPIC GLUTAMATE RECEPTORS

Yael Stern-Bach, Bernhard Bettler, Melissa Hartley and Stephen F. Heinemann.

Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, CA 92037 USA

Ionotropic glutamate receptors mediate most of the fast excitatory synaptic transmission in the central nervous system. They appear to play a key role in synaptic plasticity, such as memory formation, as well as in chronic and acute neurological disorders. Therefore, understanding how these receptors operate is crucial for the understanding of basic brain functions.

Based on their agonist affinities, ionotropic glutamate receptors have been classified into three major pharmacological subtypes: AMPA, kainate and NMDA. Recent molecular studies demonstrate that mammalian ionotropic glutamate receptor subunits are encoded by at least sixteen genes. The GluR1-4 subunits exhibit properties similar to those of native AMPA receptors, while GluR5-7 and the KA-1/KA-2 subunits assemble into receptors reminiscent of kainate-binding sites described in the brain. The NMDAR1 and NMDAR2A-2D subunits form recombinant receptors with the same properties as native NMDA receptors.

Despite extensive pharmacological and electrophysiological characterization of cloned glutamate receptors, little is known about the protein domains that determine agonist binding specificity. In order to identify such domains, a series of chimeric proteins was generated with reciprocal exchanges between an AMPA receptor subunit, GluR3, and a kainate receptor subunit, GluR6. The GluR3 and GluR6 subunits each form homomeric receptors with distinct agonist activation profiles. For example, kainate is a much more potent agonist on GluR6 than GluR3 receptors (~100 fold), while AMPA activates GluR3 but not GluR6 receptors. The results obtained with the chimeric subunits will be discussed, along with implications on the possible location of the agonist binding site and the topology of ionotropic glutamate receptors.

NATIVE KAINATE RECEPTORS IN MAMMALIAN NEURONS

J. Lerma, Dept. Neural Plasticity, Instituto Cajal, C.S.I.C., 28002-Madrid,

Cloning of glutamate receptors has provided evidence for the existence of several structurally related subunit families, each comprised of several members (1, 2). It has been proposed that KA1 and KA2 and GluR5-7 families represent subunit classes of high affinity kainate receptors and that *in vivo* different kainate receptor subtypes might be constructed from these subunits in heteromeric assembly. However, despite some indications from autoradiographic studies and binding data in brain membranes, functional glutamate receptors of the kainate type have not been detected in brain cells until very recently (3). Whole-cell patch-clamp recordings in cultured hippocampal cells, in combination with fast concentration-clamp perfusion, have demonstrated the existence of functional kainate-selective glutamate receptors which, when activated by kainate, show pronounced desensitization with fast onset and very slow recovery. They are also activated by quisqualate and domoate, but not by AMPA. Hill analysis revealed an EC_{50} for kainate of 22 μM and Hill coefficient of 1.1. Responses induced by 300 μM of kainate desensitized completely and recovered following single exponential time courses (τ_{onset}=20 ms; τ_{rec}=20 s). While the peak current varied with the concentration of glutamate, showing an EC_{50} of 400 μM, steady-state desensitization of kainate receptors was evident at low concentrations of glutamate, showing a half-maximal inactivation at $\approx$3 μM. Kainate receptors showed different pharmacology and Ca^{2+} permeability determinants than AMPA receptors. Aniracetam or cyclothiazide did not affect desensitization of kainate receptor-mediated responses, while strongly potentiated responses at AMPA receptors. Although both protein and mRNAs for kainate receptor subunits are abundant in several brain regions (4), the responsiveness of AMPA receptors to kainate makes it difficult to demonstrate the presence of functional receptors of the kainate type in native cells. In young cultures (e.g. 1-2 days in vitro) about 30% of the cells exclusively show responses of the kainate type, but the great majority (57%) express both types of non-NMDA glutamate receptors. Selective desensitization of AMPA receptors prior to the application of kainate, as described by Lerma et al. (3), revealed functional kainate receptors in such cells. However, if kainate receptors are expressed at low levels in these cells, the desensitizing response to kainate is masked by the larger and non-desensitizing response to kainate acting on AMPA receptors. This may be well the situation in more developed neurons, in which responses to kainate are mostly of the AMPA type (i.e. non-desensitizing). The lack of a specific agonist for kainate receptors precludes their functional study in more mature systems (e.g. slices or older cultures) in which their participation in synaptic transmission or plasticity can be addressed. Consequently, the functional significance of these strongly desensitizing glutamate receptors remains enigmatic. We have found that the family of new non-competitive antagonists of AMPA receptors (GYKI 52466 and 53655) minimally affects kainate-induced responses at kainate receptors while completely blocks AMPA receptor-mediated currents, making it possible for the first time to separate the responses mediated by each receptor (5). These compounds will allow determination of the role played by kainate receptors in synaptic transmission and plasticity in the mammalian brain, as well as evaluation of their involvement in neurotoxicity.

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Allosteric modulation of native and recombinant AMPA and kainate receptors.

Mark L. Mayer, Lab. Cellular & Molecular Neurophysiology, NICHD, Bldg. 49, Room 5A78, NIH, Bethesda, MD 20892.

Neurons and glia show rapidly desensitizing responses to glutamate receptor agonists, the extent of which varies from cell to cell. This reflects cell-specific expression of glutamate receptor subtypes. In hippocampal neurons responses to glutamate and AMPA, show strong desensitization, while responses to kainate are only weakly desensitizing[1]. This pattern of activity is similar to that observed for recombinant AMPA receptors expressed in HEK293 cells[2], but differs from that observed for DRG neurons[3,4], and for recombinant kainate receptors, for which kainate produces strongly desensitizing responses[5,6]. In glial O-2A progenitor cells responses to kainate show both desensitizing and non-desensitizing responses, consistent with expression of mRNA for both AMPA and kainate receptor subunits[7].

Essentially all agonists and antagonists which act at AMPA and kainate receptors show cross reactivity at individual receptor subtypes, such that the search for truly selective agonists and antagonists has yet to yield success. In contrast, we have found that cyclothiazide, and the lectin concanavalin A, which act as allosteric regulators of desensitization, show highly selective activity at native and recombinant AMPA and kainate receptors, even when both receptor populations are expressed in the same cells[7,8].

Cyclothiazide, a benzothiadiazine of similar structure to diazoxide, an allosteric modulator of AMPA receptors[1,9,10], completely abolishes desensitization evoked by brief applications of glutamate in HEK293 cells transfected with flip versions of the AMPA receptor subunits GluRA-D, but fails to block desensitization for cells expressing GluR6[11]. In contrast, concanavalin A strongly attenuates desensitization to kainate for cells transfected with GluR6, but has only weak effects in cells transfected with GluRA-D. When both AMPA and kainate receptors are expressed in the same cells, cyclothiazide selectively attenuates desensitization evoked by AMPA but not kainate, while concanavalin A selectively attenuates desensitization evoked by kainate but not AMPA. Further evidence for separate expression and assembly of AMPA and kainate receptors comes from analysis of the current voltage relationships for cyclothiazide and concanavalin A potentiated responses in oocytes injected with mixtures of glutamate receptor subunits for which kainate receptors were in their edited forms. In these experiments AMPA gave inward rectifying responses selectively potentiated by cyclothiazide, while kainate gave outward rectifying responses selectively potentiated by concanavalin A, indicating that there is no cross assembly between AMPA and kainate receptor subunits[11].

For native glutamate receptors in glia, and in hippocampal and DRG neurons, cyclothiazide and concanavalin A show selectivity of action similar to that recorded for recombinant receptors. Thus, desensitization evoked by brief applications of glutamate to hippocampal neurons is abolished by cyclothiazide, while cyclothiazide fails to modify desensitization in DRG neurons[1,4]. In contrast, concanavalin A, which has only weak effects on desensitization in hippocampal neurons, essentially abolishes desensitization in DRG neurons[3,4]. In glial O-2A progenitor cells, in which the equilibrium dose response curve for kainate is biphasic, with a high affinity component due to

activation of kainate receptors (EC50 5 μM) and a low affinity component due to activation of AMPA receptors (EC50 260 μM), concanavalin A selectively potentiates responses at the high affinity site[7], suggesting that when native AMPA and kainate receptors are expressed within the same cells, concanavalin A maintains selectivity of action. In these same cells cyclothiazide abolishes desensitization evoked by AMPA, while desensitization to kainate remains pronounced.

In recent experiments we have found that cyclothiazide can be used to discriminate amongst AMPA receptor subtypes generated by alternative splicing[2]. Both the flip and flop isoforms of AMPA receptors show modulation of desensitization by cyclothiazide[8]; however, for the flip isoforms cyclothiazide abolishes desensitization, similar to the effect observed for native AMPA receptors in hippocampal neurons[1], while for flop receptors desensitization is slowed 50-fold, but remains pronounced. In heteromeric receptors the flip isoform exerts dominant activity, such that it for native AMPA receptors it may not be possible to differentiate between native homomeric receptors generated from flip containing subunits and heteromeric receptors containing flip and flop subunits. Recent proposals by Seeburg[12] and Huganir[13] place the flip/flop module in the extracellular domain of AMPA receptors, consistent with our finding that cyclothiazide acts only from the extracellular space. This raises the intriguing possibility that the cyclothiazide binding site in AMPA receptors is generated by amino acids from the flip/flop module.

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Session IV

THE METABOTROPIC GLUTAMATE RECEPTORS: INTRA-CELLULAR DOMAINS AND G-PROTEIN COUPLING

PIN J.-P., GOMEZA J., PREZEAU L., JOLY C. AND BOCKAERT J.
UPR-CNRS 9023, CCIPE rue Cardonnille, F-34094 Montpellier Cedex5

The main fast excitatory neurotransmitter in the CNS, glutamate (Glu), acts on ionotropic and metabotropic (G-protein-coupled) receptors (mGluR). Seven cDNA coding for mGluRs have been isolated (for a review see, (Nakanishi, 1992; Pin and Duvoisin, 1994). They have no homology with any other GPCR, and form therefore a new receptor family. Based on their amino acid sequence identity, pharmacology and transduction mechanisms, they can be sub-classified into 3 groups. mGluR1 and mGluR5 which represent the first Group, are coupled to PLC, and have Quisqualate as their most potent agonist. The 2nd Group is composed of mGluR2 and mGluR3 which are coupled to Gi-proteins and have L-CCG-I as a most potent agonist. The others, mGluR4, 6 and 7 correspond to the 3rd Group, they also inhibit adenylyl cyclase but are selectively activated by L-AP4. Multiplicity in this receptor family is augmented by the existence of several splice variants for mGluR1, 5 and 4 (see (Pin and Duvoisin, 1994). Recently, the cloning from the parathyroid of a Ca-sensing receptor homologous to mGluRs revealed that this receptor family is not limited to Glu receptors. Based on the expression into Xenopus oocytes and mammalian cells of wild-type, chimeric and truncated receptors, we have examined the role of the intracellular domains of mGluRs in their specific activation of G-proteins.

By exchanging the intracellular loops and the C-terminal domain of mGluR3 which inhibits adenylyl cyclase, with the equivalent domains of the PLC-coupled mGluR1, we determined the region involved in the specific coupling of mGluRs to PLC (Pin et al., 1994). Among the chimeric receptors constructed, those able to stimulate PLC possess the second intracellular loop (i2) of mGluR1. Those having the i2 of mGluR3 do not coupled to PLC, indicating that this loop plays a critical role in the specific activation of PLC-activating G-proteins. More careful analysis of this domain indicated that the 2 third C-terminal part of this loop may be important for the specific G-protein coupling. Interestingly, this loop possesses highly amphipathic segments that likely form amphiphilic α helices and is rich in basic residues, two characteristics of G-protein interacting domains of other GPCR. These results indicate that i2 of mGluRs may be the equivalent of i3 of the well known large family of GPCR.

In contrast to any other known GPCR, PLC coupled mGluRs (mGluR1 and mGluR5) possess a long (350 residues) C-terminal intracellular domain. To elucidate the role of this domain we first analysed the function of the different splice variants of mGluR1 having different C-terminus - mGluR1a has the long C-terminus whereas mGluR1b and 1c have a short C-terminus of 67 and 57 residues respectively. When expressed into Xenopus oocytes, mGluR1a induces fast and transient activation of a

chloride channel as expected for a PLC-coupled receptor, whereas mGluR1b and c induce slow and oscillatory responses (Pin et al., 1992), typical of receptors not very well coupled to PLC (Kunkel and Peralta, 1993). When expressed into LLC-PK1 cells, mGluR1a but not mGluR1b and 1c, has a strong intrinsic activity and also stimulates adenylyl cyclase, indicating that the long C-terminus of mGluR1a plays some role in its transduction properties.

To analyse the domain within the long C-terminal tail of mGluR1a involved in its specific properties, a series of truncated receptors were constructed. As expected, deletion of most of the C-terminus of mGluR1a generates a truncated receptor having the properties of mGluR1b or c. However, truncated receptors with a C-terminus shorter than that of mGluR1b or c possess all the properties of mGluR1a. We propose that the extreme C-terminus of mGluR1b or c acts as a inhibitory domain preventing the receptor to function in the absence of agonist and to couple to adenylyl cyclase. The action of this inhibitory domain may be prevented by the long C-terminus in mGluR1a.

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MODULATION OF VOLTAGE-SENSITIVE CALCIUM CHANNELS BY METABOTROPIC GLUTAMATE RECEPTORS (mGluR2/3 AND mGluR1)

Pascale CHAVIS, Laurent FAGNI, Jean-Louis BOSSU*, Janet M. NOONEY*, Anne FELTZ*, Joël BOCKAERT

UPR CNRS 9023, CCIPE, Rue de la Cardonille, 34094 Montpellier Cedex 5, France
*Laboratoire de Neurobiologie Cellulaire, Centre de Neurochimie, CNRS, 67084 Strasbourg, France

Cloned metabotropic glutamate receptors (mGluRs) can be classified into three main groups *(Nakanishi, 1992; Pin and Duvoisin, 1994)* : Group 1 comprises mGluR1 and mGluR5 which stimulate phospholipase C (PLC). They have a high affinity for quisqualate (QA) and a low affinity for 2-(carboxycyclopentyl)-glycine (L-CCGI). Group 2 is comprised of mGluR2 and mGluR3 which inhibit cAMP production. They have a high affinity for L-CCGI (sub μM) and a low affinity for QA (> 50μM). Group 3 comprises the other mGluRs which are sensitive to L-AP4, insensitive to t-ACPD and inhibit cAMP production at least in CHO cells. Coupling to either PLC or adenylyl cyclase (AC) are the most classical ways of studying the coupling of mGluRs. However, their transducing mechanisms are much more complex. For example, mGluR1a stimulates PLC, inhibits types N- and L- VSCC, inhibits IK_M, IK_{leak}, IK_{AHP} and stimulates BK in neurons. In addition, mGluR1a stimulates PLA2 and AC (at least in transfected cells).

Their ability to control Ca^{++} entry is of particular importance since these receptors are implicated in synaptic plasticity events such as LTP and LTD *(Aniksztejn et al., 1992; Bortolotto et al., 1994; Ito and Karachot, 1990; Nakanishi, 1992; Pin and Duvoisin, 1994)*. Group 1 has already been shown to inhibit L- and N-type channels, whereas group 3 is also known to inhibit L-type channels.

Here, we report in cerebellar granule cell neurons that (1) Group 2 is able to inhibit L- and non-L-type Ca^{++} channels via a pertussis toxin-sensitive G protein. The inhibition can be detected in whole cell recording and also in cell-attached recording. Since, in this latter configuration, the agonists are added in the medium, a second messenger is likely to be implicated. We have excluded the possibility that Ca^{++}, cAMP, PKC and classical phosphorylation could be implicated. The messenger, if any, is still to be found *(Chavis et al., 1994a)*. (2) Group 1 stimulates L-type Ca^{++} channels. This can be detected in cell-attached recording, the agonist being added in the medium, and in a condition under which the cell is depolarized during the entire recording period. Here again, the messenger involved remains unknown. We have excluded that Ca^{++}, PKA and PKC could be involved. This stimulatory effect of Group 1 mGluRs on L-type channels does not involve the pertussis toxin-sensitive G protein *(Chavis et al., 1994b)*.

We are currently studying the possibility of recording the stimulatory effect in whole cell recording.

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PRESYNAPTIC GLUTAMATE RECEPTORS IN THE CONTROL OF GLUTAMATE RELEASE.

J. Sánchez-Prieto Borja,
Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense. Madrid 28040, Spain.

Knowledge of the mechanisms controlling the release of glutamate are critical to understand the long-lasting changes in synaptic plasticity as well as the neurotoxic accumulation of glutamate in the extracellular medium that occurs in certain pathological conditions. Glutamate release at a nerve terminal is triggered by the Ca^{2+} that enters through the active-zone Ca^{2+}-channels and then induces the fusion of synaptic vesicles with plasma membrane. Isolated nerve terminals, which contain all the machinery for the uptake, storage and exocytotic release of glutamate, respond to the same inhibitors and modulators as more complex preparations. Thus, in synaptosomes it has been demonstrated that the glutamate exocytosis induced by a localized increase in intracellular $[Ca^{2+}]$, is blocked by tetanus and botulinum neurotoxins, is sensitive to the blockade of Ca^{2+} channels by toxins from Agelenopsis aperta and can be modulated by a number of presynaptic receptors (for review see 1).

Protein kinase C, PKC, is present in the brain and relatively abundant in nerve terminals. Activation of PKC with phorbol esters potentiates the release evoked by 4-aminopyridine, 4AP, (2), which induces tetrodotoxin-sensitive transient depolarizations (3), but not that evoked by the clamped depolarizations induced by KCl. The facilitation of release by PKC-activation is consistent with an increase in both the depolarization and the elevation in $[Ca^{2+}]$, evoked by 4AP. Upstream of this PKC-dependent pathway there is a metabotropic glutamate receptor, mGluR, coupled to the generation of diacylglycerol, DAG, (4) that upon activation with the agonist (1S,3R) 1-aminocyclopentane-1,3-dicarboxylic acid, ACPD, potentiates the release by a mechanism that mimics that of phorbol esters (5). However, the enhanced release is observed only when the metabotropic agonist is co-applied with the putative retrograde messenger arachidonic acid (5). In phosphorylation studies we found that the role of arachidonic acid is to sensitize PKC to the DAG produced by the metabotropic receptor (6). All these data indicate that cerebrocortical nerve terminals from adult rats contain a signal transduction pathway that respond only to coincident signals with the result of a potentiation in the release of glutamate.

Strong evidence also exists for glutamate receptors activation to decrease synaptic transmission through a reduction in transmitter release. In nerve terminals from young rats we have found that L(+)-2-amino-4-phosphonobutyrate, L-AP4, and ACPD inhibit the glutamate release evoked by 4AP or KCl. The reduction of glutamate exocytosis, parallels a decrease in the rise in $[Ca^{2+}]c$ but without any change in plasma membrane depolarization. Moreover, the inhibitory action of L-AP4 is insensitive to the presence of staurosporine suggesting that protein phosphorylation is not involved in the inhibitory mechanism.

Developmental studies of the facilitatory and inhibitory pathways for glutamate release indicate the presence of inhibitory receptors in nerve terminals from young rats (1-4 weeks of age) but not in adult rats (2-3 months). In contrast, the potentiation of glutamate release via facilitatory mGluRs is very weak in nerve endings from rats of 1-2 weeks, but increases in week 3 and remains high in adult rats. Interestingly, both types of receptors coexist in the same nerve terminal as suggested by a PKC-mediated interaction by which the facilitatory mechanism suppress the inhibitory responses.

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Analysis of Physiological Functions of Glutamate Receptors at the Molecular Level

K. Moriyoshi, A. Nomura, Y. Hayashi, M. Masu, H. Iwakabe and S. Nakanishi

Institute for Immunology, Kyoto University Faculty of Medicine

Glutamate plays an essential role in the mammalian central nervous system, not only as a major excitatory neurotransmitter but also as an important signaling molecule in synaptic plasticity and neurodegeneration. In the last several years, molecular cloning studies including those from our own group (Masu et al., 1991; Moriyoshi et al., 1991) have revealed the existence of at least 20 different genes for glutamate receptors. These receptor genes are classified into 3 families, AMPA/KA receptors, NMDA receptors and metabotropic glutamate receptors. Each receptor family is subdivided into subgroups on the basis of structural and functional characteristics. The individual receptors show distinct expression pattern and molecular characteristics which may contribute to the diverse functions of glutamate (Nakanishi, 1992). To explore the physiological roles of individual receptors, we have studied the function of the glutamate receptors in different neuronal systems by combining biochemical, pharmacological, physiological and genetical approaches.

In this workshop, my presentation will focus on our recent findings indicating some crucial role of metabotropic glutamate receptors in visual and olfactory signal transducton. We have cloned 7 different subtypes of metabotropic glutamate receptors. mGluR6 is specifically localized at the postsynaptic site of the photoreceptor-bipolar synapses of retina. Our studies have indicated that mGluR6 is responsible for synaptic transmission between photoreceptor and ON-bipolar cells, thus mediating the key process in ON and OFF signal segregation in the visual system (Nomura et al., 1994).

Another subtype, mGluR2, is localized at the presynaptic site of the granule cells in the dendrodendritic synapses between the granule and mitral cells of the accessory olfactory bulb. We have identified a specific agonist for mGluR2 with the aid of Dr. Ofune in Suntory Institute. Electrophysiological study using this specific agonist shows that the mGluR2 activation suppresses the GABA-mediated transmission from granule to mitral cells and would contribute to discrimination of olfactory stimuli (Hayashi et al., 1993). The mGluR2 activation also induces neuronal plasticity responsible for olfactory memory formation in vivo (Kaba et al., 1994). Thus, our studies demonstrate that mGluRs play important and specialized roles in synaptic transmission and neural plasticity.

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NITRIC OXIDE MODULATES VISUAL PROCESSING IN THE FELINE THALAMUS BY REGULATION OF NMDA MEDIATED ACTIVITY.

J. Cudeiro*, C. Rivadulla, R. Rodriguez, S. Martínez-Conde, C. Acuña. *Dpto. Ciencias de la Salud I, Univ. de La Coruña. Dpto. Fisiología, Univ. Santiago.

Besides the complex visually specific pathways involving the dorsal lateral geniculate nucleus of the cat (LGN), where visual responses resulting from retinal stimulation are known to be the result of activation of aminoacid receptors on receiving LGN cells, there exist a large number of modulatory pathways considered to influence the activity of LGN cells more globally, having putative roles in sleep/wake and arousal states. One such pathway is the cholinergic pathway from the parabrachial nucleus of the brainstem. Interestingly, recent evidence has shown that within the LGN there is a unique distribution of nitric oxide synthase (NOS), the enzyme responsible for the production of nitric oxide (NO). Within the LGN of the cat, NOS is found exclusively within the ACh containing fibers arising in the parabrachium (Bickford et al., 1993).

We have recently presented preliminary evidence using iontophoretic application of putative inhibitors of NOS that, within the cat LGN, NO acts to enhance visual responses, specifically and selectively enhancing NMDA mediated excitation (Cudeiro et al., 1994a,b). In order to examine the mechanisms of this effect more fully, we have derived dose-response curves for excitatory aminoacids and for the non-aminoacid excitant ACh in the presence and absence of the competitive inhibitor of NOS, N^G-nitro-L-Arginine (L-NOArg). The NO donor nitroprusside was also tested. Experiments were carried out on adult cats anesthetized with a mixture of N_2O (70%), O_2 (30%) and halothane (0.1-5%) and paralyzed with gallamine (10mg/kg/h). Seven barrelled micropipettes were used for recording single-unit activity and the iontophoretic application of drugs. Each drug barrel of the electrode contained a selection of the following: 3M NaCl for extracellular recording, L-Arginine (L-Arg, 10mM, pH 6.0), physiological substrate for NOS; D-Arginine (D-Arg, 10mM,pH 6.0), the biological inactive isomer; N^G-nitro-L-arginine (L-NOArg, 10mM, pH 6.0) a competitive inhibitor of NOS; Quisqualate (Quis, 25mM, pH 8.0); α-amino-3-hydroxy-5-5-methyl-4-isoxazole-propionic acid (AMPA, 15mM, pH 8); Kainate (Kain, 0.1M, pH 8); *N*-methyl-D-aspartate (NMDA, 0.1M, pH8); Nitroprusside (10mM, pressure ejection). For each cell, after classification, visual responses were tested quantitatively before, during and after application of L-NO-Arg, alone or in combination with L-Arg. Pulsatile application of excitatory aminoacids and ACh were examine in the same manner.

In our hands, iontophoretic application of inhibitors of NOS resulted both in significant decreases in visual responses, without change in response selectivity, and decreased responses to exogenous application of NMDA. Responses to other applied excitants were largely unaffected. These effects were reversible by co-application of the natural substrate for NOS, L-arginine, but not the inactive isomer, D-arginine. Nitroprusside but not L-arginine, was able to increase markedly both spontaneous activity and the responsiveness to NMDA application. This suggests that normal the activity of NOS, a Ca^{++} and calmodulin dependent enzyme, may be a "rate limiting step" producing NO to facilitate or "permit" NMDA mediated excitation within this primary sensory nucleus. Field potential recordings obtained using electrical stimulation of the optic nerve, and the responses of cells in animals largely without afferent input to the LGN, suggest a post-synaptic site of action of NO, presumably on NMDA receptors, although actions on post-receptor mechanisms cannot be excluded. This suggests a new pathway for NO regulation of function, modulating the gain of visual signals transmitted to the cortex, operating as a "gain" machine.

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FRACTIONAL CALCIUM CURRENTS THROUGH AMPA- AND NMDA-RECEPTOR CHANNELS IN DIFFERENT TYPES OF CENTRAL NEURONS

R. Schneggenburger*, O. Garaschuk, F. Tempia** and A. Konnerth

I. Physiologisches Institut, Universität des Saarlandes, 66421 Homburg, Germany
present address: *Ecole Normale Supérieure, Lab. de Neurobiologie, 75005 Paris, France
**Dept. of Human Anatomy and Physiology, Corso Raffaello, 10125 Torino, Italy

The AMPA- and NMDA-type of glutamate receptor (GluR)-channels are permeable to monovalent and, to a varying degree, divalent cations, most noteably Ca^{2+}. A direct estimation of the fraction of Ca^{2+} to the total current through native GluR-channels was recently obtained [2] by combining whole-cell patch-clamp recordings of membrane currents with fluorometric Ca^{2+} flux measurements using saturating fura-2 concentrations [1]. Under conditions in which fura-2 is the dominant intracellular Ca^{2+}-buffer, the ratio of the Ca^{2+}-dependent fluorescence decrement over the electrical charge of the whole-cell current response (called the F/Q-ratio) was determined in neurons of the medial septum. Comparing the F/Q-ratios after activation of the GluR-channels with that of voltage-gated Ca^{2+}-currents gave an estimation of the fractional Ca^{2+}-currents of 1.4 ± 0.6 % and 6.8 ± 1.4 % for AMPA and NMDA-R channels respectively, at a membrane potential (V_m) of -80 mV and an extracellular [Ca^{2+}] of 1.6 mM [2].

To investigate possible differences in the amount of Ca^{2+}-flux through GluR-channels expressed in different types of central neurons, measurements of fractional Ca^{2+}-currents through GluR-channels were made in hippocampal CA1 neurons and in cerebellar Purkinje neurons. CA1 neurons of hippocampal slices from 10-15 d old rats were filled with 1mM fura-2 via the patch pipette. Agonists of NMDA- and AMPA-R were applied locally either to the soma or to the proximal part of the apical dendrite (40-70 µm distance from the soma) by use of ionophoretic drug application through fine pipettes. Image-analysis confirmed that the resulting Ca^{2+}-fluxes were limited to the respective neuronal compartment, when currents with a relatively small amplitude (≤ 500 pA) were evoked. The analysis of F/Q-ratios in somata and dendrites of CA1 neurons revealed fractional Ca^{2+}-currents of 0.6 ± 0.2 % and of 10.3 ± 2.2 % for the AMPA- and the NMDA-R respectively.

The fractional Ca^{2+}-current through AMPA-R in somata of Purkinje neurons was measured using cerebellar slices from young (4-7 d) rats, a developmental stage in which Purkinje neurons lack an extensive dendritic arborization. The fractional Ca^{2+}-currents after activation of AMPA-Rs were 0.6 ± 0.3 % (n=4 cells, AMPA as the agonist, V_m -60 mV) and 0.6 ± 0.1 % (n=4 cells, kainate as the agonist, V_m -80 mV). In conclusion, our results show characteristic differences of the fractional Ca^{2+}-currents through AMPA- and NMDA-R channels between different types of central neurons. The higher fractional Ca^{2+}-current of AMPA-R channels in medial septal neurons with respect to both hippocampal CA1 neurons and cerebellar Purkinje neurons is most likely due to different expression levels of the GluR2 subunit, a mechanism which has recently been described to account for a differential Ca^{2+}-permeability of AMPA-R channels in cortical neurons [3].

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Session V

Mechanosensitivity of NMDA receptors

P. Ascher and P. Paoletti

Laboratoire de Neurobiologie, Ecole Normale Supérieure

In a patch clamp study of the NMDA receptors of cultured mouse central neurones, we have observed that the NMDA responses can be modulated by a series of experimental manipulations which have in common to induce changes in membrane tension. A potentiation is observed on nucleated patches (Sather *et al.*, 1992) which are submitted to a lowered osmotic pressure. A similar potentiation is observed in nucleated patches, but also in outside-out patches, when positive pressure is applied in the recording pipette. Finally, NMDA responses can be increased by applying negative pressure to the pipette in the cell-attached mode or in inside-out patches.

All the tension changes which induce a potentiation of NMDA responses were without effect either on the cationic conductance changes induced by AMPA and kainate or on the Cl conductance changes induced by GABA and glycine in the same cells.

The modifications of the NMDA responses were more marked for lower concentrations of NMDA, but were still observed when NMDA was applied at saturating concentrations. Thus the effect of tension seems to involve both a change in affinity and a change in maximal response.

The effects of lowered osmotic pressure observed in nucleated patches were not seen in the whole-cell mode during the first minutes of recording. They only appeared after prolonged dialysis, at the time when the size of the NMDA response had decreased by about half.

"Stretch-sensitivity" has been found in many ionic channels since the initial observations of Guharay and Sachs (1984). Our observations indicate that this property can also be found in channels activated by a ligand. At the biophysical level we cannot separate effects mediated through the bilayer from effects mediated throught a possible cytoskeletal link of the NMDA receptor. However, we were not able to modify the development of the stretch-sensitivity by treating the cells with cytochalasin.

Most of the observations made on cultured mouse embryonic neurons were repeated on HEK 293 cells transfected with the two subunits NR1 and NR2A. This indicates that, if cytoskeletal elements are involved in the mechanosensitivity, these elements are not neuron-specific.

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Nitric oxide, synaptic plasticity and excitotoxicity

John Garthwaite - The Wellcome Research Laboratories, Beckenham, Kent

Nitric Oxide (NO) is a novel, diffusible type of second messenger molecule that is generated from the amino acid, L-arginine, in neurones following activation of glutamate receptors or other excitatory stimuli (Garthwaite, 1991). The synthetic enzyme, NO synthase, is Ca/calmodulin-dependent and has a widespread distribution in the CNS. In several areas, including cerebellum, hippocampus, striatum, cortex and spinal cord, NMDA receptor stimulation and the resulting rise in cytosolic Ca concentration appears to be a major effector pathway for stimulation of NO formation. Models of the diffusional spread of NO indicate that, even with brief (100 msec-sec time scale) periods of NO synthesis, and a rapid NO decay rate (0.5-5 sec half-life), NO formed from a single point source could access structures as far away as 100μm in physiologically relevant concentrations (Wood & Garthwaite, 1994). The corresponding sphere of influence enclosed about 2 million synapses. Physiologically, the major NO signal transduction pathway in target structures involves binding to a haem moiety associated with the soluble guanylyl cyclase enzyme leading to enzyme activation and cyclic GMP (cGMP) accumulation. NO-induced cGMP accumulation shows a widespread distribution in the brain that correlates with that of NO synthase (Southam & Garthwaite, 1993), reinforcing the idea that the two are functional partners.

NO has been implicated in numerous CNS functions, including synaptic plasticity, sensory signalling, control of the neuroendocrine system and the local regulation of cerebral blood flow. Its role in synaptic plasticity (LTP and LTD) remains controversial. In our experiments, NO synthase inhibitors only partly reduce tetanus-induced LTP in hippocampal slices and recent evidence indicates strongly that this component is also mediated by cGMP. The residual LTP involves neither NO nor cGMP.

Excessive glutamate receptor activation (particularly of NMDA receptors) can cause neuronal death, a phenomenon (excitotoxicity) that may contribute to several neurodegenerative disorders, including the neuronal loss associated with cerebral ischaemia. NO is a potentially toxic molecule, a property that is exploited by cells of the immune system (e.g. macrophages) as part of their repertoire for defence against invading pathogens. To test if NO contributes to glutamate neurotoxicity, two sets of experiments were performed. In slices of cerebellum and hippocampus, the acute degeneration induced by glutamate receptors stimulation (NMDA or AMPA) was unaffected by NO synthase inhibitors and it could not be duplicated by exposure to high concentrations of NO-donating drugs. In cultures of striatum, on the other hand, a component of the cell death taking place as a result of a brief (5-30 min) exposure to NMDA could be prevented by NO synthase inhibitors when applied after, but not during, the exposure to NMDA, suggesting that NO contributes to delayed degeneration in this experimental paradigm. As with LTP, NO dependent and NO-independent forms of NMDA neurotoxicity appear to exist.

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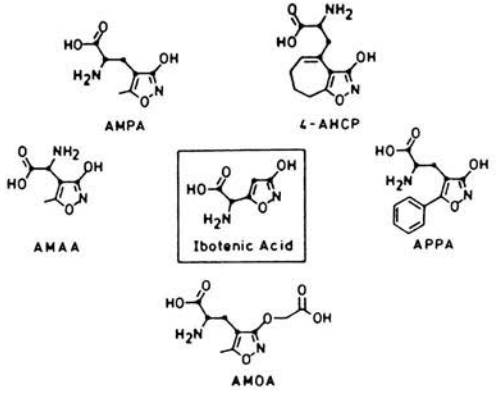
DESIGN AND MOLECULAR PHARMACOLOGY OF GLUTAMATE RECEPTOR AGONISTS, PARTIAL AGONISTS AND ANTAGONISTS

Povl Krogsgaard-Larsen

PharmaBiotec Research Center, Department of Medicinal Chemistry,
The Royal Danish School of Pharmacy, Copenhagen, Denmark

There is a rapidly growing interest in the pharmacology and therapeutic prospects of specific ligands for subtypes of central excitatory amino acid (EAA) receptors. In neurodegenerative disorders, where excessive activation of NMDA, AMPA and/or kainate receptors seems to play a key role, receptor antagonists would seem to have major therapeutic interest. Analogously, EAA receptor agonists may, at least in principle, have clinical utility in for example schizophrenia or Alzheimer's disease, where hypoactivity of central EAA pathways appears to be a factor of importance. EAA receptor antagonists as well as agonists may, however, be expected to provoke quite dramatic effects on brain functions after administration to patients, and full agonists at EAA receptors may show severe neurotoxic effects, especially after prolonged administration.



Low-efficacy partial EAA receptor agonist rather than full antagonists may be of particular interest in neurodegenerative disorders, and, similarly, partial agonists of higher efficacy may be therapeutically relevant drugs in schizophrenia or Alzheimer's disease. In these latter diseases, compounds capable of enhancing EAA receptor functions without showing direct agonistic effects may also have therapeutic prospects.

Ibotenic acid is a non-selective agonist at all subtypes of EAA receptors. In contrast, AMAA is a specific NMDA agonist,[1] whereas AMPA and 4-AHCP show specific agonist effects at AMPA receptors.[2] AMOA exhibits a unique pharmacological profile, showing antagonistic and enhancing effects, respectively, at low and high concentrations of AMPA.[3]

Whereas (*RS*)-APPA shows the characteristics of a partial AMPA receptor agonist, (*S*)-APPA has recently been shown to be a full AMPA agonist, whereas (*R*)-APPA shows AMPA

antagonist properties.[4] This highly unusual pharmacological profile has been studied in further detail,[5] and has led us to introduce the concept of "functional partial agonism", which may have general therapeutic interest in diseases characterized by dysfunctions of the central EAA neurotransmitter system.[5]

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STUDY OF THE $GABA_A$ RECEPTORS WITH SUBUNIT-SPECIFIC ANTIBODIES.

Angel L. De Blas

The $GABA_A$ receptor is a pentameric heterooligomer membrane protein constituting a Cl^- channel. Cloning studies have shown the existence of six α subunit isoforms (α_1-α_6), three ß(ß$_1$-ß$_3$), three $\gamma(\gamma_1$-$\gamma_3)$ one $\delta(\delta_1)$ and two $\rho(\rho_1$ and $\rho_2)$ in the mammalian brain. They are encoded by different genes. In addition, the two forms of the γ_2 subunit, the short (γ_{2S}) and the long (γ_{2L}), are generated by alternative mRNA splicing. Heterologous expression indicate that different combinations of the subunits can form GABA-gated Cl^- channels. Therefore, there are many (hundred of thousands) theoretically possible pentameric combinations of these subunits forming $GABA_A$ receptors in the mammalian brain.

We are addressing the peptide subunit composition of the native $GABA_A$ receptors in different regions of the brain and specific cell types by using subunit-specific antibodies. We have developed both monoclonal antibodies and polyclonal antisera to $GABA_A$ receptors subunits after immunizing mice and rabbits with i) affinity-purified $GABA_A$ receptors, ii) receptor subunit protein expressed in bacteria as fusion proteins and iii) subunit-specific synthetic peptides.

We have used the various antibodies to study the distribution of the $GABA_A$ receptor subunits in the brain by immunocytochemistry and immunoprecipitation. In addition, we have investigated the subunit composition of native brain $GABA_A$ receptors by quantitative immunoprecipitation and immunoblotting. These studies have shown that in some $GABA_A$ receptors, γ_{2S} and γ_{2L} subunits co-localize in the same pentameric receptor molecule such as in some receptors present in cerebellar granule cells. A considerable proportion of these granule cell receptors have the following pentameric subunit composition: α_1, α_6, γ_{2S}, γ_{2L} and ß$_{2/3}$. Nevertheless, there is widespread heterogeneity in the subunit composition of the $GABA_A$

receptors from granule cells which also express combinations of other subunits. We do not know yet whether different pentameric subunit combinations are expressed in the same granule cell or whether each granule cell expresses a single pentameric subunit combination. The knowledge of the subunit composition of native brain receptors will allow the study of the pharmacology and channel properties of the receptors reconstituted with the same subunit composition in heterologous expression systems.

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POSTERS

Abrupt increase in the rate of the electric organ discharge initiated by the Mauthner cell in *Gymnotus carapo*. Michel Borde, Atilio Falconi, Arturo Hernández-Cruz, Sebastian Curti and Francisco R. Morales. Laboratorio de Neurofisiología Celular, Facultad de Medicina, Facultad de Ciencias, Montevideo, Uruguay.

During experiments designed to study Mauthner cells (MC) in *Gymnotus carapo* we noticed that low intensity spinal cord stimulation sufficient to activate the Mauthner axon, induced an abrupt increase in rate (AIR) of the electric organ discharge. Intracellular direct MC stimulation produced a similar response that was subsequently named "M-AIR". Stimulation of the MC axon at the spinal cord induces a prolonged EPSP in pacemaker cells of the medullary electromotor nucleus. The short EPSP latency (4 ms) suggests that there is a paucisynaptic pathway interposed between Mauthner and pacemaker cells. Preliminary pharmacological manipulations suggests that both NMDA and NON-NMDA glutamate receptors are involved in M-AIR at the medullary pacemaker level.

We conclude that, in addition to its role as command neuron for escape response, MCs of weakly electric fish also accelerate the rate of active electrolocation during this vital motor behavior.

ENDOGENOUS NMDA RECEPTORS IN PC12 CELLS SHOW PARTICULAR FUNCTIONAL PROPERTIES.

M. Casado, A. López-Guajardo, B. Mellström, J.R. Naranjo and J. Lerma. Instituto Cajal, C.S.I.C., 28002-Madrid, Spain.

Although many human and rodent neuroblastoma cell lines are available, none has been directly shown to express functional NMDA channels. We have found that the rat pheochromocytoma cell line PC12 express functional NMDA receptors, but no kainate or AMPA type receptors. Northern blot and PCR studies show the existence of NR1 and NR2C RNA species, but no presence of NR2A, 2B or 2D. Splice variants analysis of NR1 demonstrates absence of the N-terminal insert. In patch-clamp experiments we have measured non desensitizing inward currents when PC12 cells were perfused with glutamate or NMDA in presence of glycine. Glutamate induced currents were completely blocked by APV, 7-chlorokynurenate and phencyclidine, and exhibited voltage dependent Mg^{2+} blockade. Differentiation by NGF treatment increased both membrane surface and NMDA receptor mediated responses. In the presence of low (contaminating) glycine, responses showed partial desensitization, with time constants of 1.2 s and 1.5 s for onset and recovery, respectively. Affinity constants were calculated from kinetic experiments, and were 590 nM for glutamate and 74 nM for glycine. Spermine potentiation was absent, rather spermine produced a voltage independent inhibition. Single channel recordings demonstrated at least two subconductance levels of 54.6 pS and 32.6 pS (at 0.5 mM Ca^{2+}) with frequent transitions between them. Mean open time was 0.49 ms, and opening probability was ≤ 0.001. Extracellular Ca^{2+} reduced single channel conductances but not mean open time. This demonstrates the existence of functional NMDA receptors in PC12 cells with unique properties.

IMMUNOCYTOCHEMICAL LOCALIZATION OF NMDA R1 PROTEIN IN MOUSE BRAIN

Cristina Garate, Dolores Julián*, Angelines Santa-Cecilia, Montserrat Negro, Pedro Calvo and Miguel A. Chinchetru

Departamento de Bioquímica y Biología Molecular and * Departamento de Biología Celular y Anatomía, Universidad de León, 24007 León, Spain.

N-methyl-D-aspartate (NMDA) receptors are widely expressed in the mammalian brain, and several subunits of this receptor have been identified by molecular cloning. Localization of the NMDA receptor has been carried out by radioligand and *in situ* hybridization assays. Recently, immunocytochemical localization of the NMDA R1 protein in human and rat brain has been reported, and following a similar approach, we have investigated the distribution of this protein in mouse brain.

Polyclonal antibodies were generated against a fusion protein formed by glutathione-S-transferase (GST) and the putative second intracellular domain of the NMDA R1 protein. The titre and specificity of the antibodies were determined by ELISA and western blotting assays, and they were finally purified by affinity chromatography.

Prominent immunostaining was observed in the hippocampus, specially in the stratum oriens and stratum radiatum of CA 1 region, and in the mossy fiber pathway. The protein is also highly expressed in cortex and striatum. In the cerebellum, the staining is prominent in granule and Purkinje cells. The results obtained show good correlation with previous binding and autoradiographic studies using NMDA receptor antagonists..

Molecular Pharmacology of $GABA_A$ Receptor Agonists and Partial Agonists in Oocytes Injected with different α, ß and γ Receptor Subunit Combinations.

Bjarke Ebert[2], Keith A. Wafford[1], Paul J. Whiting[1], Povl Krogsgaard-Larsen[2*] and John A. Kemp.
[1]Department of Pharmacology and Biochemistry Neuroscience Research Centre Merck Sharp and Dohme Research Laboratories Terlings Park, Eastwick Road Harlow, Essex CM20 2QR England.
[2]PharmaBiotec Research Centre, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken , DK-2100 Copenhagen, Denmark.

Using systematic combination of α1, α3 and α5, with β1, β2 and β3 together with γ1, γ2 and γ3 we have investigated the contribution of the various α, β and γ subunits to the pharma-cology of γ-aminobutyric acid$_A$ ($GABA_A$) agonists. We have characterized GABA, (RS)-dihydromuscimol (DHM), piperidine-4-sulphonic acid (P4S) and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) on recombinant human $GABA_A$ receptors expressed in Xenopus oocytes. Our observations indicate that the α subunit is the major determinant of efficay to partial $GABA_A$ agonists. When α1 and α3 or α1 and α5 are co-expressed, the α1 subunit determines the maximal efficacy, whereas the affinity is determined by the entire combination of subunits. Thus, the results of the present study demonstrates that the pharmacology of $GABA_A$ agonists is dependent on the subunit composition of the $GABA_A$ receptor complex. Functional $GABA_A$ receptors containing two different α subunits show a pharmacological profile distinctly different from receptors containing a single α subtype, indicating that two different α subunits can be co-expressed in one functional $GABA_A$ receptor complex.

Molecular Pharmacology. In press.

TAURINE FACILITATES SYNAPTIC TRANSMISSION IN THE HIPPOCAMPUS.

M. Galarreta[1], J. Bustamante[2], R. Martín del Río[1] and J.M. Solís[1].
[1]Depto. Investigación, Hosp. Ramón y Cajal, and [2]Depto. Fisiol., Fac. Medicina, U.C.M., Madrid, Spain.

Although taurine is one of the most abundant amino acids in the brain of mammals, its physiological role in the nervous system is not well defined. The best known electrophysiological effect of taurine is to act as a weak agonist on $GABA_A$ receptors.

Our purpose has been to study the actions of taurine on evoked synaptic potentials. Experiments were carried out in the CA1 region of rat hippocampal slices continuously perfused in a submerged chamber. The basic observation was that bath application of taurine (10 mM) for 30 min induced, after a transitory decrement, an enhancement of field excitatory postsynaptic potentials (fEPSPs) that lasted after taurine washout (146±9% after 1h washout, n=15). The following features related with this taurine action can be pointed out: 1) fEPSPs increase is dependent on taurine concentration (5-10 mM) and taurine perfusion time (10-30 min), 2) fEPSP potentiation develops in the presence of $GABA_A$ or NMDA antagonists, 3) N-methyltaurine, a taurine analogue, does not induce fEPSP potentiation, 4) during taurine perfusion the afferent volley (AV) also shows a long lasting increase in amplitude, 5) fEPSP enhancement is not fully explained as a function of the AV increase, as it is demonstrated by the AV/fEPSP relations before and after taurine, 6) the taurine-induced potentiation of the AV is also observed in 0 mM $Ca^{2+}$and 4 mM Mg^{2+}, 7) intracellular recordings of evoked EPSPs and monosynaptic evoked IPSPs show long term increases of both EPSPs and IPSPs amplitudes after taurine perfusion, while input resistance does not change significantly. These results could be explained, at least partially, through a taurine effect on glutamatergic and GABAergic presynaptic fibers, and show a new action of taurine consisting in a long lasting enhancement of synaptic transmission. (Supported by F.I.S.S. 93/0565; M.G. is a predoctoral fellow of the Spanish Ministry of Education and Science).

PROBING OF NMDA RECEPTOR CHANNELS BY ORGANIC CATIONS.LOCATION OF ACTIVATION AND INACTIVATION GATES .

S.Koshelev and B.Khodorov.
Institute of General Pathology and Pathophysiology,Russian Academy of Medical Sciences ,125315 Moscow ,Russia.

NMDA receptor -mediated ionic currents elicited by aspartate (ASP 100 μM ,3μM glycine ,0 Mg^{2+}) were recorded from acutely isolated hippocampal neurones by patch-clamp (whole-cell configuration) and fast perfusion techniques. A series tetraalkylammonium ions (TAA),with different length (L) of their alkyl chains (from C_1-to C_5-) ;derivatives of aminoacridine, 9-aminoacridine(9-AA) and tetrahydroacridine (THA) ,and pancuronium were used to study the gross architecture of the NMDA channel.When applied externally these drugs caused voltage-dependent blockade of open NMDA channels.The blocking potency of TAA (1/ IC_{50})increased with L,suggesting the important role of hydrophobic intercations in the mechanism of this block.Along with blockade of ion conducting pathway the organic cations affect channel gating.Thus fast blockade of NMDA channels by tetramethylammonium (TMA) and especially tetraethylammonium (TEA) is accompanied by acceleration and deepening of the current decay,which under normal conditions results from channels desensitization during a prolonged ASP challenge .Teptrapropylammonium (TPA) does not affect appreciably the current kinetics ,while tetrabutylammonium (TBA) and tetrapentylammonium (TPeA) abolish desensitization and produce a dramatic change in the time course of the tail current: a prominent transient increase in the inward current arises ("hooked tail current")upon the termination of their combined with ASP application.The amplitude of the hook increases with the blocker concentarion. All of the features of such a type of open channel blockade are compatible with the model,in which drug molecules occlude open NMDA channels,and the blocked channels could not close (deactivate)or undergo desensitization until the drug had left the blocking site in the channels .A comparison of the above effects of TAA with the effects of many other open NMDA channel blockers,led us to conclude that the size of the blocking molecule is a major factor determining its ability to interfer with channel gating.If the bulky size of a drug or even one of its dimension exceeds a certain critical value (about 11 Å),then the channel blockade leads to immobilization of gates in their open configuration.Among the drugs inducing such a "foot-in the door" type of open channel blockade proved to be 9-AA, THA,pancuronium, along with TBA and TPeA .

The following conclusion could be deduced from the results of the present study.The NMDA channel is provided with the inactivation gate along with the activation one;closing of the inactivation gate underlies channels "desensitization ".Both gates are located deep in the external channel mouth close to the binding sites for Mg^{2+} and organic blockers.Depending on a size, the blocking molecule either does not impede channel gating and therefore can be trapped in the channel after its closing or,on the contrary,it immobilizes the channel gates in the open configuration until channel unblocking occurs.

Mu and delta opioid receptors modulate the arterial hypertension induced by NMDA in the periaqueductal gray matter.

Leyva Juan, Maione Sabatino, de Novellis Vito, Pallotta Mirella, Berrino Liberato and Rossi Francesco.

Institute of Pharmacology and Toxicology, Faculty of Medicine and Surgery, 2nd University of Naples, Via Costantinopoli 16, 80138 Naples, Italy.

The periaqueductal gray (PAG) matter is located in the midbrain and plays several functions, such as nociception control, defensive behaviour and cardiovascular function. We have demonstrated an interaction of glutamergic and peptidergic systems mediated by arginine vasopressin in modulating pressor neurons within dorsal PAG matter. Since peptidergic terminals into the PAG matter are mainly of enkephalinergic type, we carried out the present study to clarify whether or not opiates may operate on pressor neurons within dorsolateral PAG matter.

Groups of 5–8 rats, anaesthetised with urethane (1.2 g/kg i.p.), have been used. After placement of catheter into femoral artery to monitoring arterial blood pressure, each animal was then placed in a stereotaxic frame for direct microinjection into the PAG area of NMDA (2nmol/rat) alone or after 5 min pretreatment with selective opioid receptor antagonists.

Animals pretreated with naloxone (NAL, 5 nmol/rat), a non selective μ receptor antagonist, or naltrindole hydrochloride (NTI, 5 nmol/rat), a selective δ receptor antagonist, significantly ($P < 0.05$) reduced NMDA-induced hypertension in the order of 31% and 37% respectively. Naloxone also significantly ($P < 0.05$) increased the latency for the maximum hypertensive effect induced by NMDA. The pretreatment with nor-binaltorphimine (BNI, 5 nmol/rat), a selective κ receptor antagonist, increased only the latency of NMDA-induced hypertension. The opioid antagonists did not alter arterial blood pressure by themselves. Microinjection of morphine (13 nmol/rat), a non selective μ receptor agonist, significantly decreased ($P < 0.05$) in the order of 57.5% the arterial hypertension induced by NMDA and this effect was prevented by a pretreatment with naloxone. Moreover, $GABA_A$ antagonist bicuculline (BIC, 2.5 nmol/rat) administered 5 min before naloxone, antagonized the effect of naloxone on the hypertension induced by NMDA. However, strychnine (STRY, 8 nmol/rat), a glycine receptor antagonist, microinjected 5 min before naloxone, did not modify basal arterial blood pressure nor naloxone-induced reduction of NMDA hypertension.

These data suggest that PAG vasopressor neurons receive both direct opioid and GABAergic inhibitory inputs. In particular, we demonstrated that both stimulation or block of opiate receptors decrease the excitability of cell bodies concerned with the modulation of sympathetic outflow. The paradoxical effect induced by opiate receptor antagonists could be a consequent disinhibition of tonically active PAG GABAergic interneurons.

GLUTAMATE RECEPTOR GENE EXPRESSION IN CEREBRAL ISCHEMIA.

Domenico E. Pellegrini-Giampietro & Flavio Moroni.
Dipartimento di Farmacologia Preclinica e Clinica, Universita' di Firenze, Viale Morgagni 65, 50134 Firenze, Italy.

Twenty four hours following transient forebrain or global ischemia in rats, mRNA levels of the GluR2 subunit which prevents Ca^{2+} permeability of AMPA/kainate receptors are markedly reduced in vulnerable CA1 pyramidal cells, whereas the Ca^{2+}-permeable subunits GluR1 and GluR3, as well as the NMDA receptor subunit NMDAR1, are little affected (Pellegrini-Giampietro et al., *Proc. Natl. Acad. Sci. USA* **89:** 10499, 1992). The switch in AMPA/kainate receptor subunit expression precedes histologically detectable CA1 neurodegeneration, suggesting that it might be causally related to post-ischemic delayed cell injury. In order to examine whether changes in subunit mRNA content correlate with altered receptor function, we determined, in cultures of rat cerebellar granule neurons, the effect of different concentrations of KCl on *i)* the amount of GluR1 and GluR2 mRNA as determined by Northern blot, *ii)* kainate-stimulated $[Ca^{2+}]_i$ rise, measured by means of fluorescent microscopy in single cells pre-loaded with fura-2, and *iii)* kainate-induced cell death. Cultures grown in low KCl (10-15 mM) displayed a lower amount of GluR2 mRNA than cultures grown in high KCl (25-30 mM), as well as a higher rise in $[Ca^{2+}]_i$ and a higher vulnerability to kainate toxicity, thus corroborating the hypothesis that the post-ischemic switch in AMPA/kainate receptor subunit expression might represent a mechanism leading to Ca^{2+}-mediated excitotoxicity.

In addition, we examined the effect of anti-ischemic drugs on glutamate receptor gene expression following global ischemia. Post-ischemic administration of the selective AMPA receptor antagonist NBQX is known to protect CA1 neurons in rats and gerbils. However, both NBQX (at a neuroprotective dose) and the NMDA receptor blocker MK-801 (not protective in the rat model) failed to alter the ischemia-induced changes in AMPA/kainate receptor subunit expression in rats, suggesting that *i)* the reduction of GluR2 expression in CA1 is not triggered by activation of AMPA/kainate or NMDA receptors, and *ii)* NBQX affords neuroprotection by a direct blockade of modified AMPA/kainate receptors, rather than by preventing the switch. 7-Cl-Thio-kynurenate, a potent antagonist at the glycine site of NMDA receptors which also inhibits lipid peroxidation (Moroni et al., *Eur. J. Pharmacol.* **218:** 145, 1992), similarly protected CA1 pyramidal cells in a gerbil model of global ischemia. *In situ* hybridization revealed that expression of NMDAR1 was severely reduced in CA1 seven days following ischemia because of pyramidal cell loss and that, in ischemic gerbils treated with 7-Cl-thio-kynurenate, protected CA1 cells were still able to express normal amounts of NMDAR1 mRNA. In conclusion, it appears that although anti-ischemic drugs can not prevent the selective reduction in the key AMPA/kainate receptor subunit GluR2 observed in vulnerable CA1 neurons prior to their degeneration, they are able to restore in rescued cells decreases in mRNA levels of glutamate receptor subunits resulting from post-ischemic cell death.

LOCALIZATION OF GLUTAMATE RECEPTORS ON LIVING CELLS USING SUBUNIT SPECIFIC ANTIBODIES AND CONFOCAL LASER SCANNING MICROSCOPY

S.A. Richmond*, A.J. Irving*, E.Molnar§, R.A.J. McIlhinney§,

J.M. Henley* and G.L. Collingridge*

*Department of Pharmacology, The Medical School, University of Birmingham Birmingham B15 2TT, U.K. & §MRC Anatomical Neuropharmacology Unit, University of Oxford Oxford OX1 3TH, U.K.

Polyclonal antibodies were produced against the N-terminus (residues 253-267) of the cloned glutamate receptor GluR1 (Hollmann et al., 1989). The antibodies were characterised both biochemically and immunocytochemically (Molnar et al., 1993). The extracellular location of the N-terminus enabled the distribution of GluR1 subunit to be examined on living hippocampal cells. Confocal laser-scanning microscopy revealed GluR1 immunofluorescence in the somato-dendritic compartment of pyramidal cells. The imunoreactivity was punctate in distribution and GluR1 hotspots were observed in dendriticprocesses. Moreover double labelling studies showed that the distribution of the subunit overlapped with the integral synaptic vesicle protein Synaptophysin. High resolution localisation of the subunit in a single cells is currently being undertaken.

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PROTEIN KINASE MODULATION OF NMDA-MEDIATED NITRIC OXIDE SYNTHESIS IN CULTURED NEURONS.

José Rodriguez[1,2], Mireille Lafon-Cazal[2] and Joël Bockaert[2].

[1]Dptq. Bioquímica y Biología Molecular, U.A.B., Barcelona, Spain and [2] UPR CNRS 9032, Montpellier, France.

Nitric oxide (NO) has been suggested to be involved in synaptic plasticity events induced by glutamate receptor stimulation like LTP and LTD. NO is synthesized by NO-synthase (NOS), and enzyme which is dependent on calcium/calmodulin and has several putative phosphorylation sites. In this study we have studied the relevance of phosphorylation by several protein kinases in the NMDA-mediated activation of NOS. Activation of NOS was monitored in terms of NO-dependent cGMP production and also by the production of superoxide radical by NOS in arginine-depleted neurons. NMDA (100 uM) stimulation of NO synthesis is partially blocked (40-60%) by several specific tyrosine kinase inhibitors like lavendustin-A, genistein, CH-26 and CH-01. Lavendustin-A was the most effective inhibitor of NMDA effect with an IC50 of 20-50 nM. On the other hand, these tyrosine kinase inhibitors were completely uneffective to block the stimulation of cGMP by NO donors (SIN-1 and SNP), the ionomycin-mediated NO production and the activation of the NMDA-receptor current. These results suggest that the inhibitors are acting at one step between the increase in intracellular calcium by NMDA and NO production by NOS. The absence of any effect of the used inhibitors of tyrosine kinases on the interaction of calcium/calmodulin with CaM-kinase II and calcium/calmodulin-sensitive adenyl cyclase suggest that the inhibition takes place at the level of NOS itself or at the level of an unknow protein regulating the activity of NOS. Since NO has been reported to be involved in synaptic plasticity events like LTP, the observed blockade of NO synthesis by tyrosine kinase inhbitors could be partially responsible for the already reported inhibition of LTP by tyrosine kinase inhibitors.

SODIUM MODULATES CA^{2+}-DEPENDENT GLUTAMATE RELEASE FROM RAT CORTICAL SYNAPTOSOMES

Marco A. Romano-Silva[1,2], Marcus V. Gomez[3] and Michael J. Brammer[1]
[1]Dept. of Neuroscience, Institute of Psychiatry, London, UK
[2]Dept.of Biochemistry and Immunology and [3]Dept. of Pharmacology, ICB, UFMG, Brazil

It is becoming clear that neurotransmitter release and its regulation are intricate processes involving the interaction of many different classes of molecules from single ions to complex proteins and organelles. Our interest was concentrated on glutamate because of its abundance and importance in the central nervous system where it is the major excitatory neurotransmitter. Glutamate release from isolated nerve endings (synaptosomes)has been shown to have Ca^{2+}-dependent and Ca^{2+}-independent components, the former being regarded as exocytotic and the latter thought to be due to reversal of the $Na^+$and K^+-dependent carrier (Nicholls, Eur.J.Biochem.(1993)212:613). Recent evidence suggests that the Ca^{2+}-dependent (exocytotic) component is possibly dependent on at least two different types of voltage sensitive Ca^{2+}-channel and that different combinations of channel types may be involved in the release of different neurotransmitters (Turner et al, Proc.Natl.Acad.Sci.USA(1993)9518). The action potential is propagated throughout the terminal by Na^+ influx through voltage sensitive Na^+-channels leading to depolarization of the membrane adjacent to the channels and opening of Ca^{2+}-channels in the active zone culminating on exocytosis. We have been using spider (PhTX2) (Romano-Silva et al, Biochem.J.(1993)296:313) and scorpion toxins (TsTX) (Romano-Silva, Neurosc.Lett.(in press)) to stimulate glutamate release from rat cortical synaptosomes. Their target is the voltage-sensitive Na^+-channel, of which they stimulate opening or inhibit inactivation. We had previously shown that TsTX was more efficient on inducing glutamate release than KCl-induced depolarization. The most important difference between these processes is that TsTX causes a large Na^+-influx. In an attempt to shed more light on the role of Na^+ we compared the effects of gramicidin D (a Na^+ ionophore) in conjunction with KCl depolarization with those of TsTX. We observed that gramicidin at a concentration of 100nM induced an increase in $[Ca^{2+}]i$ (measured on fura2-loaded synaptosomes) that was aproximately the same obtained with TsTX. However, this was accompanied by a much larger increase on $[Na^+]i$ (measured on SBFI-loaded synaptosomes) and a much smaller glutamate release than that seen with TsTX. The TsTX-induced Ca^{2+}-dependent glutamate release was significant higher than that obtained with gramicidin plus KCl. These results suggest that localized Na^+ influx through voltage dependent Na^+-channels is closely associated with Ca^{2+}-dependent glutamate release and that Na^+ must play a role in the modulation of exocytosis other than simply promoting membrane depolarization.

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THE NMDA RECEPTOR IS INVOLVED IN THE GENERATION OF A SYNCHRONIZED PATTERN OF $[Ca^{2+}]i$ OSCILLATIONS IN CEREBELLAR GRANULE CELLS

Lucía Núñez, Rosalba I. Fonteriz, Ana Sánchez and Javier García-Sancho. Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Univerdidad de Valladolid. Valladolid (Spain)

We have characterized the NMDA-induced intracellular Ca^{2+} responses in cerebellar granule cells in culture. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored by digital imaging fluorescence. Granule cells develop synaptic functional conexions after 4 days in culture. This feature goes in parallel with spontaneous, syncronized $[Ca^{2+}]_i$ oscillations, in Mg^{2+}-free medium. We have also recorded the electrical activity by using the patch-clamp thecnique in the whole cell configuration with anfotericin. In Mg^{2+}-free medium, the cells showed spontaneous membrane potencial oscillations. Both membrane potential and $[Ca^{2+}]_i$ oscillations were inhibited, in a dosis-dependent way, by several NMDA receptor antagonists including the compounds MK 801, AP-7 and ketamine. The IC_{50s} for the inhibition of the oscillations were closely related to their Ki for the NMDA receptor. $[Ca^{2+}]_i$ oscillations were also abolished in Na^+-free medium or when the Na^+ channel blocker tetrodotoxin (TTX) was added. Taken together, these results, plus the fact that Mg^{2+} abolishes both oscillation kinds, strongly suggest a role for NMDA receptors.

Inmunocytochemistry was used after imaging experiments on the same cellular fields in order to identify the nature of the oscillating cells. Only those cells stained with α-βIIItubuline (neuron positive) were able to oscillate. Moreover, only those cells responding to NMDA with $[Ca^{2+}]_i$ increases were able to oscillate in Mg^{2+}-free medium. All of this indicate the ability of isolated neurons to associate in culture and produce syncronized behaviour via NMDA receptor-mediated synaptic mechanisms.

UPREGULATION OF NMDA MEDIATED SYNAPTIC POTENTIALS OF RAT HIPPOCAMPAL NEURONES BY TRH. G. Stocca, A. Nistri*. Biophys. Lab., Int. Sch. Adv. Studies (SISSA), 34013 Trieste, Italy.

The neuropeptide TRH largely and selectively enhances responses of hippocampal neurones to NMDA. In order to investigate whether a similar potentiation may take place in the case of responses mediated by synaptically activated NMDA receptors, experiments were carried out on CA1 neurones of the adult rat hippocampal slice preparation bathed in a Mg^{2+} containing solution. Intracellular recording with 3 M KCl electrodes were performed during focal electrical stimulation of afferent fibres (0.05 Hz; 50 μs)which evoked minimal EPSPs (1-2 mV) with frequent failures. In the presence of pharmacological block of AMPA and GABA receptors by a combination of CNQX (10 μM), bicuculline (20 μM) and CGP35348 (1 mM), evoked EPSPs were considered to be due to glutamate induced activation of NMDA receptors. In 7 cells at -73±3 mV membrane potential bath-applied TRH (tartrate monohydrate; 10-15 μM) enhanced the peak amplitude of EPSPs by 36±20% and their area by 49±28% without any detectable change in resting potential or input resistance. The rate of EPSP decay was prolonged as its monoexponential τ increased by 42±29% In some cells the rise in EPSP amplitude was large enough to reach spike threshold. On the same cells after 1h washout of TRH, replacing CNQX with CPP (10 μM) allowed detection of AMPA receptor mediated EPSPs (1-5 mV) with a much faster timecourse. These latter EPSPs were not enhanced by TRH as their peak, area and τ values were 97±4, 98±10 and 122±19% of controls, respectively. These data suggest that TRH can selectively facilitate excitatory synaptic transmission mediated by glutamate via NMDA receptors. This phenomenon may be relevant to account for some behavioural effects of the neuropeptide.

IDENTIFICATION OF A CaM-KINASE II REGULATORY PHOSPHORYLATION SITE IN NON-NMDA GLUTAMATE RECEPTORS. J.L. Yakel#, P. Vissavajjhala, V.A. Derkach, D.A. Brickey & T.R. Soderling. Vollum Institute, OHSU, Portland, OR 97201, USA.

Glutamate receptor (GluR) ion channels are colocalized in postsynaptic densities with CaM-kinase II, an enzyme which has previously been shown to phosphorylate and strongly enhance non-NMDA GluR currents in cultured rat hippocampal neurons (McGlade-McCulloh et al., 1993, *Nature* **362**:640). We wanted to extend these findings by demonstrating the regulatory effect of CaM-kinase II on expressed non-NMDA (i.e. AMPA-type) GluRs, and to identify the regulatory site phosphorylated by CaM-kinase II. In this study, the intracellular application of activated CaM-kinase II into cultured mammalian 293 cells expressing GluR6 or into *Xenopus* oocytes expressing GluR1 enhanced the GluR current activated by kainate. This effect of CaM-kinase II was further investigated in the *Xenopus* oocyte/GluR1 system which gave a 1.5-fold increase in kainate current with no effect on the sensitivity of the receptor to kainate. When Ser627, which is located in the major intracellular loop of GluR1, was mutated to Ala, the expressed mutant no longer responded to the CaM-kinase II-induced enhancement of kainate current but was normal with respect to current-voltage relationship and kainate sensitivity. Since all non-NMDA GluR ion channels have a Ser in an analogous position, this regulatory phosphorylation site may be of general importance in enhancing postsynaptic responsiveness as occurs in long-term potentiation.

Supported in part by NIH grant 27037

Current address: NIEHS, Box 12233, Research Triangle Park, NC 27709

List of Invited Speakers

Workshop on

MOLECULAR MECHANISMS OF SYNAPTIC FUNCTION

List of Invited Speakers

P. Ascher — Laboratoire de Neurobiologie, Ecole Normale Supérieure, 49 rue d'Ulm, Paris 75005 (France).
Tel.: 33 1 44 32 37 44
Fax : 33 1 44 32 38 87

M.V.L. Bennett — Department of Neuroscience, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Ave, Bronx, NY. 10461 (USA).
Tel.: 1 718 430 25 36
Fax : 1 718 824 30 58

A.L. de Blas — Division of Molecular Biology & Biochemistry, School of Biological Sciences, University of Missouri-Kansas City, 5100 Rockhill Road, Kansas City, MO. 64110-2499 (USA).
Tel.: 1 816 235 22 47
Fax : 1 816 235 51 58

J. Bockaert — UPR CNRS 9023, CCIPE, Rue de la Cardonille, 34094 Montpellier Cedex 5 (France).
Tel.: 33 67 14 29 30
Fax : 33 67 54 24 32

G.L. Collingridge — Department of Pharmacology, The Medical School, University of Birmigham, Edgbaston, Birmingham B15-2TT (U.K.).
Tel.: 44 21 414 45 06
Fax : 44 21 414 45 09

M.G. Darlison — Institut für Zellbiochemie und Klinische Neurobiologie, Universitäts-Krankenhaus Eppendorf, Universität Hamburg, Martinistrasse 52, 20246 Hamburg (Germany).
Tel.: 49 40 47 17 45 51
Fax : 49 40 47 17 45 41

J. Garthwaite — The Wellcome Research Laboratories, The Wellcome Foundation Ltd., Langley Court, South Eden Park Road, Beckenham-Kent BR3 3BS (U.K.).
Tel.: 44 81 639 51 78
Fax : 44 81 639 75 34

C.E. Jahr — Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University L-474, Portland, OR. 97201-3098 (USA).
Tel.: 1 503 494 54 71
Fax : 1 503 494 69 72

K. Keinänen — VTT Biotechnology and Food Research, P.O. Box 1500, 02044 Espoo (Finland).
Tel.: 358 0 456 51 42
Fax : 358 0 455 21 03

P. Krogsgaard-Larsen — PharmaBiotec Research Center, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, 2100 Copenhagen (Denmark).
Tel.: 45 35 37 08 50
Fax : 45 35 37 22 09

J. Lerma — Departamento de Plasticidad Neural, Instituto Cajal, CSIC, Dr. Arce 37, 28002 Madrid (Spain).
Tel.: 34 1 585 47 10
Fax : 34 1 585 47 54

M.L. Mayer — Lab. Cellular & Molecular Neurophysiology, NICHD, Bldg. 49, Room 5A78, NIH, Bethesda, MD. 20892 (USA).
Tel.: 1 301 402 47 75
Fax : 1 301 402 47 77

K. Moriyoshi — Institute for Immunology, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606 (Japan).
Tel.: 81 75 753 44 07
Fax : 81 75 753 44 04

R.A. Nicoll — Department of Pharmacology, University of California, San Francisco, CA. 94143-0450 (USA).
Tel.: 1 415 476 20 18
Fax : 1 415 476 52 92

J.P. Pin — UPR-CNRS 9023, CCIPE, rue Cardonnille, 34094 Montpellier Cedex 05 (France).
Tel.: 33 67 14 29 33
Fax : 33 67 54 24 32

J. Rossier — Institut Alfred Fessard, CNRS, 91198 Gif-sur-Yvette Cedex (France)
Tel.: 33 1 69 82 34 36
Fax : 33 1 69 82 43 43

J. Sánchez-Prieto — Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid (Spain).
Fax : 34 1 394 39 09

P. H. Seeburg — Laboratory of Molecular Neuroendocrinology, University of Heidelberg, Center for Molecular Biology, (ZMBH), Neuenheimer Feld 282, 6900 Heidelberg (Germany).
Tel.: 49 6221 56 68 91
Fax : 49 6221 56 58 94

Y. Stern-Bach — Molecular Neurobiology Laboratory, The Salk Institute, 10010 N Torrey Pines Rd. La Jolla, CA. 92037 (USA).
Tel.: 1 619 453 41 00
Fax : 1 619 450 21 72

G.L. Westbrook — Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University L-474, Portland, OR. 97201-3098 (USA).
Tel.: 1 503 49 45 429
Fax : 1 503 49 46 972

List of Participants

Workshop on

MOLECULAR MECHANISMS OF SYNAPTIC FUNCTION

List of Participants

M. Borde — Laboratorio de Neurofisiología Celular, Facultad de Medicina, Gral. Flores 2125, 11800 Montevideo (Uruguay).
Tel.: 598 2 94 35 44
Fax : 598 2 94 35 44

M. Casado — Instituto Cajal, CSIC, Avenida Dr. Arce 37, 28002 Madrid (Spain).
Tel.: 34 1 585 47 10
Fax : 34 1 585 47 54

M.A. Chinchetru — Departamento de Bioquímica y Biología Molecular, Universidad de León, 24007 León (Spain).
Tel.: 34 87 29 12 23
Fax : 34 87 29 12 26

J. Cudeiro — Departamento de Ciencias de la Salud I, Universidad de La Coruña, 15071 La Coruña (Spain).
Tel.: 34 81 10 20 57 - 10 40 80
Fax : 34 81 10 40 58

B. Ebert — PharmaBiotec Research Centre, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy,2 Universitetsparken, 2100 Copenhagen (Denmark)
Tel.: 45 35 37 08 50
Fax : 45 35 37 22 09

M. Galarreta — Departamento de Investigación, Hospital Ramón y Cajal, Crta. Colmenar Km. 9, 28034 Madrid (Spain).
Fax : 34 1 336 90 16

G. Gasic — CELL, Editorial Offices, 50 Church Street, Cambridge, MA. 02138 (USA).
Tel.: 1 617 661 70 57
Fax : 1 617 661 70 61

I. Herrero — Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid (Spain).
Fax : 34 1 394 39 09

R. Howlett — NATURE, 4 Little Essex Street, London WC2R 3LF (U.K.).
Tel.: 44 71 836 66 33 - Ext. 2150
Fax : 44 71 836 99 34

B. Khodorov — Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, 125315 Moscow (Russia).
Fax : 70 95 151 95 40

D.M. Kullmann	Department of Clinical Neurolcgy, Institute of Neurology, University of London, Queen Square, London WC1N 3BG (U.K.). Tel.: 44 071 837 36 11 Fax : 44 071 278 56 16
J. Leyva	Institute of Pharmacology and Toxicology, Faculty of Medicine and Surgery, 2nd University of Naples, Via Costantinopoli 16, 80138 Naples (Italy). Tel.: 39 81 566 58 78 Fax : 39 81 564 04 96
A. López Guajardo	Instituto Cajal, CSIC, Lab. B-18, Avenida Dr. Arce, 37, 28002 Madrid (Spain). Tel.: 34 1 585 47 50 Fax : 34 1 585 47 54
G. Maguire	Sensory Sciences Center, University of Texas, 6420 Lamar Fleming Avenue, Room 316, Houston, TX. 77030 (USA). Tel.: 1 713 792 86 50 Fax : 1 713 792 45 13
M. Morales	Instituto Cajal, CSIC, Lab. B-18, Avda. Dr. Arce 37, 28002 Madrid (Spain). Tel.: 34 1 585 47 50 Fax : 34 1 585 47 54
D.E. Pellegrini-Giampietro	Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G.B. Morgagni 65, 50134 Firenze (Italy). Tel.: 39 055 423 74 11 Fax : 39 055 436 16 13
S.A. Richmond	Department of Pharmacology,The Medical School, University of Birmingham, Birmingham B15 2TT (U.K.). Tel.: 44 21 414 45 07 Fax : 44 21 414 45 09
R. Rivosecchi	Medizinische Fakultät der Universität des Saarlandes, 66421 Homburg/Saar (Germany). Tel.: 49 6841 166 484 Fax : 49 6841 166 468
J. Rodríguez	Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Autónoma de Barcelona, 08913 Bellaterra, Barcelona (Spain). Tel.: 34 3 581 15 25 Fax : 34 3 581 15 73
M.A.Romano-Silva	Department of Biochemistry and Imunology, ICB, UFMG, Belo Horizonte (Brazil). Tel.: 55 31 441 56 11 Fax : 55 31 441 59 63

D. Ruano — Institut Alfred Fessard (CNRS), 91198 Gif-sur-Yvette (France).
Tel.: 33 1 69 82 34 23
Fax : 33 1 69 82 43 43

J. Sallés — Departamento de Farmacología, Facultad de Farmacia, Universidad del País Vasco, c/Marqués de Urquijo s/nº, 01006 Vitoria, Alava (Spain).
Tel.: 34 45 14 00 05 Ext. 366
Fax : 34 45 13 07 56

A. Sánchez — Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, Valladolid (Spain).
Tel.: 34 83 42 30 85
Fax : 34 83 42 35 88

R. Schneggenburger — Ecole Normale Supérieure, Lab. de Neurobiologie, 46 rue d'Ulm, 75005 Paris (France).
Tel.: 33 1 44 32 37 44
Fax : 33 1 44 32 38 87

G. Stocca — Biophys. Lab., SISSA, 34013 Trieste (Italy).
Tel.: 39 40 378 72 27
Fax : 39 40 378 75 28

A. Valero — Istituto Cajal, CSIC, Avda. Dr. Arce 37, 28006 Madrid (Spain).
Tel.: 34 1 585 47 10
Fax : 34 1 585 47 54

J. Vitorica — Departamento de Bioquímica, Bromatología y Toxicología, Facultad de Farmacia, Universidad de Sevilla, c/Profesor García González s/nº, 41012 Sevilla (Spain).
Fax : 34 5 423 37 65

J.L. Yakel — NIEHS, P.O. Box 12233, Research Triangle Park, NC. 27709 (USA).
Tel.: 1 919 541 14 07
Fax : 1 919 541 18 98

R.S. Zukin — Department of Neuroscience, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Ave, Bronx, NY. 10461 (USA).
Tel.: 1 718 430 25 36
Fax : 1 718 824 30 58

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Organized by A. Klug and J. A. Subirana. Lectures by F. Azorín, D. M. Crothers, R. E. Dickerson, M. D. Frank-Kamenetskii, C. W. Hilbers, R. Kaptein, D. Moras, D. Rhodes, W. Saenger, M. Salas, P. B. Sigler, L. Kohlstaedt, J. A. Subirana, D. Suck, A. Travers and J. C. Wang.

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5 **Workshop on Structure of the Major Histocompatibility complex.**
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8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
Organized by C. Gancedo and J. M. Gancedo. Lectures by T. G. Cooper, T. F. Donahue, K.-D. Entian, J. M. Gancedo, C. P. Hollenberg, S. Holmberg, W. Hörz, M. Johnston, J. Mellor, F. Messenguy, F. Moreno, B. Piña, A. Sentenac, K. Struhl, G. Thireos and R. S. Zitomer.

10 **Workshop on Engineering Plants Against Pests and Pathogens.**
Organized by G. Bruening, F. García-Olmedo and F. Ponz. Lectures by R. N. Beachy, J. F. Bol, T. Boller, G. Bruening, P. Carbonero, J. Dangl, R. de Feyter, F. García-Olmedo, L. Herrera-Estrella, V. A. Hilder, J. M. Jaynes, F. Meins, F. Ponz, J. Ryals, J. Schell, J. van Rie, P. Zabel and M. Zaitlin.

11 **Lecture Course on Conservation and Use of Genetic Resources.**
Organized by N. Jouve and M. Pérez de la Vega. Lectures by R. P. Adams, R. W. Allard, T. Benítez, J. I. Cubero, J. T. Esquinas-Alcázar, G. Fedak, B. V. Ford-Lloyd, C. Gómez-Campo, V. H. Heywood, T. Hodgkin, L. Navarro, F. Orozco, M. Pérez de la Vega, C. O. Qualset, J. W. Snape and D. Zohary.

12 **Workshop on Reverse Genetics of Negative Stranded RNA Viruses.**
Organized by G. W. Wertz and J. A. Melero. Lectures by G. M. Air, L. A. Ball, G. G. Brownlee, R. Cattaneo, P. Collins, R. W. Compans, R. M. Elliott, H.-D. Klenk, D. Kolakofsky, J. A. Melero, J. Ortín, P. Palese, R. F. Pettersson, A. Portela, C. R. Pringle, J. K. Rose and G. W. Wertz.

13 **Workshop on Approaches to Plant Hormone Action.**
Organized by J. Carbonell and R. L. Jones. Lectures by J. P. Beltrán, A. B. Bleecker, J. Carbonell, R. Fischer, D. Grierson, T. Guilfoyle, A. Jones, R. L. Jones, M. Koornneef, J. Mundy, M. Pagès, R. S. Quatrano, J. I. Schroeder, A. Spena, D. Van Der Straeten and M. A. Venis.

14 **Workshop on Frontiers of Alzheimer Disease.**
Organized by B. Frangione and J. Avila. Lectures by J. Avila, K. Beyreuther, D. D. Cunningham, A. Delacourte, B. Frangione, C. Gajdusek, M. Goedert, Y. Ihara, K. Iqbal, K. S. Kosik, C. Milstein, D. L. Price, A. Probst, N. K. Robakis, A. D. Roses, S. S. Sisodia, C. J. Smith, W. G. Turnell and H. M. Wisniewski.

15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
Organized by J. M. Mato and A. Ullrich. Lectures by M. Barbacid, A. Bernstein, J. B. Bolen, J. Cooper, J. E. Dixon, H. U. Häring, C.- H. Heldin, H. R. Horvitz, T. Hunter, J. Martín-Pérez, D. Martín-Zanca, J. M. Mato, T. Pawson, A. Rodríguez-Tébar, J. Schlessinger, S. I. Taylor, A. Ullrich, M. D. Waterfield and M. F. White.

16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

17 **Workshop on Cell Recognition During Neuronal Development.**
Organized by C. S. Goodman and F. Jiménez. Lectures by J. Bolz, P. Bovolenta, H. Fujisawa, C. S. Goodman, M. E. Hatten, E. Hedgecock, F. Jiménez, H. Keshishian, J. Y. Kuwada, L. Landmesser, D. D. M. O'Leary, D. Pulido, J. Raper, L. F. Reichardt, M. Schachner, M. E. Schwab, C. J. Shatz, M. Tessier-Lavigne, F. S. Walsh and J. Walter.

18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
Organized by C. Nathan and A. Celada. Lectures by D. O. Adams, M. Aguet, C. Bogdan, A. Celada, J. R. David, A. Ding, R. M. Fauve, D. Gemsa, S. Gordon, J. A. M. Langermans, B. Mach, R. Maki, J. Mauël, C. Nathan, M. Röllinghoff, F. Sánchez-Madrid, C. Schindler, A. Sher and Q.-w. Xie.

19 Workshop on Viral Evasion of Host Defense Mechanisms.
Organized by M. B. Mathews and M. Esteban. Lectures by E. Domingo, L. Enjuanes, M. Esteban, B. N. Fields, B. Fleckenstein, L. Gooding, M. S. Horwitz, A. G. Hovanes-sian, M. G. Katze, I. M. Kerr, E. Kieff, M. B. Mathews, G. McFadden, B. Moss, J. Pavlovic. P. M. Pitha, J. F. Rodríguez, R. P. Ricciardi, R. H. Silverman, J. J. Skehel, G. L. Smith, P. Staeheli, A. J. van der Eb, S. Wain-Hobson, B. R. G. Williams and W. Wold.

20 Workshop on Genomic Fingerprinting.
Organized by McClelland and X. Estivill. Lectures by G. Baranton, D. E. Berg, X. Estivill, J. L. Guénet, K. Hayashi, H. S. Kwan, P. Liang, M. McClelland, J. H. Nadeau, D. L. Nelson, M. Perucho, W. Powell, J. A. Rafalski, O. A. Ryder, R. Sederoff, A. J. G. Simpson, J. Welsh and H. Zischler.

21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

22 Workshop on Molecular Bases of Ion Channel Function.
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

23 Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.
Organized by C. M. Thomas, E. M. H. Wellington, M. Espinosa and R. Díaz Orejas. Lectures by J. C. Alonso, F. Baquero, P. A. Battaglia, P. Courvalin, F. de la Cruz, D. K. Chattoraj, E. Díaz, R. Díaz Orejas, M. Espinosa, J. C. Fry, K. Gerdes, D. R. Helinski, B. Hohn, E. Lanka, V. de Lorenzo, S. Molin, K. Nordström, R. W. Pickup, C. M. Thomas, J. D. van Elsas, E. M. H. Wellington and B. Wilkins.

24 Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.
Organized by P. D. Coleman, F. Mora and M. Nieto-Sampedro. Lectures by J. Avila, Y.-A. Barde, P. D. Coleman, C. W. Cotman, Y. Lamour, M. P. Mattson, A. Matus, E. G. McGeer, B. S. Meldrum, J. Miquel, F. Mora, M. Nieto-Sampedro, V. H. Perry, M. P. Rathbone, G. S. Roth, R. R. Sturrock, H. B. M. Uylings and M. J. West.

25 Workshop on Genetic Recombination and Defective Interfering Particles in RNA Viruses.
Organized by J. J. Bujarski, S. Schlesinger and J. Romero. Lectures by V. Agol, P. Ahlquist, J. J. Bujarski, J. Burgyán, E. Domingo, L. Enjuanes, S. P. Goff, T. C. Hall, A. S. Huang, K. Kirkegaard, M. M. C. Lai, T. J. Morris, R. F. Ramig, D. J. Robinson, J. Romero, L. Roux, S. Schlesinger, A. E. Simon, W. J. M. Spaan, E. G. Strauss and S. Wain-Hobson.

26 Workshop on Cellular Interactions in the Early Development of the Nervous System of Drosophila.
Organized by J. Modolell and P. Simpson. Lectures by S. Artavanis-Tsakonas, J. A. Campos-Ortega, S. B. Carroll, C. Dambly-Chaudière, C. Q. Doe, R. G. Fehon, M. Freeman, A. Ghysen, V. Hartenstein, D. Hartley, Y. N. Jan, C. Klämbt, E. Knust, A. Martínez-Arias, M. Mlodzik, J. Modolell, M. A. T. Muskavitch, B.- Z. Shilo, P. Simpson, G. M. Technau, D. van Vactor and C. Zuker.

27 Workshop on Ras, Differentiation and Development
Organized by J. Downward, E. Santos and D. Martín-Zanca. Lectures by A. Balmain, G. Bollag, J. Downward, J. Erickson, L. A. Feig, F. Giráldez, F. Karim, K. Kornfeld, J. C. Lacal, A. Levitzki, D. R. Lowy, C. J. Marshall, J. Martín-Pérez, D. Martín-Zanca, J. Moscat, A. Pellicer, M. Perucho, E. Santos, J. Settleman, E. J. Taparowsky and M. Yamamoto.

28 Human and Experimental Skin Carcinogenesis
Organized by A. J. P. Klein-Szanto and M. Quintanilla. Lectures by A. Balmain, F. F.

Becker, J. L. Bos, G. T. Bowden, D. E. Brash, A. Cano, C. J. Conti, G. P. Dotto, N. E. Fusenig, J. L. Jorcano, A. J. P. Klein-Szanto, E. B. Lane, H. Nakazawa, G. Orth, M. Quintanilla, D. R. Roop, J. Schweizer, T. J. Slaga and S. H. Yuspa.

29 **Workshop on the Biochemistry and Regulation of Programmed Cell Death.**
Organized by J. A. Cidlowski, H. R. Horvitz, A. López-Rivas and C. Martínez-A. Lectures by J. C. Ameisen, R. Buttyan, J. A. Cidlowski, J. J. Cohen, M. K. L. Collins, T. G. Cotter, A. Eastman, D. R. Green, E. A. Harrington, T. Honjo, H. R. Horvitz, J. T. Isaacs, M. D. Jacobson, P. H. Krammer, A. López-Rivas, C. Martínez-A. S. Nagata, G. Núñez, R. W. Oppenheim, S. Orrenius, J. J. T. Owen, M. C. Raff, L. M. Schwartz and J. Yuan.

30 **Workshop on Resistance to Viral Infection.**
Organized by L. Enjuanes and M. M. C. Lai. Lectures by M. Ackermann, R. Blasco, L. Enjuanes, M. Esteban, P. A. Furth, F. L. Graham, L. Hennighausen, G. Keil, M. M. C. Lai, S. Makino, J. A. Melero, K. Olson, J. Ortín, E. Paoletti, C. M. Rice, H. L. Robinson, J. F. Rodríguez, B. Roizman, J. J. Rossi, P. Roy, S. G. Siddell, W. J. M. Spaan, P. Staeheli and M. Del Val.

31 **Workshop on Growth and Cell Survival Factors in Vertebrate Development**
Organized by M. C. Raff and F. de Pablo. Lectures by Y.- A. Barde, H. Beug, Y. Courtois, A. Efstratiadis, F. Giraldez, J. B. Gurdon, T. M. Jessell, N. Le Douarin, G. Martin, A. McMahon, D. Metcalf, E. Mitrani, F. de Pablo, P. H. Patterson, M. C. Raff, M. M. Rechler, D. B. Rifkin, J. P. Thiery and M. L. Toribio.

32 **Workshop on Chromatin Structure and Gene Expression**
Organized by F. Azorín, M. Beato and A. P. Wolffe. Lectures by F. Azorín, M. Beato, E. M. Bradbury, B. R. Cairns, F. J. Campoy, V. G. Corces, C. Crane-Robinson, E. Di Mauro, S. C. R. Elgin, G. Felsenfeld, W. T. Garrard, M. Grunstein, W. Hörz, J. T. Kadonaga, U. K. Laemmli, A. Ruiz-Carrillo, M. A. Surani, F. Thoma, J. O. Thomas, A. A. Travers, B. M. Turner, K. E. van Holde, A. P. Wolffe and J. L. Workman.

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Instituto Juan March de Estudios e Investigaciones
Castelló, 77 • Teléfono 34 - 1 - 435 42 40 • Fax 576 34 20
28006 Madrid (España)

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