SYNAPSE FORMATION AND FUNCTION: THE NEUROMUSCULAR JUNCTION AND THE CENTRAL NERVOUS SYSTEM

Organizers: Stanley Froehner and James Patrick

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Function of Neurotransmitter Receptors

T 001 CALCIUM MODULATION AND HIGH CALCIUM PERMEABILITY OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS, Steven Vernino, Mariano Amador, and John A. Dani, Division of Neuroscience and Dept. of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030-3498.

Two properties were found to distinguish neuronal from muscle nicotinic acetylcholine receptors (nAChRs). First, neuronal nAChRs have a greater Ca^{2+} permeability. The high Ca^{2+} flux through neuronal nAChRs activates a Ca^{2+}-dependent Cl^- conductance, and the Ca^{2+} to Cs^+ permeability ratio (P_{Ca}/P_{Cs}) is 7 times greater for neuronal than for muscle nAChRs. Single-channel measurements and permeation models indicated that about 2% of the current through muscle nAChRs is carried by Ca^{2+} in physiologic solutions. Measurements with the Ca^{2+} indicator, fura-2, show that the fraction of current carried by Ca^{2+} through neuronal nAChRs is much greater than for muscle nAChRs. Activity-dependent Ca^{2+} influx through neuronal nAChRs may produce an important intracellular synaptic signal. A second difference between the receptor types is that neuronal nAChRs are potently modulated by physiological levels of external Ca^{2+}. Extracellular Ca^{2+} enhances currents through neuronal nAChRs and decreases currents through muscle nAChRs. The decrease in current through muscle nAChRs by extracellular Ca^{2+} is consistent with single-channel measurements (1), which demonstrated that Ca^{2+} competes with Na^+ and K^+ for occupancy of the channel. Then, Ca^{2+} moves through the channel more slowly than the monovalent cations. Therefore, Ca^{2+} decreases the macroscopic currents through the muscle nAChR channels by decreasing the underlying single-channel conductance. In contrast, currents through neuronal nAChRs are enhanced by extracellular Ca^{2+} even though the single-channel conductance decreases. Our results suggest that external Ca^{2+} may directly modulate neuronal nAChRs. Modulation by Ca^{2+} is seen when cells are internally perfused by Ca^{2+} chelators and is seen at voltages that would allow little Ca^{2+} entry into the cell. Therefore, an intracellular Ca^{2+}-dependent enzyme cascade is a very unlikely mechanism, but membrane-bound second messenger systems in close proximity to the intracellular face of the receptor channel cannot be excluded. The results indicate that changes in extracellular Ca^{2+} modulate neuronal nAChRs and may modulate cholinergic synapses in the central nervous system.

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T 002 GLUTAMATE RECEPTOR KINETICS AND SYNAPTIC FUNCTION, Robin A. J. Lester, John D. Clements, Gary L. Westbrook, and Craig E. Jahr, Vollum Institute, OHSU, Portland, OR 97201.

Many excitatory synapses in the central nervous system depend on the activation of two types of postsynaptic ligand-gated ion channels by presynaptically released L-glutamate. Even though the same exocytotic event can activate both receptor types, the component of the synaptic conductance due to NMDA receptors has much slower onset and decay kinetics than the AMPA receptor component. NMDA receptors have much higher affinity for L-glutamate than AMPA receptors and thus the differences in time course may be accounted for by prolonged occupancy of NMDA receptors by L-glutamate leading to long bursts of channel openings whereas AMPA channel activity is quickly curtailed by rapid unbinding. Consistent with these suppositions, we have shown that competitive NMDA antagonists, such as D-AP5, can inhibit synaptic responses of hippocampal neurons only if it is present before presynaptic stimulation. If D-AP5 is rapidly applied after the onset of the synaptic conductance, no inhibition of the epsc is observed indicating that rebinding of transmitter is not required for long lasting activation and suggesting that the lifetime of free transmitter in the cleft is short. In support of this, short applications of L-glutamate to outside-out patches from hippocampal neurons evoke prolonged NMDA channel activity that is not curtailed by subsequent application of D-AP5. Both the onset and decay times of the NMDA channel currents in patches are the same as those of the NMDA component of the epsc indicating that diffusion of transmitter to extrasynaptic sites is not necessary to account for the slow onset. Rather, a slow opening rate and prolonged bursts of openings delay the time to peak and result in a slow decay phase. Agonists of NMDA receptors with lower affinities than L-glutamate, such as L-aspartate and L-cysteate, evoke currents in patches that decay much faster than those due to L-glutamate because their dissociation rates are several fold faster. If an excitatory amino acid other than glutamate were used as a neurotransmitter, we would expected that the NMDA component of the epsc would decay with a time constant consistent with its affinity.

Structure of Neurotransmitter Receptors

T 003 STRUCTURAL ORGANIZATION OF FUNCTIONAL DOMAINS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR, Jonathan B. Cohen, Michael P. Blanton, David C. Chiara, Shannan D. Sharp, and Benjamin H. White, Department of Anatomy & Neurobiology, Washington University Sch. Med., St. Louis, MO.

To describe the mechanism of permeability control by Torpedo nicotinic acetylcholine receptors (AChR), we have been using affinity labeling techniques to identify regions involved in the binding of agonists and in ion permeation and also to describe differences in AChR structure characteristic of the different receptor functional states. The general approach is to covalently incorporate radiolabeled ligands into native AChR, to then isolate labeled subunits and to identify sites of specific labeling by N-terminal sequence analysis of peptides generated by proteases and isolated by SDS-PAGE or HPLC. Previous studies have identified a short segment of α-subunit (α-190-200) as the primary site of labeling by competitive antagonist affinity labels. Based upon an analysis of the sites of labeling by agonist ([^{3}H]nicotine and [^{3}H]acetylcholine mustard ([^{3}H]AChM)) and competitive antagonist ([^{3}H]tubocurarine ([^{3}H]dTC)) affinity labels, we have now identified other regions of α-subunit as well as regions within γ- and δ- subunits that contribute to the agonist binding site. [^{3}H]AChM was used to identify regions of the binding site interacting with the positive charge of ACh. In its reactive form, AChM has the same structure as ACh but contains the positive charge within a reactive, quaternary aziridium ion well-suited to react with nucleophiles in the binding site. Specific labeling of AChR is restricted to α-subunit, and within α-subunit [^{3}H]AChM reacts selectively with Tyr-93. The lack of reactivity with tyrosyl or acidic side chains within α190-200 reveals a selective orientation of ACh within its binding site, and the general lack of reactivity with acidic side chains establishes the importance of aromatic interactions for the binding of the quaternary ammonium group. In contrast to [^{3}H]AChM, when photoincorporated into AChR, [^{3}H]nicotine reacts primarily with αTyr-198 and also with Tyr-190 and Cys-192, but not with Tyr-93 (1). As is generally true for competitive antagonists but not agonists, dTC is bound nonequivalently by the 2 ACh sites on the AChR (K_1 = 35 nM, K_2 = 2 μM). Upon UV irradiation, [^{3}H]dTC is specifically photoincorporated into AChR α-,γ-, and δ- subunits, with a concentration dependence that places the high and low affinity sites at the α-γ and α-δ interfaces, respectively (2). Within α-subunit, [^{3}H]dTC incorporation is restricted to α190-200. Within γ- and δ-subunits, the principle sites of incorporation occur at homologous residues γ Trp-55 and δ-Trp-57. These results provide the first identification of regions of γ- and δ- subunits contributing to the ACh binding site, and comparison of aligned sequences of ligand-gated ion channels identifies conservation patterns in this region indicating a more general involvement in ligand binding domains.

To characterize agonist-induced changes of AChR structure, we have mapped the sites of incorporation of a novel, uncharged photoaffinity non-competitive antagonist, [^{125}I]TID (3). [^{125}I]TID binds with similar affinity to AChR in the absence or presence of antagonist, but agonist inhibits photolabeling of all AChR subunits by >75%. In the absence of agonist, [^{125}I]TID specifically labels β-subunit Leu-257 and Val-261 as well as homologous residues in the M2 region of each other subunit. In the presence of agonist, that labeling is reduced by $\simeq$90% and, in addition, labeling is broadened to include a homologous set of serine residues at the N terminus of M2 (in β-subunit, Ser-250 and Ser-254). This pattern of labeling supports an α-helical model for M2 and provides a first indication of differences in structure in the M2 region between closed and desensitized states. The sites of non-specific incorporation of [^{125}I]TID within the AChR define regions in contact with lipid. The primary sites of nonspecific labeling are found within the M4 segment, and the periodicity and distribution of labeled residues establish that in both resting and desensitized states, the M4 segment is α-helical in nature and presents a broad face to membrane lipid.

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Termination of Neurotransmitter Activity

T 004 NEUROTRANSMITTER REUPTAKE: MECHANISM, ISOLATION AND RECONSTITUTION OF THE TRANSPORTERS, AND MOLECULAR CLONING, Baruch I. Kanner[1], Shoshi Keynan[1], Nicola Mabjeesh[1], Gilia Pines[1], Niels C. Danbolt[2], Jon Storm-Mathisen[2], Young-Jin Suh[3] and Gary Rudnick[3], [1]Dept. of Biochemistry, Hadassah Medical School, The Hebrew University, Jerusalem, Israel, [2]Anatomical Institute, University of Oslo, Norway, [3]Dept. of Pharmacology, Yale University School of Medicine, New Haven, CT 06510.

Synaptic transmission at most vertebrate synapses is thought to be terminated by a rapid uptake of the neurotransmitter into presynaptic nerve terminals or glial cell processes. Such uptake is catalyzed by sodium-coupled neurotransmitter transporter systems. We have studied the uptake systems for GABA and L-glutamate, the major inhibitory and excitatory neurotransmitters in the central nervous system. The GABA transporter catalyzes cotransport of sodium, chloride and GABA. The protein was purified from rat brain and reconstituted into liposomes in an active form. This glycoprotein migrates as an 80 kDa polypeptide on SDS-PAGE. Polyclonal antibodies prepared against this 80 kDa band immunoprecipitate GABA transport activity. Using these antibodies the distribution of the transporter in rat brain has been studied by immunocytochemistry. It was found to be associated with neuronal GABA-ergic terminals. Kinetic and pharmacological studies suggest the presence of a variety of GABA transporter subtypes. There are at least two types of high-affinity GABA transporters. One is inhibited by ACHC and seems to be neuronal. The other is more sensitive to β-alanine and is suspected to be of glial origin. Low affinity GABA transport has also been observed. The uptake system for the neurotransmitter L-glutamate (Glu) catalyses electrogenic cotransport of this amino acid with sodium and potassium (the latter travelling in the opposite direction. Recently we have reported on the isolation and reconstitution of the Glu-Tp. The transporter was purified to a high degree and it exhibited, upon reconstitution, the same properties as the native transporter. The major polypeptide present in this purified preparation had an apparent molecular mass of 80 kDa. Antibodies raised against this polypeptide, immunoprecipitate the L-glutamate transport activity - as assayed upon reconstitution from a partially purified detergent extract. The immunocytochemical localization of the transporter was studied at the electron microscopic level in hippocampal as well as cerebellar cortex of rat. It was found to be located in glial cell processes rather than neurons. Recently we have, in collaboration with the labs of H. Lester and N. Nelson, isolated and expressed a cDNA clone encoding for the GABA transporter (Guastella et al., 1990). Subsequently we studied the transient and stable expression of the cloned transporter in mammalian cells, and have been able to define unambiguously the subtype of the cloned transporter, to study the effect of N-glycosylation and to provide evidence that low affinity GABA transport is not a property of the cloned high-affinity transporter. Antibodies were raised against synthetic peptides corresponding to several regions of the GABA transporter. All the antibodies recognised the intact transporter on Western blots. Upon papain or pronase treatment a reconstitutively active transporter can be isolated upon lectin chromatography. On SDS-PAGE these fragments exhibit increased mobility, indicating an apparent reduction of 20 kDa in molecular mass. The fragment obtained after pronase digestion reacts with the antibodies against the internal loops but not with those raised against the carboxy terminal. We conclude that the amino- and carboxy-terminal parts of the transporter, possibly including transmembrane α-helices 1, 2 and 12, are not required for the transport function.

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T 005 GENE STRUCTURE AND REGULATION OF EXPRESSION OF ACETYLCHOLINESTERASE, P. Taylor, Y. Li, S. Camp, D. Getman, M.E. Fuentes, T. Rachinsky, Z. Radic and D. Vellom. Dept. of Pharmacology, University of California, San Diego, La Jolla, CA 92093.

A single gene exists for acetylcholinesterase with defined chromosomal locations in mouse and human, and expression of the molecule is precisely regulated in neurons, muscle and hematopoietic cells. In fact, the regulation of gene expression associated with development and synaptogenesis often shares common properties with receptors and channels which typically are encoded by polygenic systems. The expression of acetylcholinesterase appears to be controlled at three levels. Transcription is affected by the presence of alternative cap sites and alternative mRNA splicing in the 5' non-translated region. Alternative exons corresponding to the very carboxy terminus of the protein encode differences in sequence. The sequence differences yield monomeric enzyme species, a sulfhydryl-containing carboxy terminus allowing for disulfide linkages to structural subunits and a unique hydrophobic sequence allowing for attachment of a glycophospholipid. The 3'-untranslated region also contains alternative polyadenylation signals which affect mRNA stability. Studies in muscle cells show that enhanced mRNA stability is critical to the increased expression seen with muscle cell differentiation. RNase protection, primer extension, transfection and expression of minigene constructs will be used to show the various control points for AChE expression. Supported by grants from USPHS and MDA.

Post-Translational Modifications of Synaptic Molecules

T 006 NATIVE ACETYLCHOLINE RECEPTORS ON NEURONS: COMPOSITION, FUNCTION, AND REGULATION BY CELL-CELL INTERACTIONS. Darwin K. Berg, Sukumar Vijayaraghavan, William G. Conroy, Ann B. Vernallis, Roderick A. Corriveau, Phyllis C. Pugh, and Zhong-wei Zhang; Department of Biology, UC San Diego; La Jolla, CA 92093.

A family of genes encoding subunits of nicotinic acetylcholine receptors (AChRs) on neurons has been cloned from several species. Expression studies in *Xenopus* oocytes have demonstrated that the encoded subunits can assemble in various combinations to produce receptors with distinctive physiological features. While it is likely that specific AChR gene products can have a dramatic impact on the fate and function of receptor subtypes *in vivo*, little is known about the subunit composition of native AChRs and even less is known about the role of composition in determining receptor location, function, and regulation in neurons.

Chick ciliary ganglion neurons have at least 2 classes of AChRs. One class binds the monoclonal antibody mAb 35, is concentrated predominantly in postsynaptic membrane on the neurons, and generates nicotinic responses mediating synaptic transmission through the ganglion (mAb 35-AChRs). The other class binds α-bungarotoxin, is located predominantly in non-synaptic membrane on the neurons, and, until recently, had no known function (αBgt-AChRs). Northern blot analysis and RNAase protection experiments demonstrate that ciliary ganglia contain moderate amounts of at least 5 AChR gene products. Immunoprecipitation experiments and immunoblot analysis with subunit-specific monoclonal antibodies show that each of 3 subunits tested is segregated either to mAb 35-AChRs or to αBgt-AChRs. The unique combination of subunits comprising mAb 35-AChRs may dictate a synaptic location.

By loading neurons with a calcium-sensitive fluorescent dye, it can be shown that activation of αBgt-AChRs elevates the intracellular level of free calcium. The pharmacology of the response agrees well with that predicted for αBgt-AChRs from binding studies and distinguishes it from the response of mAb 35-AChRs on the cells. This establishes native αBgt-AChRs as being functional nicotinic receptors. Their non-synaptic location and effects on calcium levels suggest that αBgt-AChRs may participate in a novel form of cholinergic signalling with important regulatory consequences for the neuron.

Several lines of evidence support the contention that information from the postsynaptic target tissue can regulate neurotransmitter receptors on ganglionic neurons. Axotomy sharply and specifically reduces the number of mAb 35-AChRs and αBgt-AChRs in ciliary ganglia. The levels of AChR gene transcripts in the ganglion increase substantially when the neurons contact target tissue in the eye during normal development. A component(s) of about 50 kD from the target tissue increases the acquisition of ACh sensitivity by the neurons in culture and also enables the neurons to enhance their ACh responses acutely in a cAMP-dependent manner. Such components represent candidates for target-derived molecules that act via a retrograde pathway to regulate neurotransmitter receptors on the innervating neurons.

T 007 REGULATION OF NEUROTRANSMITTER RECEPTORS BY SERINE AND TYROSINE PROTEIN PHOSPHORYLATION, Richard L. Huganir, HHMI, Neuroscience Department, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Neurotransmitter receptors mediate signal transduction at the postsynaptic membrane of synaptic connections between cells in both the central and peripheral nervous systems. This pivotal role in the mechanism of synaptic transmission suggests that neurotransmitter receptors may be potential targets at which synaptic plasticity could occur. Modulation of the function, expression or density of neurotransmitter receptors in the postsynaptic membrane could have profound effects on the efficacy of synaptic transmission. A variety of data now suggest that protein phosphorylation of neurotransmitter receptors is a primary mechanism for the regulation of neurotransmitter function. We have used the nicotinic acetylcholine receptor as a model system to study the role of protein phosphorylation in the regulation of neurotransmitter receptor and ion channel function. The nicotinic acetylcholine receptor is a pentameric complex of four types of subunits the α, β, γ and δ in the stoichiometry of $\alpha_2\beta\gamma\delta$. The nicotinic receptor is multiply phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and a protein tyrosine kinase (PTK). PKA phosphorylates the γ and δ subunits, PKC phosphorylates the δ subunit while the PTK phosphorylates the β, γ and δ subunits. The phosphorylation of these subunits occurs on the major intracellular loop of each subunit between the third and fourth transmembrane domains. Phosphorylation of the nicotinic acetylcholine receptor by all of these protein kinases appears to regulate the rate of the desensitization of the receptor. The phosphorylation of the nicotinic receptor is under the control of a variety of nerve derived factors including acetylcholine itself and the neuropeptide calcitonin gene-related peptide. Recent studies have also shown that tyrosine phosphorylation of the nicotinic receptor is regulated in vivo and in vitro by neuronal innervation of muscle. Experiments to characterize the components of nerve involved in the regulation of tyrosine phosphorylation have demonstrated that agrin, a neuronal extracellular matrix protein, regulates tyrosine phosphorylation in a manner similar to neuronal innervation. Agrin is thought to be released from the neuron and to mediate the nerve-induced clustering of the nicotinic acetylcholine receptor at the neuromuscular junction. These results suggest that tyrosine phosphorylation of the receptor may be involved in the regulation of agrin and nerve-induced clustering of the receptor at synapses. In addition, in recent studies, we have begun to examine the role of protein phosphorylation in the regulation of the major inhibitory and excitatory neurotransmitter receptors in the nervous system, the $GABA_A$ and glutamate receptors. These receptors are also phosphorylated by a variety of protein kinases including PKA, PKC and PTKs. The phosphorylation of these receptors also occurs on the major intracellular loop of the receptor subunits and appears to be involved in the regulation of desensitization of the receptor. These studies suggest that protein phosphorylation of neurotransmitter receptors is a major mechanism for the regulation of their function and thus may play an important role in the regulation of synaptic plasticity.

Synaptic Organization of Neurotransmitters Receptors and Ion Channels

T 008 TOPOGRAPHY AND SORTING OF VOLTAGE-DEPENDENT ION CHANNELS AND RECEPTORS IN NEURONS AND POLARIZED CELLS, Kimon J. Angelides, Eun-hye Joe, and Jose Velazquez, Departments of Molecular Physiology and Biophysics and Neuroscience, Baylor College of Medicine, Houston, TX 77030.

Neurons are highly polarized cells characterized by a cell surface organization in which proteins are segregated and maintained in discrete functional domains. A fundamental problem in neurobiology is how neurons establish their patterned plasma membranes. Voltage-dependent calcium channels and GABA receptors are distributed in high density on cell bodies and dendrites and voltage-dependent sodium channels (NaChs) are localized at nodes of Ranvier in axons. In myelinated nerve, clustering of NaChs at nodes of Ranvier creates sites of large inward sodium current and is crucial in facilitating saltatory conduction down the axon. Although it is not currently known how proteins are synthesized and delivered to different membrane domains of the neuron, in other polarized cell types it is thought that the different membrane domains are constructed as a result of targeted transport of proteins. However, the problem faced by even the simplest neuron in establishing its mosaic surface membrane is many times more complex than for almost any other cell. While it is not known how specific cell surface domains are created in neurons, recent work with viral and glycosylphosphatidylinositol-linked proteins expressed in neurons and epithelial cells suggest that similar mechanisms for protein sorting could operate in both cell types.

In nerve, NaChs are composed of a protein of 260 kDa (α-subunit) and in some laboratories additional subunits of 36 kDa (β_1) and 33 kDa (β_2) accompany the large polypeptide. In brain, three distinct NaCh genes have been found and there is evidence that these NaCh proteins are differentially localized to cell bodies and axons. The GABA receptor is a pentameric complex each of whose subunits are encoded by multiple genes which can form active GABA receptor complexes.

To gain insight into the subunit requirements and signals that target NaCh subtypes and specific GABA receptor to distinct synapses on dendrites and cell bodies, we examined the sorting of specific combinations of GABA receptor α- and β-subunit subtypes and rat brain NaChs after transfection of their cDNAs into polarized epithelial cells and neurons. Digital and confocal microscopy with NaCh specific antibodies and NaCh specific fluorescent neurotoxins show that NaChs II and III, isoforms primarily segregated to axons, are targeted to the apical membrane of polarized epithelia and distributed in patches. In addition, in those cells expressing apical NaChs, both ankyrin and spectrin, cytoskeletal elements that associate with NaChs *in vitro*, accumulate with NaCh patches. Differential detergent extraction and fluorescence photobleach recovery measurements of the lateral mobility of fluorescently labeled NaChs shows that >90% of the NaChs expressed in the apical membrane are immobile. Using GABA receptor subunit-specific antibodies, depending on their subunit composition, defined receptor assemblies composed of α- and β-subunit subtypes are specifically routed to either apical or basolateral membranes. The results indicate that a signal contained in the NaCh α-peptide primary sequence is sufficient to target NaChs to a specific membrane domain and to induce NaCh-cytoskeleton assembly, while the acquisition of quaternary structure of GABA receptor complexes composed of specific subunit combinations may determine the differential targeting of GABA receptors to either dendrites, cell bodies, or to specific synapses within these regions. Supported by the NIH.

T 009 THE SUBMEMBRANE MACHINERY FOR ACETYLCHOLINE RECEPTOR CLUSTERING, Stanley C. Froehner, Department of Physiology, University of North Carolina, Chapel Hill, NC 27599

At the vertebrate neuromuscular junction, nicotinic acetylcholine receptors (AChR) are localized almost exclusively to the postsynaptic membrane. At these sites, the density of AChR is remarkably high - 8-10,000 receptors per μ^2 of membrane surface. Since AChR are transmembrane proteins inherently capable of diffusion within the plane of the membrane, they must interact with other proteins that serve as synaptic anchors in order to be maintained in this specialized distribution. We are interested in the mechanisms by which AChR are anchored in the postsynaptic membrane and how this process is regulated during synapse formation and remodeling. By analogy to mechanisms used by other cells to form specialized membrane domains, it seems likely that the postsynaptic cytoskeleton is involved in anchoring AChR at the synapse. A peripheral membrane protein of M_r 43,000 (43K protein) is closely associated with AChR in muscle and has been implicated in the receptor clustering phenomenon. The 43K protein is present in amounts stoichiometric with the AChR and is unrelated in primary structure to any other cytoskeletal protein. To test directly the involvement of the 43K protein in AChR clustering, we have examined the distribution of AChR in the presence and absence of the 43K protein. *Xenopus* oocytes injected with *in vitro*-synthesized RNA encoding the α, β, γ, and δ subunits of the mouse muscle AChR express receptors on the cell surface in a diffuse distribution. When coinjected with RNA encoding the mouse 43K protein, AChR are organized into small clusters. These clusters also contain the 43K protein, indicating that the 43K protein plays a direct role in the clustering, probably by interaction directly with the AChR. Finally, when expressed alone, the 43K protein forms clusters of similar size and shape, even in the absence of the receptor. These results provide direct evidence for a role for the 43K protein in clustering of AChR. We are currently using a similar approach to determine how other proteins of the postsynaptic cytoskeleton assemble. Among other proteins closely associated with postsynaptic sites is one of M_r 58,000 (58K protein). Biochemical studies demonstrate that the 58K protein is complexed with dystrophin and possibly with other members of the dystrophin/spectrin family of proteins. Isolation and sequencing of cDNA clones encoding the Torpedo and mouse 58K proteins indicate that these proteins are unrelated to any other sequence in the protein data banks. Coexpression of the 58K protein with AChR and/or the 43K protein will allow us to study the assembly of these postsynaptic components and begin to understand how this process is regulated during neuromuscular synaptogenesis.

T 010 REGULATION OF ACHR DEGRADATION AT VERTEBRATE NEUROMUSCULAR JUNCTIONS, Miriam M. Salpeter, Dept. Neurobiology & Behavior, Cornell University, Ithaca.

The metabolic turnover rate of AChRs at the vertebrate neuromuscular junction (nmj) varies with the state of innervation, and can sometimes (but not always) be regulated by cAMP and/or muscle activity. However, a basic mechanism for this regulation, and the circumstances under which the AChR turnover can be regulated has not yet been determined. Work from our laboratory has suggested that there are two populations of AChR at the nmj, (called R_S and R_F) which differ in their degradation rate and in their responses to innervation and other factors. Other laboratories have explained the existing experimental results without invoking two AChR populations. The alternate models and their corroborating evidence will be discussed.

T 011 NICOTINIC ACETYLCHOLINE RECEPTORS ON AUTONOMIC NEURONS, Peter Sargent, Guy Bryan, Evelyn Garrett, Danny Pang, and Laura Streichert, Depts. of Stomatology and Physiology, Univ. of California, San Francisco, CA 94143.

We examined the effects of innervation upon the distribution and number of nicotinic acetylcholine receptors (AChRs) on the surface of autonomic neurons in the frog cardiac ganglion. AChRs were monitored either with anti-electric organ AChR monoclonal antibody 22 (a gift from Dr. Jon Lindstrom) or with neuronal-bungarotoxin (n-BuTX). Electron microscopic examination of immunoperoxidase-stained ganglia indicates that AChRs are clustered at synaptic sites on normally innervated neurons, where they are found on the postsynaptic surface opposite active zones. Surprisingly, some AChR clusters (20% of the total) are also found in the extrasynaptic membrane. No extrasynaptic AChR clusters are found in frog skeletal muscle using identical methods for visualizing AChRs. Denervation of cardiac ganglia for 2-3 weeks results in a 4-fold reduction in the average size of AChR clusters and a marked change in their distribution; AChR clusters on denervated neurons are often distributed randomly on the cell body surface, whereas those on normal cells are concentrated at the base of the cell, where most of the synaptic boutons lie. The original synaptic clusters of AChRs thus do not survive denervation. By contrast, immunoperoxidase-stained AChR clusters in frog skeletal muscle are retained at their original position and at their original size for 2-3 weeks in the absence of innervation. The effects of denervation upon the number of AChRs in the cardiac ganglion was examined using ^{125}I-n-BuTX. Bath application of n-BuTX indicates that the toxin blocks both EPSPs and ACh potentials at 5-20 nM. ACh potentials on both normal and denervated cells are reduced by more than 80% in the presence of 20 nM n-BuTX. Binding assays using ^{125}I-n-BuTX and cardiac ganglion homogenates indicate the presence of two classes of ^{125}I-n-BuTX binding sites, a high-affinity site with a K_d of 1.7 nM and a B_{max} of 3.8 fmol/ganglion and a low-affinity site with a K_d of > 100 nM. The high-affinity site is likely to represent the functional AChR. Denervation of cardiac ganglia for 2-3 weeks does not change the number of high-affinity sites in the ganglion, suggesting that it does not change the number of AChRs. AChRs were also visualized autoradiographically after incubating intact tissue with ^{125}I-n-BuTX. ^{125}I-n-BuTX binds selectively to synaptic sites on the ganglion cell surface, and the binding occurs with high affinity (K_d=1.3 nM) and shows nicotinic pharmacology. Denervation does not alter the number of autoradiographic grains on the cell surface. The failure of denervation to increase the number of AChRs on the surface of cardiac ganglion cells is curious, given the increase in ACh sensitivity of these neurons induced by denervation. To learn whether this denervation supersensitivity to ACh might be caused by changes in extracellular acetylcholinesterase (AChE), we measured sensitivity of both normal and denervated cells to both ACh and carbachol, a non-hydrolyzable analog of ACh. Denervation increases sensitivity to ACh but not to carbachol. The denervation-induced increase in ACh sensitivity is not observed if ganglia are pre-treated with an irreversible inhibitor of AChE. Denervation supersensitivity to ACh is therefore not caused by a change in the number or properties of AChRs but rather by a reduction in the effectiveness of AChE. These results suggest that AChRs on neurons are regulated differently than those on muscle fibers.

Use- Dependent Development of Synaptic Structure

T 012 SYNAPTIC COMPETITION AT THE NEUROMUSCULAR JUNCTION. Jeff W. Lichtman, Rita Balice-Gordon, Peter van Mier, Adam Brown. Washington University School of Medicine, St. Louis, MO 63110.

Postnatal elimination of synaptic connections between motor neurons and muscle fibers occurs at virtually all developing neuromuscular junctions in mammals. Before the period of synapse elimination every muscle fiber is innervated by multiple motor axons (often two) whose synapses are interspersed at the same site on the muscle fiber. However, following the loss, each muscle fiber retains innervation from exactly one motor neuron. A number of lines of evidence indicate that the transition from multiple to single innervation is due to a loss of axonal branches within the muscle rather than death of motor neurons. Thus, each motor neuron initially innervates more muscle fibers than it will remain in contact with. In many ways this segregative event is analogous to changes in axonal projections in the postnatal central nervous system.

We have followed synapse elimination at individual neuromuscular junctions over time in living mice. By using one vital fluorescent probe for nerve terminals (4-Di-2-ASP) and another for postsynaptic acetylcholine receptors (fluorescently tagged α-bungarotoxin) we have watched the events underlying synapse withdrawal. Surprisingly, the loss of nerve terminals coincides with a loss of postsynaptic receptors at the same sites. We have found no evidence that axons compete for occupation of the same sites. Rather, as synapse elimination proceeds each junction undergoes a series of pre- and postsynaptic receptor site disappearances. The postsynaptic losses are correlated with a step by step withdrawal of overlying synaptic boutons from one of the two axonal inputs. Only when the junctions are finally singly innervated does the loss of postsynaptic receptor sites cease.

Our evidence suggests that the postsynaptic receptor changes begin before the overlying synapse is withdrawn. Fluorescent recovery after photobleaching and other experiments suggest to us that receptor loss is due at least in part to migration of receptors to other sites on the muscle fiber membrane. The precocious postsynaptic changes that precede synapse loss may mean that the signal that instigates synapse withdrawal emanates from the postsynaptic cell. If the postsynaptic cell mediates synaptic competition between axons the mechanism must permit the postsynaptic cell to distinguish between the synaptic contacts that will be maintained from those synapses of the other axon that are interspersed with it at the same junction. One difference between inputs that the postsynaptic cell could use to distinguish the synapses of one axon from those or another is their activity patterns. To test the potential role of activity differences as a stimulus for synaptic competition we have followed adult junctions in which synaptic transmission from one axonal branch has been effectively blocked. By locally puffing a saturating dose of α-bungarotoxin onto the postsynaptic receptors at one part of an adult neuromuscular junction, one branch of an axon's terminal arbor on that muscle fiber is made ineffective. By following such junctions over several weeks we could assess if the postsynaptic cell can sense that some of its receptor sites are inactive at the time other receptor areas are functional. The results were dramatic: within 2-3 days of such partial postsynaptic blockade, nerve terminal branches over the blocked sites began to disappear. The entire nerve terminal area overlying the blocked receptors was resorbed over the course of 7-10 days. Furthermore, postsynaptic receptors at the blocked site were also eliminated and no new receptors took their place. Thus, this experimental result mimics the loss of nerve terminals and receptors seen during competitive synapse elimination between different axons. This result shows that postsynaptic activity has a destabilizing effect on receptor sites that did not contribute to the postsynaptic activity. This sort of mechanism could also explain some competitive events between axons in the developing central nervous system.

T 013 THE ROLE OF NMDA RECEPTORS IN DEVELOPMENT OF ORDERLY BINOCULAR CONNECTIONS IN XENOPUS. Susan B. Udin, State University of New York, Buffalo, NY 14214.

Visual input plays a pivotal role in the establishment of matching binocular maps in the tectum of *Xenopus* frogs. In adult *Xenopus*, each tectal lobe receives a direct retinotectal projection from the contralateral eye. The ipsilateral eye's input is relayed indirectly, via the nucleus isthmi. When the frog has been reared with normal visual input, these two representations are in register so that each position in the binocular portion of the visual field is represented at a single location via both eyes in each tectal lobe. The essential role of visual experience in establishing this registration is demonstrated by the fact that the ipsilateral map fails to organize properly if the animal is reared in the dark. Moreover, abnormal visual experience resulting from surgical rotation or realignment of the contralateral eye during development induces an orderly rearrangement of the ipsilateral map, reestablishing registration with the contralateral projection.

The process that brings the ipsilateral map into register with the contralateral map involves a Hebbian mechanism, whereby axons with similar firing patterns stabilize one another when they converge on common postsynaptic dendrites. The NMDA-type glutamate receptor is appropriate for such a process, since retinotectal axons release glutamate and activate this receptor, inducing calcium influx into tectal cells. We suggest that the calcium triggers release of a retrograde messenger. Ipsilateral axons with correlated firing activity would be stabilized by the synergistic action of this messenger and the elevated intracellular calcium associated with the axons' firing. Evidence for this hypothesis comes from experiments showing that blockade of NMDA receptors with chronic application of the antagonist APV prevents realignment of the ipsilateral map with the contralateral map after eye rotation during development.

Changes in NMDA receptors may underlie the loss of plasticity seen during normal development. The changes described above typically occur only during a critical period of development. However, plasticity in adults can be restored by chronic treatment with low doses of NMDA. Three months of NMDA treatment in frogs which have had one eye rotated after the normal end of the critical period induces rearranged connections. The exogenous NMDA may boost the activation of a diminished population of receptors such that the threshold for activity-dependent alteration of synaptic connections is reached.

Changes in plasticity also are associated with changes in expression of the polysialylated neural cell adhesion molecule NCAM. We find high levels of immunostaining for this molecule in the tecta of developing frogs but not in normal adults. In dark-reared adults, in which plasticity is maintained at juvenile levels, immunostaining is higher than in age-matched controls. In adult frogs unilaterally treated with NMDA, the NMDA-treated side reacquires a pattern of high staining while the control side does not. These results suggest that the activity-dependent mechanisms associated with plasticity may affect connectivity in part by altering expression of variants of NCAM.

Synaptic Regulation of Gene Expression

T 014 STRUCTURE AND ASSEMBLY OF THE MAMMALIAN MUSCLE ACETYLCHOLINE RECEPTOR. Zach W. Hall, Xiao Mei Yu, Shahla Verrall, Raymond A. Chavez, Department of Physiology, University of California, San Francisco, CA 94143.

We have used an in vitro translation system to examine the transmembrane topology of newly-synthesized α and δ subunits of the mouse muscle AChR. By introducing novel glycosylation sites and by using a fusion protein in which prolactin, used as an antigenic marker, is attached to N-terminal fragments of the subunits, we determined which particular segments are on the cytoplasmic or lumenal side of the microsomal membrane. Our results are consistent with a model in which the entire N-terminal domain preceding M1 is translocated into the lumen, the C-terminus is lumenal, and M1-M4, but not MA, are transmembrane domains.

We have also carried out experiments in COS cells that transiently express receptor subunits to investigate which parts of the subunits mediate specific recognition steps during receptor assembly. We have found that chimeric subunits, in which the N- and C-terminal regions are from the ϵ subunit and the intervening portion from the β subunit, are able to substitute for the ϵ, but not the β, subunit during AChR assembly. We have also used a dominant-negative assay to show that fragments of the α, γ and δ subunits that contain the N-terminal region, but lack all transmembrane domains block heterodimer formation and the surface expression of the AChR. Sucrose gradient experiments also show that a fragment of the α subunit containing the N-terminus forms a specific complex with the δ subunit. On the basis of these observations, we conclude that the N-terminal domain contains the information necessary for specific subunit association. (Supported by grants from the NIH and the Muscular Dystrophy Association).

T 015 SYNAPTIC REGULATION OF TRANSCRIPTION: FROM ONCOGENES TO TRANSGENES, Richard J. Smeyne, Karl Schilling, John Oberdick, *Daniel Luk, *Linda Robertson, *Tom Curran and James I. Morgan. Departments of Neuroscience and *Molecular Oncology and Virology, Roche Institute of Molecular Biology, Nutley, N.J. 07110.

Excitation of neurons both in vivo and in culture results in the rapid transcriptional activation of a class of genes, known generically as cellular immediate-early (cIE) genes. This induction occurs even in the absence of ongoing protein synthesis and represents the earliest transcriptional consequences of cell stimulation. Many (but not all) cIE genes encode known or putative transcription factors that are supposed to alter the expression of target genes, thereby providing an initial link between a stimulus and long term changes in cellular phenotype. One group of cIE genes encode proteins of the fos and jun families that form homo- and heterodimers with one another and exhibit sequence specific DNA binding activity to the AP-1 consensus (TGACTCA). In the brain following seizure, it is possible to show elevation of c-fos and c-jun mRNA as well as mRNA and protein for other fos- and jun-related family members. This induction occurs in a staggered manner such that there is a dynamic appearance and disappearance of these proteins but a protracted elevation of AP-1-like DNA binding activity. I.e. a relatively brief period of stimulation not only elicits a rather long-term elevation of AP-1 binding activity but also the molecular composition of these AP-1 complexes varies in a time-dependent manner. Many types of stimuli have been shown to elicit c-fos induction in brain; including, electrical and chemoconvulsant seizures, cerebral ischaemia, nerve and brain trauma, painful and tactile stimuli, osmotic stress, photic, olfactory and acoustic stimuli, and administration and withdrawal of drugs of abuse such as amphetamines, cocaine and morphine. This has resulted in the widespread use of Fos immunohistochemistry and in situ hybridization for c-fos mRNA to map the pathways and sites of action of neurophysiologically and neuropathologically relevant agents and conditions. Since these methods are somewhat tedious and prone in some cases to ambiguity because of cross-reactivity of reagents we have developed a new Fos mapping strategy based upon transgenic mice. Bacterial lacZ has been inserted, in-frame, into the fourth exon of a c-fos genomic fragment that contains all known 5' and intragenic regulatory elements, introns, exons and 3' mRNA regulatory sequences. The fusion protein encoded by this gene is translocated to the nucleus and can be readily detected by its galactosidase activity. Transgenic mice have been generated using this fusion gene which is shown to recapitulate normal c-fos regulation both in terms of basal and stimulated expression. Constitutive expression of this fos-lacZ transgene (and cognate c-fos) is seen to be associated with cells that are undergoing terminal differentiation and that will subsequently die. Whether expression of c-fos is causally related to programmed cell death is unknown at the present. During development of the CNS, constitutive fos-lacZ expression is seen in neurons in a number of brain regions at times when active synaptogenesis is under way. Instances include, mitral cells of the olfactory bulb, pyramidal neurons in the distal region of the CA1 layer of hippocampus and the parafascicular nucleus of the thalamus. The transgene can also be induced transiently in brain with differential patterns of expression by diverse physiological and pharmacological stimuli that will be discussed. In addition, many neurotransmitters and growth factors induce the transgene in primary dissociated cultures of the fetal nervous system.

Molecules Involved in Synaptic Differentiation

T 016 THE PUTATIVE AGRIN RECEPTOR Justin. R. Fallon, Jian-Yi Ma and Mary A. Nastuk, Neurobiology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA. 01545

Agrin is a component of the synaptic basal lamina that induces the clustering of acetylcholine receptors (AChRs) on cultured myotubes and is likely to play a central role in orchestrating postsynaptic differentiation at the neuromuscular junction. However, little is known about how agrin interacts with the cell surface, or the nature of its putative membrane receptor. To address these questions, we have characterized agrin binding to cultured myotubes and to purified plasma membrane fractions from *Torpedo* electric organ. Immunofluorescence microscopy shows that on myotubes incubated with agrin at 4°C (resting myotubes), agrin binds in a uniform, finely punctate pattern that correlates poorly with the distribution of AChRs. Immunoelectron microscopy shows that agrin binds to a component closely associated with the plasma membrane; binding to formed elements of the extracellular matrix is not observed. Myotubes stimulated with agrin at 37°C for ≥2hr show a co-clustering of agrin binding sites and AChRs. By contrast, if anti-AChR antibodies are used either to cluster or to internalize AChRs, the distribution and number of agrin binding sites remains unchanged. Agrin binding to myotubes is saturable as measured by RIA and, like agrin-induced AChR clustering, requires extracellular calcium. To further investigate the biochemical nature of the putative agrin receptor, we turned to *Torpedo* electric organ membrane fractions. Agrin binds to these membranes as judged by immunofluoresence microscopy, RIA, and depletion of bioactive agrin from solution. Binding measured by all three assays requires calcium and is abolished by pretreatment of the membranes with trypsin. Half-maximal binding is observed at subnanomolar agrin concentrations. Agrin binds selectively to membrane fractions enriched in synaptic components: membranes from non-synaptic regions of electric organ membranes or liver show low or undetectable levels of agrin binding. Agrin binding to *Torpedo* membranes is unaffected by removal of peripheral membrane proteins by alkaline treatment. The level of agrin binding to solubilized preparations is unaffected by depletion of AChR by α-bungarotoxin affinity chromatography. These data indicate that the putative agrin receptor is: 1) an integral membrane protein distinct from the AChR; 2) highly concentrated at postsynaptic-like specializations on myotubes and in postsynaptic membranes from electric organ; and 3) binds agrin in a calcium dependent fashion. The aggregation and calcium dependence of the putative agrin receptor may represent important control points in postsynaptic differentiation.

Use Dependent Modification of Synaptic Function

T 017 INDUCTION MECHANISMS FOR NMDA-RECEPTOR INDEPENDENT LTP IN HIPPOCAMPUS, David B. Jaffe, Stephen H. Williams, Richard Gray, Nelson Spruston, and Daniel Johnston, Division of Neuroscience, Baylor College of Medicine, Houston, TX 77030

The induction of LTP at mossy fiber synapses in the CA3 region of hippocampus is independent of the activation of NMDA receptors. We have tested the hypotheses that induction is dependent on a rise in intracellular Ca^{2+} and on the membrane potential in the postsynaptic neuron. Using electrophysiological techniques in rat slices, we found that the injection of BAPTA, a Ca^{2+} chelator, into CA3 neurons blocked the induction of mossy fiber LTP. In other experiments, we found that strong hyperpolarization during the tetanus train blocked, while depolarization during the train enhanced, LTP induction. These results suggest that a rise in $[Ca^{2+}]_i$ is required for LTP and that induction (e.g. the influx of Ca^{2+}) depends on the membrane potential of the postsynaptic neuron. Induction also appears to require high-frequency synaptic activity in addition to postsynaptic depolarization because depolarization alone had no effect on LTP. We propose that an additional factor, which is released with high-frequency stimulation, facilitates the induction of mossy fiber LTP. We have also begun to explore the postsynaptic mechanisms of Ca^{2+} entry into pyramidal neurons using fluorescent imaging techniques. Voltage-gated Ca^{2+} channels appear to be located throughout the dendrites and are activated by action potentials that partially invade the dendrites. These results may help explain the Hebbian nature of both NMDA-receptor dependent and independent LTP.

T 018 BIOCHEMICAL MECHANISMS CONTRIBUTING TO LONG-TERM POTENTIATION, Eric Klann, Dane Chetkovich, Shu-Jen Chen, Craig Powell, J. Suzanne Sessoms, Morganne Gower, and J. David Sweatt, Baylor College of Medicine, Houston, TX 77030.

The induction of one form of long-term potentiation (LTP) in the CA1 region of hippocampus is known to be dependent upon the activation of NMDA receptors. However, the biochemical events subsequent to NMDA receptor activation that are required for LTP induction and maintenance are not well-defined. We have observed that NMDA receptor activation and LTP-inducing tetanic stimulation elicits an increase in cyclic AMP levels in the CA1 region of hippocampus. The NMDA receptor mediated increase in cyclic AMP appears to be due to calcium influx and subsequent activation of calmodulin-sensitive adenylyl cyclase. These results suggest the possibility that activation of the cyclic AMP cascade may be one of the biochemical sequelae of NMDA receptor activation that contributes to the induction of NMDA receptor dependent LTP. Previous pharmacological and electrophysiological studies have suggested that persistent activation of protein kinases contributes to the maintenance of LTP. We have directly assayed hippocampal slices for long-lasting increases in protein kinase activity after LTP-inducing stimuli. We have found that the maintenance phase of LTP is associated with a calcium-independent, persistently activated form of protein kinase C. The induction of this effect is blocked by NMDA receptor antagonists. This finding compliments previous studies and strongly supports the hypothesis that persistent activation of protein kinase C contributes to the maintenance of LTP.

Role of the Extracellular Matrix in Synaptogensis

T 019 THE ACTIVITY-DEPENDENT CNS PROTEOGLYCAN RECOGNIZED BY MONOCLONAL ANTIBODY CAT-301 IS STRUCTURALLY AND FUNCTIONALLY RELATED TO AGGRECAN, S. Hockfield and H. Fryer, Section of Neurobiology, Yale University School of Medicine, New Haven, CT.

Evoked neuronal activity plays an important role in sculpting the final morphological and physiological features of neurons in the mammalian central nervous system (CNS). Activity-dependent alterations in neuronal phenotype occur predominantly during circumscribed periods in development, which have been called "critical" or "sensitive" periods. We have been studying molecular properties of neurons that correlate with, and might subserve, the acquisition of mature neuronal phenotype during developmental critical periods.

The chondroitin sulfate proteoglycan (CSPG) recognized by monoclonal antibody Cat-301 is expressed as a perisynaptic, surface antigen on subsets of neurons in many areas of the CNS. In three systems we have demonstrated that the expression of the Cat-301 CSPG is regulated by neuronal activity early in the postnatal period. Deprivation of normal patterns of neuronal activity early in development inhibits the expression of the proteoglycan. The restoration of normal activity late in development does not restore normal levels of expression. Deprivation in adult animals does not reduce proteoglycan expression. Surgical, environmental and pharmacological manipulations during the early postnatal period (but not in adults) can all affect the level of Cat-301 expression.

We have examined the relationships between the Cat-301 CSPG and proteoglycans from other tissues. Cat-301 recognizes a high molecular CSPG from cartilage, which we have demonstrated to be aggrecan. Like aggrecan, the Cat-301 CSPG from brain binds hyaluronic acid. However, aggrecan and the Cat-301 CSPG from brain are not identical in that aggrecan has keratan sulfate substitution, while the CNS CSPG does not. The Cat-301 CSPG also has a lower buoyant density than aggrecan. These biochemical data suggest that the Cat-301 CSPG represent another member of the family of high molecular weight CSPGs which bind to and aggregate with hyaluronic acid.

Biochemical and ultrastructural studies have provided evidence that the Cat-301 CSPG is an element of an extracellular matrix in the mature mammalian CNS. Taken together, our experiments suggest that the late expression of extracellular matrix molecules may play a role in stabilizing the mature synaptic structure of CNS neurons.

Late Abstract

GLUTAMATE-GATED ION CHANNELS IN THE BRAIN: GENETIC MECHANISMS FOR GENERATING MOLECULAR AND FUNCTIONAL DIVERSITY, P.H. Seeburg, Ph.D., B. Sommer, Ph.D., W. Wisden, Ph.D., H. Monyer, M.D., T.A. Verdoorn*, Ph.D., N. Burnashev*, Ph.D., B. Sakmann*, M.D., Ph.D., Center for Molecular Biology, University of Heidelberg, 6900 Heidelberg, Germany and *Max-Planck Institut für Medizinische Forschung, 6900 Heidelberg, Germany

L-glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS). It gates cationic channels which mediate fast excitatory synaptic responses and participate in mechanisms underlying higher neural functions. These channels also mediate neuronal death following excessive glutamate release in the CNS secondary to acute pathological situations. Furthermore, a variety of chronic neurological disorders may be generated by toxins acting as agonists at glutamate-gated receptor channels. There exist distinct ionotropic glutamate receptor subtypes, named according to the agonists, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainate (KA). The latter two are referred to as non-NMDA receptors. The channels intrinsic to all subtypes conduct Na^+ ions but NMDA-gated channels also possess Ca^{2+} conductance deemed to play a role in neuronal plasticity and neurotoxicity. AMPA receptors are the main mediators of fast excitatory neurotransmission and are expressed throughout the CNS. These channels can also be gated by KA. However, there appears to exist a separate class of glutamate receptor channels which bind KA with high affinity and which seem to be insensitive to AMPA.

Recently we and others have cloned a growing family of related glutamate receptor subunits of rat brain, that form functional cation channels *in vitro*. These subunits can be grouped into those belonging to the AMPA selective glutamate receptor (4 subunits, GluR-A, -B, -C, and -D) and to a KA selective receptor subtype. All subunits are approximately 900 amino acids in length and are predicted to contain four membrane spanning regions (TMI to TMIV) which determine the transmembrane topology of these polypeptides. All subunits show a considerable sequence conservation across their transmembrane regions, including the intracellular portion flanked by TMIII and TMIV. The subunits differ most in their putative extracellular regions (470 residues) where AMPA receptor subunits share approximately 60%, and KA receptor subunits 75% sequence identity. Pairwise comparisons between subunits of the two receptor classes reduce identity to about 25% in this domain.

Structure function studies with recombinantly expressed AMPA receptor subunits have revealed the importance of an arginine residue within channel segment TMII in determining ion transport properties. This arginine is only found in the TMII region of the GluR-B subunit, whereas a glutamine residue is present instead in the other three subunits. In consequence, homo-oligomeric GluR-B channels, and heteromeric assemblies with this subunit show different current-voltage relationships than do channels reconstituted with GluR-A, -C, or -D. Also, a Ca^{2+} conductance through AMPA channels can be observed, but only when these channels are assembled in the absence of GluR-B. Experiments involving site-directed mutagenesis have shown that such disparate channel properties are independent of the subunits themselves but depend on the positively charged arginine in the TMII segment. The current properties through glutamate-gated channels in cultured hippocampal neurons and in hippocampal slices suggest that most natural AMPA receptors incorporate the GluR-B subunit. Hence, the GluR-B subunit determines the properties of AMPA receptors in the CNS.

As shown by gene isolation, sequencing and cDNA analysis, the arginine residue in the Q/R site of the TMII segment in GluR-B is not encoded by the gene but appears to be introduced by RNA editing . This process is highly selective as demonstrated by our finding that GluR-A, -C and -D subunits are not targeted, and is highly efficient since no Q/R site glutamate containing GluR-B is found in the adult brain. However, RNA editing appears to be a developmentally regulated process since GluR-B(Q) subunits coexist with GluR-B(R) subunits in the fetal rodent brain. Hence, AMPA selective glutamate-gated ion channels in fetal brain may be expected to display different monovalent and divalent ionic permeabilities and conductance properties than in the adult.

Ion Channels and Presynaptic Mechanisms

T 100 EXPRESSION OF HUMAN NEURONAL VOLTAGE-DEPENDENT CALCIUM CHANNEL IN TRANSFECTED HEK293 CELLS, P. Brust, A. Maroufi, M. Akong, D. Feldman, S. Ellis, G. Velicelebi and M. Harpold, SIBIA, 505 Coast Blvd. S., La Jolla, CA, 92037

Recently, we reported isolation of the cDNAs encoding three subunits (α_{1D}, α_{2b}, β_2) of a human neuronal voltage-dependent calcium channel [Williams, M., Feldman, D.F., McCue, A.F., Brenner, R., Velicelebi, G., Ellis, S.B. and Harpold, M.M. (1992) Neuron, in press]. Recombinant expression of these subunits in Xenopus oocytes and pharmacological characterization of the voltage-dependent Ba^{2+} currents indicated that the α_{1D},α_{2b}, and β_2 subunits, together, comprise a neuronal subtype of a DHP-sensitive L-type VDCC. We report here recombinant expression of the $\alpha_{1D}\alpha_{2b}\beta_2$ channel in HEK293 human embryonic kidney cells. pcDNA1 and pCMV expression vectors were used to direct both transient and stable expression of the three subunits in HEK293 cells. To establish stable transfectants, the pSV2neo selectable marker was co-transfected. Functional expression of the recombinant channel was determined through: (a) measurement of Ca^{2+} flux, and (b) measurement of voltage-dependent Ba^{2+} currents. To detect increases in calcium flux, the cells were first loaded with fluo-3, a calcium-sensitive fluorescent dye, prior to depolarization of the cell membrane by the addition of KCl. Cells which had been transfected with the vectors containing the α_{1D},α_{2b}, and β_2 cDNAs responded to depolarization by an increase in fluorescence, indicating an increase in intracellular Ca^{2+}. This response was enhanced by the DHP-agonist Bay K 8644 and inhibited by the DHP-antagonist nifedipine. No significant fluorescence response was detected in untransfected cells. High-voltage activated currents of 25-60 pA were detected in the transfected cells through measurement of inward Ba^{2+} currents in response to electrical polarization by whole-cell patch recording. Application of Bay K 8644 increased the magnitude of the currents approximately three-fold and resulted in the appearance of prolonged tail currents. More detailed characterization of the recombinant calcium channels is in progress.

T 101 SYNAPTOGLYCAN: A KERATAN SULFATE PROTEOGLYCAN OF SYNAPTIC VESICLES, S.S. Carlson, M. Iwata, and T. Scranton. Dept. of Physiology and Biophysics, University of Washington, Seattle WA. 98195 We have determined that synaptic vesicles contain a vesicle-specific keratan sulfate integral membrane proteoglycan. This is probably the major proteoglycan of electric organ synaptic vesicles. It exists in two forms, a low molecular weight (L Form) of about 100 kd and a higher molecular weight form (H form), about 250 kd on SDS-PAGE. Both forms contain SV2, an epitope on the cytoplasmic side of the vesicle membrane. The SV2 protein is non-covalently complexed to 4 proteins with molecular weights of 23 kd, 35kd, 39 kd, and 45 kd. In addition to electric organ, we have analyzed the SV2 antigen in vesicle fractions from two other sources; electric fish brain and rat brain. Both the H and L forms of SV2 are present in these vesicles and all are keratan sulfate proteoglycans. Unlike previously studied synaptic vesicle proteins, this proteoglycan contains a marker specific for a single group of neurons. While the SV2 protein is present in all neurons of the fish brain, a unique keratan sulfate epitope, SV1, of this proteoglycan is not. SV1 is specific for the cells innervating the electric organ and is not found in other neurons of the electric fish brain. We propose the name synaptoglycan for this synaptic vesicle keratan sulfate proteoglycan.

T 102 HABITUATION OF NEUROTRANSMITTER RELEASE IN PC12 CELLS, Laura Cheever and Daniel E. Koshland, Jr. Division of Biochemistry and Molecular Biology, Department of Molecular and Cellular Biology, University of California at Berkeley, Berkeley, CA 94720. Habituative learning and memory were studied using neuronally differentiated PC12 cells as a model system. These clonal cells secrete norepinephrine in response to stimulation with ATP or acetylcholine. With repetitive stimulation, the amount of norepinephrine released decreases, meeting many of the criteria for habituative learning. Retention of changes in cellular behavior following habituation depends on the number of habituating stimuli. For stimulation with ATP, habituation results from receptor inactivation, demonstrating that a common phenomenon such as receptor inactivation can potentially give rise to learning and memory. Kinetic equations based on a hypothetical stimulation-dependent inactivation of the response machinery are used to analyze the observed habituation.

T 103 IMMUNOCHEMICAL IDENTIFICATION OF AN N-TYPE AND AN L-TYPE CALCIUM CHANNEL FROM RAT BRAIN, Johannes W. Hell[1], Ruth E.Westenbroek[1], Michael K. Ahlijanian[1], Terry P. Snutch[2], and William A. Catterall[1], [1]Department of Pharmacology, University of Washington, Seattle, WA 98195 and [2]Biotechnology Laboratory, University of British Columbia, Vancouver, B.C., V6T 1W5. Polyclonal antibodies were raised against synthetic peptides, which correspond to sequences of multiple calcium channel α1 subunits cloned from rat brain. In immunoprecipitation experiments, one antiserum (CNB1) specifically recognized only the ω-CgTx GVIA-receptor (N-type channel), but not the dihydropyridine-receptor (L-type channel). In contrast, antiserum CNC 1 immunoprecipitated only the dihydropyridine-receptor, but not the ω-CgTx GVIA -receptor. Neither of these receptors could be precipitated using the third antiserum CNA 1. This suggests, that CNB 1 is specific for the N-type channel and CNC 1 for the L- type channel , whereas CNA 1 may recognize the ω-CgTx GVIA- and dihydropyridine-insensitive P-type channel. This conclusion was further supported by immunohistochemical investigations, where all three antisera showed a differential cellular and subcellular distribution in rat brain. Immunoblotting and immunoprecipitation experiments are in progress to identify and compare the protein components of these brain calcium channels.

T 104 POTENTIATION OF TRANSMITTER SECRETION AT DEVELOPING NEUROMUSCULAR SYNAPSES BY ACTIVATION OF PROTEIN KINASE C. Ann M. Lohof, Joan Rothlein and Mu-ming Poo, Department of Biological Sciences, Columbia University, New York, NY 10027.

We have investigated the possible role of protein kinase C (PKC) activation in regulating transmitter release at developing neuromuscular synapses in nerve-muscle cultures prepared from Xenopus embryos. Synaptic currents were recorded from myocytes which had been contacted by a growing neurite (natural synapse) or which had been brought into contact with a neuron (manipulated synapse), using whole-cell patch-clamp recording method. Local perfusion of the synapse with medium containing *20-300 nM of 12-O-tetradecanoyl-phorbol-13-acetate* (TPA) resulted in a marked increase in the frequency of spontaneous synaptic currents (SSCs). Similar treatment with the inactive analogue 4-alpha-phorbol (100 - 200 nM) produced no increase in the SSC frequency. TPA also produced a small increase in the mean SSC amplitude, apparently due to more frequent large-amplitude currents. The potentiation was apparently due to a presynaptic action of TPA on the secretion mechanism, since TPA had little effect on the postsynaptic response to iontophoretic application of ACh. After brief treatment with TPA, the potentiation usually persisted for as long as the recording was made. Interestingly, only brief activation of protein kinase C appears to be necessary to trigger a long-term potentiation of spontaneous secretion. Application of the kinase inhibitors staurosporine (0.25 - 1 μM) and H-7 (100 μM) before TPA application prevented TPA-induced potentiation. However, these drugs produced only a transient inhibitory effect, if any, on transmitter secretion when applied after the TPA-induced potentiation was established. This observation suggests that initiation of down-stream event(s) following PKC activation may lead to a persistent potentiation which becomes independent of PKC activity itself. Identification of processes affected by PKC within the presynaptic nerve terminal may reveal cellular events important for synapse development and plasticity.

T 105 IDENTIFICATION OF PROTEINS REQUIRED FOR ATP-DEPENDENT AND Ca^{2+}-ACTIVATED STEPS IN THE REGULATED SECRETORY PATHWAY, Thomas F.J. Martin, Bruce W. Porter, Jane H. Walent and Jesse C. Hay, Department of Zoology, University of Wisconsin, Madison, WI 53706 Neural, endocrine and exocrine cells possess a Ca^{2+}-activated secretory pathway by which the exocytotic release of neurotransmitters, hormones and enzymes is regulated. Ca^{2+}-activated secretion can be reconstituted in depleted permeable cells by the addition of cytosolic proteins. We have undertaken a characterization of the cytosolic proteins which are responsible for the reconstitution of ATP-dependent, Ca^{2+}-activated norepinephrine (NE) secretion from permeable PC12 cells (J. Biol. Chem. 264:10299, 1989). A protein which exhibits substantial activity alone in the reconstitution assay has recently been purified from rat brain cytosol. This protein, termed CAPS (Ca^{2+}-dependent Activator Protein for Secretion), was purified 500-fold to homogeneity in a 5-step protocol. The CAPS protein is a dimer of two 140kD subunits and exhibits hydrophobic properties, including Ca^{2+}-dependent interaction with a hydrophobic resin. Rabbit antisera to the CAPS protein have been generated and shown to recognize a single 140kD protein in immunoblotting studies of rat brain cytosol resolved by SDS PAGE. CAPS antisera specifically block ATP-dependent, Ca^{2+}-activated NE release from permeable PC12 cells. Immunoblotting studies of tissue extracts indicate that the CAPS protein is expressed in high concentrations in neural tissues, protein-secreting endocrine tissues, exocrine tissues and in mast cells. In contrast, the protein is either not detected or is present at very low levels in many other tissues, including liver, muscle, kidney and spleen. Partial protein sequences obtained from the CAPS protein suggest that it is a novel rat brain protein. Further structural characterization of the protein may provide clues to the nature of the biochemical process activated by Ca^{2+} during secretion.

In recent studies, it has been possible to demonstrate that the ATP-dependent and Ca^{2+}-triggered steps of NE release can be staged in separate incubations. Preincubations with ATP and cytosolic proteins enhance the rate of ATP-independent, Ca^{2+}-activated NE release. The purified CAPS protein was found to support the Ca^{2+}-triggered release step but not the ATP-dependent priming step. Conversely, a low molecular mass (20 kD) protein was partially purified from rat brain cytosol and shown to exhibit ATP-dependent priming activity but not Ca^{2+}-triggering activity. This protein has been termed PEP (Priming in Exocytosis Protein). Further characterization of the PEP protein may reveal the nature of the requirement for ATP in Ca^{2+}-regulated secretion.

T 106 ESTABLISHMENT OF SYNAPTOTAGMIN (P65)-DEFICIENT VARIANT SUBCLONES OF PC12 RAT PHEOCHROMOCYTOMA CELLS, Takahashi, M., Shoji-Kasai, Y., Yoshida, A., Ogura, A., Sato, K., Kondo, S., Hoshino, T., Fujimoto, Y., Kuwahara, R., Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan

Synaptotagmin (p65) is a novel phosphoprotein localized in secretory vesicular and presynaptic membranes of various neurons and some endocrine cells. Synaptotagmin has characteristic structural domains including a small amino-terminal region, a single transmembrane region, and a large cytoplasmic domain containing two repeats homologous to the regulatory region of protein kinase C. To define the specific roles of synaptotagmin in neurotransmitter and hormone release , we isolated variant subclones of PC12 cell deficient in synaptotagmin by "cell-killing technique".

An antibody recognizing the amino-terminal peptide of synaptotagmin specifically bound to the synaptic button-like structure of cultured neurons without permeabilizing the cell membrane with detergents, indicating that the amino-terminal portion of synaptotagmin in the plasma membrane was exposed to extracellular region. Synaptotagmin-expressing cells were killed by treating them with the antibody followed with a complement. The population of cells not stained by the antibody increased significantly after repeating the cell killing ten times. We isolated individual single cells with a micropipette, and checked their immunoreactivities. Finally we obtained three clones negative to anti-synaptotagmin antibody out of these nearly thousand of clones. Western blot, Northern blot, and PCR analyses revealed that these variant clones were deficient in synaptotagmin protein and the mRNA. On the other hand, these cells retained many properties exhibited by wild type PC12 cells, including ultrastructural morphology, NGF-responsiveness. We are presently comparing the exocytotic activities of catecholamine between the wild type and variants. These variant cells should be useful for the elucidation of the physiological roles of synaptotagmin in the secretion of neurotransmitters and hormones.

T 107 SYNTHETIC ACTION POTENTIALS: SYNCHRONOUS BIOCHEMICAL DEPOLARIZATIONS ON A MILLISECOND TIME SCALE. Timothy J. Turner & Kathleen Dunlap, Department of Physiology, Tufts University School of Medicine, Boston, MA 02111.

We are interested in mechanisms of neurosecretion in central neurons, especially with regard to the properties and regulation of presynaptic Ca channels. We have used synaptosomes, a preparation of rat brain enriched in nerve terminals, to study ^{3}H-glutamate release on a subsecond time scale approaching the time course on which synaptic events are observed to occur. A major limitation of such studies is the requirement for chemical depolarizing stimuli such as elevated KCl solutions or Na channel alkaloids. Such stimuli are greatly prolonged relative to the action potential duration. We have developed a stimulation paradigm that theoretically can be as brief as several milliseconds through the concerted use of the Na channel activator batrachotoxin and the pore blocker tetrodotoxin. The synaptosomes are loaded with ^{3}H-glutamate and are suspended in a Na-free saline (N-methylglucamine substituted) that contains 5 μM BTX. This toxin causes activation of Na channels at resting potentials and blocks inactivation, so that at rest there is a population of Na channels that is persistently activated. The membrane permeability is dominated by Na^+, but it remains polarized since in the absence of external Na^+, E_{Na} is negative. When the terminals are superfused with a solution containing Na^+, the value for E_{Na} becomes positive and the membrane is depolarized in proportion to $[Na^+]_e$, resulting in Ca-dependent release of ^{3}H-glutamate. The duration of the depolarization can be limited by simultaneously superfusing with a solution containing Na^+ and TTX. As TTX binds to the Na channel it blocks the pore, allowing repolarization as K reclaims its role as the dominant permeant ion. Because TTX associates with its receptor in a first-order fashion, the rate of block is directly proportional to the [TTX]. Based on the kinetics of ^{3}H-TTX binding to the Na channel, the rate of block of Na channels is 30 $\mu M^{-1}\ s^{-1}$, predicting that the depolarization will last 10 ms at 10 μM TTX. Using this approach, we find that ^{3}H-glutamate release outlasts the predicted duration of the synthetic action potential. We will present data to compare the rate of ^{3}H-glutamate release, $^{45}Ca^{+2}$ uptake, and ^{14}C-guanidinium uptake to establish the relationship between the duration of presynaptic depolarization and neurosecretion. Our ultimate goal is to use this paradigm to study the regulation of neurosecretion at central synapses and determine whether mechanisms which modulate Ca currents in cell bodies also regulate neurotransmitter release from terminals.

T 108 CHARACTERIZATION OF ^{125}I-ω-CONOTOXIN BINDING SITES IN HUMAN NEUROBLASTOMA CELL LINES, G. Velicelebi, S. Simerson and D.Feldman, SIBIA, 505 Coast Blvd S., La Jolla, CA 92037

Three human neuroblastoma lines, IMR32, SHSH5Y, and SMS-SAN cells, were analyzed for specific binding of ^{125}I-ω-conotoxin (^{125}I-ω-CgTx) in the uninduced state and after induction for neuronal differentiation with either 10 μM retinoic acid (RA) or with a combination of 1mM dibutyryl cyclic AMP (Bt_2cAMP) and 2.5 μM 5-bromodeoxyuridine (5-BrdUr) for ten days. The number of ^{125}I-ω-CgTx binding sites (B_{max}) and the dissociation constant (Kd) were determined through Scatchard analysis of saturation binding data and compared for each cell line for the three sets of experimental conditions. No specific binding was detected in membranes prepared from uninduced IMR32 and SHSY5Y cells in contrast to SMS-SAN cells, where approximately 5000 sites were calculated per cell. Induction with either RA or a combination of Bt_2cAMP and 5-BrdUr affected the values of K_D and B_{max} in significantly different ways in each cell line. The results are summarized below:

Cell Line	Induction	K_d, pM	B_{max}, R/cell
SMS-SAN	none	35 ± 7	5312 ± 539
SMS-SAN	RA	24 ± 3	1494 ± 186
SMS-SAN	Bt_2cAMP+5-BrdUr	41 ± 4	7950 ± 150
SHSY5Y	none		not detected
SHSY5Y	RA	16 ± 1	2298 ± 138
SHSY5Y	Bt_2cAMP+5-BrdUr	115 ± 26	3040 ± 428
IMR32	none		not detected
IMR32	RA	44 ± 7	1070 ± 330
IMR32	Bt_2cAMP+5-BrdUr	22 ± 1	6428 ±1228

The results of ^{125}I-ω-CgTx binding studies were correlated with those from electrophysiological recordings of voltage-dependent calcium channels in the SMS-SAN cell line. Whereas uninduced SMS-SAN cells displayed small (10-60 pA) Ba^{2+} currents, cells that had been induced with Bt_2cAMP and 5-BrdUr exhibited 5-to-10 fold larger currents. RA induction had little or no effect on the magnitude of the Ba^{2+} current. Approximately 80% of the Ba^{2+} current was blocked by ^{125}I-ω-CgTx in either case. The effects of the two induction protocols on the electrophysiological properties of the Ba^{2+} current in these cell lines is being investigated further.

Receptor Structure and Function

T 200 CHRONIC ELEVATION OF PROTEIN KINASE A (PKA) ENHANCES $GABA_A$ RECEPTOR CURRENTS COMPOSED OF αβγ SUBUNITS AND NOT αβ SUBUNITS

T.P. Angelotti*, M.D. Uhler+, and R.L. Macdonald#@ Depts. of Pharmacology*, Biochemistry+, Neurology#, and Physiology@, University of Michigan Medical School, Ann Arbor, MI 48109.

The role of PKA in the steady-state expression and function of $GABA_A$ receptors was examined by studying recombinant receptors expressed in three cell lines which possessed various levels of activated kinase. These included the parental cell line, L929 (intermediate kinase), and two derivative cell lines, Cα12 (high kinase) and RAB10 (low kinase). Whole-cell and single-channel GABA-receptor currents were recorded 48 hrs post-transfection, from single cells transiently expressing various combinations of $GABA_A$ receptor subunit cDNAs. These included wild-type (WT) bovine α_1, β_1, or human γ_{2S} or mutant (MUT) bovine β_1 (Ser^{409} -->Ala^{409}), lacking the PKA consensus phosphorylation site.

In all cell types, expression of WT β_1 with α_1 and γ_{2S} subunit cDNAs produced similar GABA-receptor whole-cell current concentration-response curves with respect to Hill slope, EC_{50} value, and modulation by co-administered diazepam. Single channel current amplitudes also were not different between cell types. One difference between cell types was the overall magnitude of the GABA-receptor whole-cell currents; $GABA_A$ receptor currents expressed in Cα12 cells were 2 - 3 times larger than those expressed in L929 or RAB10 cells. Expressed receptors composed of α_1 and β_1 subunits had the expected pharmacology, yet receptor currents were not enhanced when expressed in Cα12 cells. Expression of the MUT β_1 subunit along with WT α_1 and γ_{2S} subunits produced functional receptors suggesting that the mutation did not prevent expression. Further characterization of the mutant β_1 subunit in Cα12 cells will be necessary to determine if the modulation of receptor currents by overexpression of PKA occurs via this site.

(This work was supported by a grant from the Lucille Markey Foundation)

T 201 EXPRESSION OF *CAENORHABDITIS ELEGANS* NEUROTRANSMITTER RECEPTORS IN *XENOPUS* OOCYTES. Joseph P. Arena, Ken K. Liu, Philip S. Paress, and Doris F. Cully. Dept. Biochemical Parasitology, Merck Sharp and Dohme Research Laboratories, P.O. Box 2000, Rahway, N.J. 07065-0900. The free-living nematode *C. elegans* is used as a model for differentiation, development, and morphology of simple nervous and muscular systems. The small size of *C. elegans* has precluded the electrophysiologic experiments needed to characterize the properties, pharmacology, and function of neurotransmitter receptors in this organism. As an alternate approach we used the surrogate expression system of *Xenopus laevis* oocytes to express, and study the properties of, *C. elegans* neurotransmitter receptors. Oocytes were injected with 50 ng of poly (A)+ RNA and membrane current at -80 mV was measured 2-3 days later. Glutamate activated an inward membrane current that desensitized in the continued presence of glutamate. Glutamate-receptor agonists quisqualate, kainate, and NMDA were inactive. The glutamate-sensitive current had a reversal potential of -22 mV, and the reversal potential was dependent on external chloride. The chloride channel blockers flufenamate and picrotoxin inhibited glutamate-sensitive current. Ibotenate also activated a picrotoxin-sensitive chloride current. Ibotenate was inactive when current was previously desensitized with glutamate, and the responses to low concentrations of glutamate and ibotenate were additive. Glutamate-sensitive current was also activated with the anthelmintic/insecticide, avermectin. The glutamate/ibotenate/avermectin responses were insensitive to intracellular injection of EGTA. However, in oocytes from approximately 60 % of the donors glutamate also activated an oscillatory component of current that was EGTA-sensitive. In addition, acetylcholine activated an oscillatory current component that was sensitive to atropine and intracellular EGTA. We conclude that *C. elegans* RNA encodes for a glutamate receptor/chloride channel complex. In addition, the data suggest expression of muscarinic acetylcholine receptors, and a second glutamate receptor linked to a rise in intracellular calcium.

T 202 CHARACTERIZATION OF A THIRD GLUR 4 ISOFORM, A. Buonanno*, V. Gallo, L.W. Upson, W.P. Hayes, C.A. Winters and L. Vyklicky Jr., Laboratory of Developmental Neurobiology, NIH, Bethesda, MD 20892

The flip and flop isoforms of glutamate receptor (GluR) subunits are generated by differential RNA splicing (Sommer *et. al.*, Science 249, 1583, 1990). We have isolated cDNAs coding for a third isoform of the GluR-4 subunit which differ from the previously described flip and flop variants in the C-terminal 36 amino acids, this isoform was named GluR-4c. The novel C-terminus was found in GluR-4-flip and -flop cDNAs, and the sequence has been conserved through evolution since it is homologous to that found in the GluR-2 and GluR-3 subunits. *Xenopus* oocytes injected with transcripts synthesized from a GluR-4c cDNA express functional homooligomeric channels activated by glutamate, kainate, quisqualate and AMPA, and blocked by CNQX. Responses to quisqualate or AMPA are potentiated by either aniracetam or wheat germ agglutinin. The I/V relationship, recorded using 100 μM kainate, shows inward rectification at holding potentials between -60 to -10 mV. Northern blot analysis using the cDNA probe revealed that GluR-4 transcripts (6.2, 4.2 and 3.0 kb) are predominantly expressed in cerebellum and their levels increase maximally between P1 and P8, a time that coincides with proliferation of granule cells. Cultured cerebellar granule cells also express the 3 transcripts, whereas astrocytes fail to accumulate detectable levels of the 3.0 kb RNAs. Using oligonucleotide probes specific for either the flip, flop or the GluR-4c C-terminus, we found that these probes hybridize selectively to the 6.2 and 4.2 kb transcripts. The same probes were also used for *in situ* hybridization histochemistry to study the cellular distribution of GluR-4 transcripts during cerebellar development. We found in sections from P1 and P8 cerebella that granule cells are labelled by the flop and C-terminus probes in the external germinal layer prior to their migration into the internal granule cell layer; labelling was not detected with the GluR-flip probe. Differential expression of GluR-4 transcripts was also observed in cerebella from adult mice where we found that the 3 probes labelled the Bergmann glial cells, whereas only the flop and C-terminus probes labelled strongly the granule cell layer.

T 203 CLONING, CHARACTERIZATION, AND EXPRESSION OF cDNAs CODING FOR HUMAN GLUTAMATE RECEPTORS. N.G. Mayne, Y. M. Snyder, R.L. Sharp, and J. P. Burnett, Lilly Research Laboratories, Indianapolis, IN 46285.

cDNAs coding for members of several different types of rat glutamate receptors have been cloned (Hollman et al. Nature 342,643,1989; Keinanen et al. Science 249,556,1990; Egebjerg at al. Nature 351, 745, 1991; Masu et al. Nature 349, 760,1991; Moriyoshi et al. Nature 354, 31,1991) Among these are proteins which can be expressed in cell culture and/or oocytes to produce receptors which correspond pharmacologically to all of the major types of glutamate receptors: AMPA- , kainate, NMDA- and metabotropic- type receptors. Hood et al. (PNAS 88 7557,1991) have described one member of the human glutamate receptor family of the AMPA type.

We have cloned and characterized cDNAs for several members of the human glutamate receptor family which are of the AMPA and metabotropic types . Analysis of these demonstrates that , like the rat, multiple subtypes of these receptor proteins exist in humans. Some of these are closely related to the previously described rat clones, but others are significantly different.

These data confirm and extend the evidence for diversity of excitatory amino acid receptors in the brain.

T 204 DIFFERENCES IN THE ACTION OF NONCOMPETITIVE INHIBITORS ON MUSCLE AND ELECTRIC ORGAN ACETYLCHOLINE RECEPTORS, V.A. Eterovic[1], L. Li[2], P. A. Ferchmin[1], Y.H. Lee[2], R.M. Hann[1], A.D. Rodriguez[3] and M.G. McNamee[2], [1]Dept. of Biochemistry, Univ. Central del Caribe, Bayamon, PR 00960, [2]Dept. of Biochemistry and Biophysics, Univ. of California, Davis, CA 95616, and [3]Dept. of Chemistry, Univ. of Puerto Rico, Rio Piedras, PR 00931

Muscle-type acetylcholine receptor (AChR) from BC3H1 cells and the receptor from electric organ of *Torpedo californica* were expressed in *Xenopus laevis* oocytes and studied with a two-electrode whole-cell voltage clamp. The positively-charged channel blockers, phencyclidine (PCP) and tetracaine (TTC), displayed a much lower potency on muscle receptor than on the electric organ receptor. The IC_{50} for both blockers at the electrocyte receptor was close to 1 μM at -60 mV, and lower at more hyperpolarized voltages. In contrast, with muscle receptor IC_{50}'s varied between 20 and 70 μM at -60 or -80 mV. Eupalmerin acetate (EUAC), a new, uncharged noncompetitive inhibitor that displaces [^{3}H]PCP from its high-affinity binding site, inhibited both receptors with similar potency: IC_{50} 4.9 μM and 6.4 μM for electrocyte and muscle receptors, respectively. Another positively-charged channel blocker, Mg^{++} also inhibited both receptors with similar potency (IC_{50} 0.5-1.0 mM). Unlike PCP, Mg^{++} block was voltage-independent (-100 to -20 mV). Furthermore, the degree of inhibition by 0.5 μM PCP was the same at 1 or 5 mM Mg^{++}.

Noncompetitive inhibitors of the PCP and TTC families are believed to interact with a polar subsite and a hydrophobic subsite located on the M2 helices of the five receptor subunits. The uncharged inhibitor EUAC would be expected to interact mostly with the hydrophobic subsite. Our observations with these three inhibitors suggest that the polar subsite is less polar in muscle receptor, while the hydrophobic subsite does not differ significantly. This is supported by comparison of amino acid sequences forming the proposed noncompetitive inhibitor site. On the other hand, Mg^{++} seems to bind to sites somewhat distant from the membrane bilayer, and not to the PCP binding site.

T 205 A METABOTROPIC NICOTINIC AcCho RECEPTOR EXPRESSED IN MOUSE MYOTUBES, Fabrizio Eusebi, Francesca Grassi, Elisabetta Mattei, Anna M. Mileo and Aldo Giovannelli. Laboratorio di Biofisica, CRS Istituto Regina Elena Via delle Messi d'Oro 156 - 00158 Rome, Italy

Electrophysiological, biochemical and Ca^{2+} imaging studies of cultured mouse myotubes were used to investigate whether the neurotransmitter acetylcholine (AcCho) causes a rise in intracellular Ca^{2+} level through activation of a second messenger system. Bath applications of AcCho to myotubes (i) elicited a significant membrane current even in a sodium-free Ca^{2+}-medium, when the current was carried mainly by calcium ions; (ii) caused a rapid and transient cytosolic accumulation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) ; (iii) evoked a conspicuous a-bungarotoxin-sensitive long-lasting intracellular Ca^{2+} enhancement even in the presence of cadmium; (iv) transiently increased the intracellular calcium content when cells were equilibrated in a Ca^{2+}-free, atropine-containing medium. We propose that, in addition to acting on ionotropic nicotinic receptor, AcCho induces an intracellular calcium mobilization by activating a metabotropic nicotinic receptor that could be either the same or a different molecule in respect to the AcCho ionotropic receptor-channel.

This work was supported partially by FIDIA and by CNR.

T 206 SITE-SPECIFIC MUTAGENESIS OF THE CAMP-DEPENDENT PHOSPHORYLATION SITES OF THE NICOTINIC ACETYLCHOLINE RECEPTOR, Peter W. Hoffman, Arippa Ravindran and Richard L. Huganir, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Department of Neuroscience, Baltimore, MD 21205. The nicotinic acetylcholine receptor (nAChR) is a pentameric complex of four types of subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. All of the subunits have been shown to contain sites of protein phosphorylation located in the major intracellular loop between the third and fourth transmembrane α-helixes. Phosphorylation sites for protein kinase C (on the α and δ subunits), cAMP-dependent protein kinase (on the γ and δ subunits), and a protein tyrosine kinase (on the β, γ, and δ subunits) have been described in the nAChR of Torpedo. We have studied the phosphorylation of the nAChR by expression of wildtype and mutant receptors in Xenopus oocytes. When wildtype nAChR is expressed in oocytes the receptor is phosphorylated solely at the cAMP-dependent protein kinase phosphorylation sites. This phosphorylation is not significantly enhanced by incubation with forskolin and cAMP analogs or when the catalytic subunit of cAMP-dependent protein kinase is co-expressed with the nAChR. These results demonstrate that the basal level of cAMP-dependent protein kinase in oocytes is sufficient to phosphorylate the nAChR to completion. Employing in vitro mutagenesis we have mapped the sites of phosphorylation and have found that two serine residues on the γ and δ subunits are phosphorylated. We have also expressed mutant receptors in which all known sites of phosphorylation have been removed. These mutant receptors are not phosphorylated and assemble and express normally. Using patch-clamp and rapid perfusion techniques, we have analyzed the desensitization of wildtype and mutant channels expressed in Xenopus oocytes. Out-side out patches were placed in the path of a rapid perfusion system in which the solution superfusing the patch can be changed within 1-3 ms. Deletion of the phosphorylation sites slowed the rate of desensitization of the receptor at all ACh concentrations tested. Moreover, mutation of the phosphorylated serine residues to a negatively charged amino acid such as apartatic acid appeared to mimic the phosphorylation producing a mutant nAChR with the desensitization rate of the phosphorylated wildtype receptor. These results provide additional evidence that cAMP-dependent phosphorylation increases the desensitization rate of the nAChR and that it may play an important role in modulating synaptic function.

T 207 THE *TRK* FAMILY OF NEUROTROPHIN RECEPTORS
Fabienne Lamballe, Rüdiger Klein, Peter Tapley, Shuqian Jing, Venkata Nanduri and Mariano Barbacid, Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543.
The NGF family of neurotrophic factors (NGF, BDNF, NT-3 and NT-4) recognizes at least two distinct classes of cell surface receptors. One of these receptors is a cysteine-rich glycoprotein, $p75^{LNGFR}$, which contains a short cytoplasmic domain devoid of informative structural motifs. $p75^{LNGFR}$ binds each of the above neurotrophins with the same low affinity ($K_d \sim 10^{-9}$ M) and does not mediate most of their biological responses. Recent studies have indicated that the NGF family of neurotrophins also binds to the *trk* family of tyrosine protein kinase receptors. The *trk* gene was first identified as the normal allele of an oncogene present in a human colon carcinoma biopsy. The related *trk*B and *trk*C genes were subsequently isolated by screening brain cDNA libraries at low stringency. Unlike $p75^{LNGFR}$, these *trk* receptors show binding specificity. Whereas NGF only binds to the product of the *trk* proto-oncogene, $gp140^{trk}$, BDNF specifically recognizes $gp145^{trkB}$, the tyrosine protein kinase receptor encoded by the *trk*B locus. The *trk*C gene product, $gp145^{trkC}$, appears to be the primary receptor for NT-3, a neurotrophin that can also interact with $gp140^{trk}$ and $gp145^{trkB}$. Recent studies regarding the binding specifity of the recently identified NT-4 protein will be presented.
The interaction between these neurotrophins and their cognate *trk* receptors elicits a variety of biological responses including (i) phosphorylation of the receptor in tyrosine residues; (ii) induction of c-Fos expression; (iii) induction of DNA synthesis (iv) survival and neurite outgrowth in PC12 cells and (v) morphologic transformation of NIH3T3 cells. None of these biological responses requires the presence of $p75^{LNGFR}$. Moreover, they are not affected by co-expression of this low affinity receptor. Finally, each of the *trk* tyrosine kinases binds their cognate neurotrophin(s) with high affinity ($K_d \sim 10-11$ M), either in the presence or absence of $p75^{LNGFR}$. These findings strongly suggest that the *trk* family of tyrosine protein kinases are the high affinity receptors that mediate the known neurotrophic properties of the NGF family of neurotrophins.

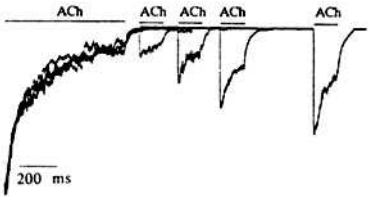
T 208 PROMISCUITY OF THE CLONED MOUSE 5-HT_{1C} RECEPTOR FOR G PROTEINS AS SHOWN BY EFFECTS ON ADENYLATE CYCLASE AND PHOSPHOLIPASE C, V.L. Lucaites[1], D.L. Nelson[1], L. Yu[2] and M. Baez[1], [1]Lilly Res. Labs., Lilly Corporate Center, and [2]Dept. Med. & Mol. Gen., I.U. School of Med., Indpls, IN 46285
The mouse 5-HT_{1C} and rat 5-HT_2 cloned receptors were transfected into Syrian hamster tumor cells (AV12-664). These two receptors define a subclass of 5-HT receptors and couple to phospholipase C. Unexpectedly, the cloned 5-HT_{1C} receptor was also found to inhibit the formation of cAMP. The effects of 5-HT were dose-dependent, producing EC_{50} values of 0.49±0.07 nM and 47.6±11.5 nM for stimulating IP formation and inhibiting cAMP formation respectively. Thus, 5-HT had relatively potent effects on both second messenger systems. In contrast to the 5-HT_{1C} receptor, the closely related 5-HT_2 receptor stimulated inositol phosphate (IP) formation, but had no effect on cAMP production. Pretreatment with as little as 20 ng/ml pertussis toxin (PTX) in cells expressing the 5-HT_{1C} receptor reduced the ability of 5-HT to inhibit cAMP formation by at least 70%. However, concentrations of PTX ranging from 20 to 1000 ng/ml had no significant effect on 5-HT-stimulated IP formation. This suggested that the 5-HT_{1C} receptor was producing its effects by direct coupling to two different G proteins rather than by crosstalk. After PTX inhibition of the inhibitory G protein, a small, but reproducible, 5-HT_{1C} receptor-mediated stimulation in cAMP formation was revealed. Although the 5-HT_{1C} and 5-HT_2 receptors are structurally similar, there is little homology within the third intracellular loop. This loop, which has been implicated in G protein coupling, could therefore account for the differences in the ability of these two receptors to interact with different G proteins. Future studies of the 5-HT_{1C} receptor may help to dissect the structural features that determine coupling to different G proteins.

T 209 MAPPING OF THE LIGAND BINDING SITE OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS USING CHIMERIC SUBUNITS, Charles W. Luetje, Marietta Piattoni and Jim Patrick, Dept. of Molecular and Cellular Pharmacology, University of Miami, Miami, FL 33101 and Division of Neuroscience, Baylor College of Medicine, Houston TX 77030.
Nicotinic acetylcholine receptors (nAChR) are found at the neuromuscular junction and throughout the nervous system. These receptors are formed by various combinations of homologous subunits, yielding a family of acetylcholine (ACh) gated ion channels with diverse physiological and pharmacological properties. While several recent affinity labelling studies have identified amino acid residues which may form portions of the ligand binding site common to all nAChR, we have attempted to identify residues responsible for pharmacological diversity among nAChR. We constructed a series of chimeric receptor subunits composed of portions of two pharmacologically dissimilar neuronal nAChR subunits, α2 and α3. Receptors formed in *Xenopus* oocytes by α2, in combination with β2, are insensitive to blockade by neuronal bungarotoxin (NBT) (IC50>1μM), and are more sensitive to nicotine than to ACh. In contrast, $\alpha3\beta2$ is highly sensitive to NBT blockade (IC50<10nM), and is less sensitive to nicotine than to ACh. Analysis of receptors formed by chimeric α subunits, in combination with β2, allowed us to map the location of amino acid residues responsible for this pharmacological diversity. Residues responsible for the difference in NBT sensitivity were localized to regions 84-121, 121-181 and 195-215. Residues responsible for the differences in agonist sensitivity were localized to regions 1-84 and 195-215. Within region 195-215, we used site-directed mutagenesis to identify the residue at position 198 (gln in α3, pro in α2) as important in determining agonist and antagonist sensitivity.

T 210 DESENSITIZATION OF NICOTINIC ACH RECEPTORS IS ALTERED FOLLOWING GAMMA-EPSILON SUBUNIT SUBSTITUTION, David Naranjo & Paul Brehm, Department of Neurobiology, SUNY at Stony Brook, Stony Brook, NY 11794

We utilized fast perfusion of ACh to outside-out membrane patches derived from oocytes in which either mouse αβδγ or αβδε ACh receptors were expressed. Rapid jumps in ACh concentration from 0 to 100 μM (within < 1msec) resulted in currents which corresponded to the activation of as few as 1 channel in some patches to >100 channels in others (see Figure). The macroscopic and single channel ensemble currents from αβδγ receptors desensitize with at least two time constants (40ms & 80ms) which are voltage-independent between -80 mV and +40 mV. Desensitization is concentration-dependent with 8 fold increases in half-time of decay as the concentration is decreased from 100 to 1 μM. The time constants for channel desensitization exceed those for channel closure (7-15 msec) measured after jumps to ACh-free solution. Half-recovery from desensitization occurs within 500msec for αβδγ receptors but with repeated application of ACh an incomplete recovery is observed corresponding to channel 'drop out'. Analysis of patches containing a single αβδγ channel show at least two kinetic burst modes, one with a 0.9 Po (open probability) and a second with 0.5 Po. This indicates that a single protein is capable of switching between two modes of operation.

Comparisons between αβδγ and αβδε receptors show that the latter desensitize 10 times faster as well as 'drop out' faster. Single αβδε receptor currents exhibit 'flicker' behavior at 100 μM ACh indicating a fast channel block at this concentration. This behavior was not observed for αβδγ. Also, mean open times are reduced and conductance is increased following substitution of gamma by epsilon subunit. In summary, we conclude that substitution of the gamma subunit by epsilon leads to faster desensitization and more effective block of the ACh receptor channel by ACh.



T 211 TIME-DEPENDENT CHANGES IN THE FUNCTIONAL PROPERTIES OF ACETYLCHOLINE RECEPTORS IN A MOUSE MUSCLE CELL LINE, Dawn Shepherd and Paul Brehm, Department of Neurobiology and Behavior, SUNY at Stony Brook, Stony Brook, NY 11794.

Extensive changes in functional properties of acetylcholine receptors (AchRs) have been shown to occur during development of mammalian skeletal muscle *in-vivo* and amphibian skeletal muscle both *in-vivo* and *in-vitro*. Aneural mammalian primary muscle cultures undergo only limited changes in AchR properties during development in culture, but we have found extensive time-dependent changes using a fusing mouse muscle cell line, C2. AchRs were detected using single-channel recordings on cell-attached patches, with pipette [Ach] of 100-300 nM or 10-20 μM. Channel openings were detected from 2-3 days following myotube formation, with the major channel class having mean open time of 12-15 ms, and single-channel conductance of 50 pS. Ach-activated channels ranging in conductance from 12-40 pS, and with mean open times of greater than 10 ms also began to be detected at this time. Seven to ten days following cell fusion, channels were detected having 50 pS conductance, but mean open time of only 5-6 ms. This was concurrent with the first appearance of "adult" type AchRs, of 70 pS conductance, and mean channel open time of 3 ms. Channel openings by both the shorter mean open time 50 pS channel and the 70 pS channel were increasingly common with longer times after myotube formation. In addition, the proportion of openings by the 70 pS channel increased with time in culture, representing 40-50% of channel openings two weeks after fusion.

We are now using desensitizing concentrations of acetylcholine (10-20μM) to distinguish between the openings of long and short open time 50 pS channels. Open probability (P_o) measured within bursts shows marked differences between the short and long open time 50 pS channels. We are attempting to determine whether this reflects a shift in kinetic modes of a single channel type or a difference in subunit composition between two different channels. Comparisons of P_o for 50pS and 70 pS channels indicate that the P_o characteristic of the short open time 50 pS channel resembles that measured for the adult type 70pS channel. This finding suggests that the short open time 50 pS channel may represent an intermediate between the immature and adult phenotypes, sharing conductance properties with the "embryonic" 50 pS channel, and kinetic properties with the "adult" 70 pS channel.

T 212 SEQUENCES ON THE N-TERMINUS OF ACH RECEPTOR SUBUNITS REGULATE THEIR ASSEMBLY, Katumi Sumikawa, Department of Psychobiology, University of California, Irvine, CA 92717 - 4550

A functional nicotinic acetylcholine receptor (AChR) consists of five subunits of four different types arranged as $\alpha 2, \beta, \gamma$, and δ. Each of the four subunits is encoded by a separate mRNA. The synthesis of functional AChRs therefore involves complex processes including assembly of different types of subunits in a particular order. It was thought that assembly of subunits was not directed by particular sequences on individual subunits, but was simply dependent on the correct folding of subunits. Recent findings suggest that assembly of AChR subunits appears to occur in the endoplasmic reticulum without requiring N-glycosylation and disulfide bond formation, both thought to be important for proper folding. These findings indicate that correct folding may not be required for assembly, although it is necessary for their transport to the plasma membrane. In order to search for "assembly signals" on the subunit of AChRs, mutant α subunits of *Torpedo* AChR were constructed and expressed in *Xenopus* oocytes together with other normal subunits. I have found that chimeric α subunits, consisting of the N-terminal extracellular domain of the AChR α subunit, followed either by the hydrophobic transmembrane segments of $GABA_A$ receptor or glutamate receptor subunits, were still recognized as the AChR subunit and associated with co-expressed other normal AChR subunits. Furthermore, a truncated α subunit, consisting of about one half of the N-terminal extracellular domain (amino acid residues 1-128) without a conserved N-glycosylation site and disulfide bond between Cys-128 and Cys-142, was still able to associate with other normal subunits, suggesting that this part of the N-terminal extracellular domain contains "assembly signals".

T 213 THREE DOMAINS OF THE α SUBUNIT OF THE GLYCINE RECEPTOR FORM THE STRYCHNINE BINDING SITE.

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The inhibitory glycine receptor (GlyR) is a member of the ligand-gated ion channel receptor superfamily. Glycine activation of the receptor is competitively antagonized by the convulsant alkaloid, strychnine. Using site-directed mutagenesis we have identified several amino acid residues on the α1 subunit of the GlyR that are involved in the formation of the strychnine binding site. These residues were identified using transient expression of each of the mutated cDNAs in mammalian (293) cells and examination of [^{3}H]strychnine binding, glycine displacement of bound [^{3}H]strychnine and the electrophysiological responses to the application of glycine and strychnine. These studies revealed that residues from three separate domains within the extracellular region of the α1 subunit contribute to the formation of the binding site for strychnine. The first domain includes aspartate 148 within the disulfide loop found in all subunits of the ligand-gated ion channel superfamily. The second domain includes glycine 160 and tyrosine 161, and the third domain includes lysine 200 and tyrosine 202 and is close to the first transmembrane region. These results, combined with analyses of other ligand-gated ion channel receptors, suggest a conserved tertiary structure and a common mechanism for competitive antagonism in this receptor superfamily.

T 214 HIGH CALCIUM PERMEABILITY AND CALCIUM MODULATION OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS, Steven Vernino, Mariano Amador and John A. Dani, Division of Neuroscience and Department of Physiology, Baylor College of Medicine, Houston, TX, 77030

Two properties were found to distinguish neuronal from muscle nicotinic acetylcholine receptors (nAChRs). First, neuronal nAChRs have a greater Ca^{++} permeability. The Ca^{++} flux through neuronal nAChRs activates a Ca^{++}-dependent Cl^- conductance, the Ca^{++} to Cs^+ permeability ratio is 7 times greater for neuronal than for muscle nAChRs, and neuronal nAChRs mediate a larger intracellular Ca^{++} increase per unit current than muscle receptors.

A second difference between the receptor types is that neuronal nAChRs are potently modulated by physiological levels of external Ca^{++}. Neuronal nAChR currents are enhanced specifically by extracellular Ca^{++} in a dose-dependent manner. This modulation is independent of the receptor's ability to flux calcium.

The results indicate that changes in extracellular Ca^{++} modulate the neuronal receptor and may modulate nicotinic synapses in the nervous system. In addition, activation of neuronal nAChRs produces significant Ca^{++} influx that could be an important intracellular signal.

T 215 POTENTIATION OF THE GABA-A RECEPTOR BY ETHANOL REQUIRES PHOSPHORYLATION OF SERINE 343 OF THE γ2L SUBUNIT.

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γ-aminobutyric acid ($GABA_A$) receptors are GABA gated chloride channels which are located at inhibitory synapses in the mammalian CNS and the function of which can be modulated by a number of agents including benzodiazepines, barbiturates, neurosteroids and ethanol. Molecular cloning approaches have revealed that a family of GABA-A receptor subunits exist (α1-6, β1-3, γ1-3, δ, σ) which presumably coassemble to form numerous receptor subtypes. Further diversity is created by alternative splicing, leading to the formation of 2 forms of the γ2 subunit (γ2S and γ2L) which differ by the presence of an extra 8 amino acids in the putative large cytoplasmic loop region of γ2L.

Recently it has been shown that, when coexpressed in *Xenopus* oocytes with an α and a β subunit, γ2L but not γ2S, confers ethanol sensitivity upon the receptors. In this study we have used *Xenopus* oocyte expression of recombinant bovine $GABA_A$ receptor and site directed mutagenesis to further dissect the molecular basis of ethanol potentiation. The extra 8 amino acids found in γ2L (LLRMFSFK) contains a protein kinase C consensus phosphorylation site at the serine residue. Deletion of FSFK and RMFSFK sequences prevented the ethanol potentiation of α1β1γ2L receptors, while having no effect upon the potentiation of receptors by flunitrazepam. A similar effect was found by mutating the serine, the putative phosphate acceptor, to an alanine, and by mutating the lysine (required for the consensus phosphorylation site) to a leucine. Additionally, overnight incubation of α1β1γ2L injected oocytes in the protein kinase inhibitor H7 abolished the ethanol potentiation of these receptors.

These data strongly suggest that phosphorylation at residue serine 343 of γ2L is a requirement for ethanol potentiation of GABA-A receptors.

Synapse Formation and Function

T 300 DEVELOPMENTAL REGULATION OF AN INNERVATION-DEPENDENT, JUNCTION-SPECIFIC MUSCLE PROTEIN, Stephanie H. Astrow and Wesley J. Thompson, Department of Zoology, University of Texas, Austin, TX 78712.

We have generated a monoclonal antibody (mAb 3G2) which recognizes a protein at the neuromuscular junction in adult rats. The antigen appears to be unique to muscle, as immunohistochemical staining is not detectable in other tissues including smooth muscle, cardiac muscle, spinal cord or autonomic ganglia. The epitope for mAb 3G2 is intracellular, as labeling of intact muscle fibers requires permeabilization. MAb 3G2 immunoreactivity lies beneath the acetylcholine-rich synaptic gutters, but is also present in sarcoplasmic protrusions located between, or at the edges of, the synaptic gutters. Deeper within fibers, mAb 3G2 immunoreactivity surrounds sole plate nuclei and is associated with perijunctional Z-discs. At birth, mAb 3G2 immunoreactivity is not restricted to neuromuscular junctions, but is widely distributed within muscle fibers in regions not occupied by myofibrils or nuclei. Some small (presumably secondary) muscle fibers contain immunoreactivity along their entire length, while larger fibers have patches of immunoreactivity in which longitudinal filaments or transverse bands with a sarcomeric periodicity are seen. Localization of mAb 3G2 immunoreactivity to neuromuscular junctions begins postnatally, several days after acetylcholine receptor clusters can be detected in hindlimb musculature. The establishment of this synaptic localization is neurally regulated, as neonatal denervation prevents its occurrence. In adults, denervation results in a loss of synaptic immunoreactivity which returns upon reinnervation. MAb 3G2 is also found at the myotendinous junction (its localization here is innervation-independent) and in cultured rat myotubes. MAb 3G2 recognizes a 41 kD protein on immunoblots of extracts of newborn muscle. The protein is highly insoluble and present in low abundance. Based on its distribution within muscle fibers, its developmental and neural regulation, and its molecular weight, the 41 kD protein can be distinguished from other known postsynaptic proteins. The solubility characteristics of the 41 kD protein taken together with its association with Z discs at neuromuscular and myotendinous junctions suggest that the 41 kD protein may be a cytoskeletal protein. Furthermore, its neural dependence and developmental regulation suggest that it may participate in synaptic stabilization, perhaps as a member of a complex of proteins that tether the nerve terminal to the junctional site in the postsynaptic membrane.

T 301 ACTIVITY-DEPENDENT METABOLIC STABILITY OF END-PLATE ACETYLCHOLINE RECEPTORS IS MEDIATED BY CALCIUM INFLUX AND BY PROTEIN PHOSPHORYLATION.

H.R. Brenner*, S. Rotzler* and P. Caroni§, *Department of Physiology, University of Basel, CH-4051 Basel, and §Friedrich Miescher Institute, CH-4002 Basel, Switzerland.

Upon denervation, the metabolic half-life $t_{1/2}$ of endplate acetylcholine receptors (AChR's) decreases, but AChR's can be restabilized by direct stimulation of denervated muscle in culture (Rotzler et al., Nature 349: 337-339, 1991). We have now attempted to characterize the signalling pathway linking muscle activity and AChR stabilization. Muscles were organ cultured after 17-40 days of denervation *in vivo*, when $t_{1/2}$ of the synaptic AChR's had declined to about 2-3 days, and were then subject, in organ culture, to stimulation and/or pharmacological treatments affecting intracellular signalling systems. After labeling AChR's with ^{125}I-α-bungarotoxin, the apparent half-lives $t_{1/2}$ of AChR's in the endplate membrane were estimated, by quantitative autoradiography, from the loss of radioactivity as a function of culturing time. We found that metabolic stabilization of AChR's was dependent on the pattern of muscle activity, with short, high frequency stimulation being more effective than long, low frequency stimulation; stimulation-induced AChR stabilization was prevented by the presence of Ca^{++} channel blockers (+)PN200-110 or of D600. The Ca^{++} channel activator (+)SDZ202-791 when applied in culture medium containing 15 mM K^+ induced AChR stability in the absence of muscle activity; in agreement with results by Shyng et al. (Neuron 6: 469-475, 1991), AChR stabilization could also be induced in the absence of muscle activity by treatment of muscles with membrane permeant cAMP-analogue but not with cGMP; cAMP levels in muscle were not affected by muscle activity, however; activity-dependent AChR stabilization was not prevented by cycloheximide, suggesting its independence of protein synthesis; blockade of protein phosphatase activity by treatment with okadaic acid reduced the amount of activity required to produce AChR stabilization; treatment of unstimulated muscles with the phorbol ester TPA had no effect on AChR stability, and stimulation-induced AChR stabilization was not prevented by stauroporine; both activity-dependent Ca^{++}-influx and cAMP treatment induce the phosphorylation of myosin light chain isoforms; no phosphorylation of 43K protein nor of AChR subunits could be resolved by muscle stimulation causing the stabilization of synaptic AChR's. These findings are consistent with the notion that muscle activity causes AChR stabilization via the activation of calcium-dependent protein phosphorylation reactions.

T 302 COMPLIANCE OF HIPPOCAMPAL NEURONS TO PATTERNED SUBSTRATE NETWORKS, Gregory J. Brewer*, Joseph M. Corey+ and Bruce C. Wheeler+, *Southern Illinois University School of Medicine, Springfield, IL 62704 and +University of Illinois, Urbana, IL 61801

In vitro techniques for the study of synaptogenesis and the specificity of synaptic connections is difficult because of the large number of neurons and the multitude of synapses they form with other cells surrounding them in many directions. A reduction in the number of neurons and connections and controlled direction of process growth would produce simpler networks more conducive to monitoring synapse formation and patterns of synaptic specificity. We have established a method for patterning rat hippocampal neurons at densities low enough to observe individual contacts between neurons. Growth of these neurons at these low densities was made possible by use of a serum-free medium and reduced oxygen (Brewer and Cotman, 1989). Substrates were patterned by etching polylysine coated glass substrates with a UV laser through a quartz mask or by photolithography with aminosilane chemistry to create linewidths of 3, 5 and 10 μm, with nodes of 5, 10 and 20 μm diameter separated either 80, 120 or 160 μm. Not only did somae strongly prefer the unablated polylysine or aminosilane areas, but they also migrated to loci where the local area of adhesion was higher. Maximum migration to nodes of >88% occurred for a combination of 5 μm pathwidth, 20 μm node diameter and 80 μm pathlength. Daily observations indicated active migration to larger adhesive areas, which explains the differential compliance. These substrate networks should facilitate electrical and morphological studies of synaptogenesis.

T 303 COMPARISON BETWEEN THE EXPRESSION OF ACETYLCHOLINE RECEPTOR α-SUBUNIT GENE AND MYOGENIC GENES DURING NEUROMUSCULAR JUNCTION FORMATION.

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During vertebrate muscle development, the expression of the acetylcholine receptor (AChR) genes becomes progressively restricted to junctional nuclei. We investigate the molecular mechanisms responsible for this compartimentalized gene expression by examining the distribution of the mRNAs coding for two of the regulatory factors of the chicken α-subunit gene : the chicken MyoD1 (CMD1) and myogenin. At early stages of chicken muscle development, CMD1 mRNA is detected in the somites just before the apparition of α-subunit mRNA ; myogenin mRNA, contrary to what was observed in the mouse, is detected slightly later. At embryonic day 14 (ED14), CMD1 and myogenin mRNAs are expressed like the α–subunit mRNA along the entire length of the muscle fibers. Then, as α-subunit mRNA disappears from extrajunctional areas, myogenin mRNA decreases and becomes undetectable at ED19 while only a few clusters of CMD1 mRNA remain at the same stage. These results reinforce the hypothesis that the decrease in myogenin and CMD1 mRNA could play a role in the repression of AChR genes in extrajunctional areas. The mechanisms responsible for the persistence of AChR genes at the junctional nuclei remain to be identified.

T 304 TARGETING OF THE 43 KD PROTEIN TO THE POSTSYNAPTIC MEMBRANE AND ITS ROLE IN AChR AGGREGATION. Emma K. Dutton, James E. Yeadon and Steven J. Burden, Department of Biology, MIT, Cambridge, MA 02139.

One of the earliest events of neuromuscular synapse formation is the accumulation or aggregation of acetylcholine receptors (AChRs) to the nerve-muscle contact site. AChRs, initially evenly distributed throughout the myofiber, become highly concentrated and localized in the postsynaptic membrane. This accumulation of AChRs at the synaptic site is due to a posttranslational mechanism that triggers a redistribution of AChRs already present in the myofiber membrane at the time of nerve-muscle contact. In addition, associated with AChR aggregates is a submembrane cytoskeletal protein of M_r 43,000 (43 kd protein), and recent evidence suggests that the 43 kd protein is involved in the mechanism of AChR aggregation (Phillips et. al., *Science* 251, 1991; Froehner et. al., *Neuron* 5, 1990). We have developed a system to investigate targeting of the 43 kd protein to the postsynaptic membrane and the role of the 43 kd protein in AChR aggregation. A plasmid directing the expression of a full-length mouse 43 kd protein tagged with an immunoreactive epitope from influenza heamagglutinin has been transfected into primary myoblast/fibroblast cultures. We have demonstrated that the tagged 43 kd protein is targeted to the membrane and associated with endogenous AChR aggregates in primary rat myotubes. In contrast, transfected myoblasts/fibroblasts show a different phenotype. This system is being used to determine the region of the 43 kd protein that is necessary to target the protein to the membrane and to investigate the role of the 43 kd protein in AChR aggregation.

T 305 Isolation and Characterization of cDNAs Encoding Mouse 58K Protein. T. M. Dwyer, M. E. Adams, and S. C. Froehner. Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755-3844.

The 58K protein (M_r 58,000) was first identified in *Torpedo* electric organ and subsequently shown to be concentrated at the postsynaptic membrane of the mammalian neuromuscular junction. It is a cytoplasmic protein peripherally associated with the membrane and may be involved in clustering acetylcholine receptors. The 58K protein has recently been shown to associate with dystrophin and the 87K postsynaptic protein (Butler, M.H., K. Douville, A.A. Murnane, N.R. Kramarcy, J.B. Cohen, R. Sealock, and S.C. Froehner. *J. Biol. Chem.* submitted). To obtain the primary sequence of the mouse 58K protein a cDNA encoding a portion of *Torpedo* 58K (Butler, M.H., A.A. Murnane, S.C. Froehner. 1989. *J. Cell Biol.* 109:143a) was used to screen mouse cDNA libraries. Three overlapping cDNAs of a combined length of 3.3kb were isolated: m12a (1.8kb), m6a (1.4kb), and b5a (1.0kb). m12a is almost entirely 3' untranslated sequence but does not contain a polyadenylation signal sequence. Together b5a and m6a contain a single open reading frame of 1.7kb that encodes > 90% of the expected mouse 58K protein. The combined nucleotide sequence shows 54% identity to *Torpedo* but no significant similarity to any entry in the Genbank or EMBL sequence databases. Northern blot analysis of poly A^+ RNA isolated from mouse C2 cells using the m6a cDNA as a probe showed a single band of 5kb. Preliminary Southern blot analysis of mouse genomic DNA indicates that the gene encoding 58K protein exists in low copy number in the mouse genome.

T 306 DISSECTION OF SIGNALLING PATHWAYS IN NEURONAL CELLS WITH RECOMBINANT INHIBITORS OF PROTEIN KINASES, Grigori Enikolopov and Natalia Peunova, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Protein kinases are directly involved in the modulation of synaptic plasticity in the brain. We are using specific recombinant inhibitors of various protein kinases as tools to dissect particular signalling events and to investigate the points of overlap between different signalling pathways in neuronal cells. We have prepared a series of recombinant expression clones fusing various cellular protein domains with peptide sequences known to act as highly specific pseudosubstrate inhibitors of PKA, PKC and CaMKII. These chimeric inhibitors were used to test the requirement for various kinases in signal transduction in the neuronal cell line PC12, monitored by induction of expression of co-transfected variants of the fos-gene. Our results indicate that depolarisation-induced gene activation is mediated by CaMKII and PKC, acting through different regulatory DNA elements. We are currently exploiting this approach to investigate other Ca^{2+}-mediated events in neuronal cells, including the intracellular Ca^{2+} release and synergistic interactions of the Ca^{2+}- and cAMP-dependent signalling pathways.

T 307 MECHANISM OF ACTIVITY DEPENDENT STABILIZATION OF AChR DEGRADATION AT DENERVATED ENDPLATES, Guido Fumagalli, Jacopo Andreose, Terje Lømo* and Miriam M. Salpeter**, Dept. of Medical Pharmacology, University of Milano, Italy; *Institute of Neurophysiology, University of Oslo, Norway; **Dept. of Neurobiology and Behavior, Cornell University, Ithaca NY.

Acetylcholine receptors (AChRs) at the neuromuscular junction (nmj) of innervated muscles have a stable degradation rate (t/2 about 10 days). After denervation there is a progressive increase in the degradation rate of the total population of junctional AChR which approaches a final t/2 of about 1-2 days (Levitt et al., Science 210: 550-51, 1980). Chronic electrical stimulation of long term denervated rat soleus muscles restores normal AChR stability (Fumagalli et al., Neuron 4: 563-69, 1990). Thus neurally evoked muscle activity is a factor in regulating AChR stability. Two distinct population of junctional AChR co-exist at the denervated nmj: AChR of the first population, called slowly degrading (R_S) AChRs, are synthesized in innervated muscles, accelerate following denervation from a t/2 of about 10 days to one of about 3 to 4 days, and are stabilized again after reinnervation. This modulation of degradation rate occurs while the R_S AChR are in the plasma membrane and requires no synthesis of new AChRs. The AChRs of the second population, called rapidly degrading (R_f) AChRs are synthesized in denervated muscles and replace the R_S as they degrade. The degradation t/2 of the R_f is about 1-2 days and appears not to be stabilized by reinnervation (Shyng and Salpeter, J. of Neuroscience 10: 3905-15, 1990). The increasing ratio of R_f/R_S AChRs contributes to the progressive increase in degradation rate seen after denervation. To determine the mechanism of AChR stabilization by muscle activity we asked whether electrical stimulation could stabilize the R_f AChRs. Both soleus muscles of Wistar rats were denervated and 25 days later, when the R_f had replaced the R_S AChRs, they were labeled with ^{125}I α-bungarotoxin by injection. Electrical stimulation of the right soleus muscle was started 6 hours after labeling, while the left muscle remained as an unstimulated control. Two patterns of stimulation were used: 60 pulses, 100 Hz every 100 sec, and 10 pulses, 20 Hz every 10 sec. Muscles were removed 1, 3 or 5 days after stimulation and the degradation rate determined. We found no significant difference between the stimulated and unstimulated muscles. Both had a t/2 of about 2-3 days. We conclude that electrical stimulation, like reinnervation, does not stabilize the degradation rate of R_f AChRs. The stabilization of junctional AChRs after electrical stimulation must therefore occur due to a replacement of the R_f AChR by the stable AChR. This could occur due by the down regulation of R_f relative to R_S synthesis, or by an induction of the stable R_S population.

T 308 43KD PROTEIN IS NOT SUFFICIENT FOR ACETYLCHOLINE RECEPTOR CLUSTERING. Herman Gordon and Zach Hall. Dept. of Anatomy, Univ. of Arizona, Tucson, AZ 85724 and Dept. of Physiology, Univ. of California, San Francisco, CA 94143.

Acetylcholine receptors (AChRs) in the surface membranes of cultured and embryonic myotubes spontaneously cluster into patches ranging from several to several tens of microns in extent. AChRs on prefusion myoblasts and in isolated expression systems do not spontaneously cluster. AChR clustering is thus thought to involve additional molecules that can interact with either the intracellular or extracellular sides of the AChRs. These clustering molecules are likely to play key roles in the development and maintenance of the neuromuscular junction *in vivo*. Molecules that may be involved in the clustering of AChRs include the 43kd protein in the cytoplasm and basal lamina components such as agrin, heparan sulfate proteoglycans, and s-laminin on the extracellular face.

We have used the S27 genetic variant of the C2 mouse muscle cell line to investigate whether the 43kd protein is required for AChR clustering. S27 myotubes express AChRs, but do not cluster them as do myotubes of the parental C2 line. Immunoprecipitation and immunofluorescence reveal that the 43kd protein is expressed by cultured S27 myotubes and that it is spread diffusely over the surface of the cells. In contrast, 43kd protein is associated with clusters of AChR in wild type C2 cells. Thus, the 43kd protein is not sufficient for AChR clustering although it may be necessary. Co-expression of 43kd protein with AChR subunits in Xenopus oocytes (Froehner et al. (1990), Neuron 5: 403) and in quail fibroblasts (Phillips et al. (1991), Science 251: 568) gives rise to extensive AChR clusters. Thus, these cells may contain additional components that are required for AChR clustering. We suggest that the S27 variant does not contain at least one of the required additional components. We have found that the S27 variant synthesizes shortened glycosaminoglycan chains, and it is possible that normal glycosaminoglycans may be required for AChR clustering.

T 309 Agrin Isoforms Induce Clustering Of Acetylcholine Receptors On Rat Myotubes, Werner Hoch, Fabio Rupp, James T. Campanelli, Thane Kreiner and Richard Scheller, Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Beckman Center, Stanford University, Stanford CA 94305

Agrin, a component of the synaptic basal lamina, is predominantly expressed during early development in neurons of the CNS and in myotubes, to a lesser extent. This protein has been purified from its richest source, the electric organ of Torpedo Californica. Using monoclonal antibodies against agrin, cDNAs encoding parts of agrin have been obtained from a marine ray expression library, and subsequently by homology screening of rat spinal cord libraries (Rupp et al., Neuron 6, 811).

Recently, we have identified several isoforms of agrin, which differ by the presence or absence of three small inserts in the C-terminal part of the protein as a consequence of alternative splicing. We constructed cDNAs containing the complete coding region of four rat agrin isoforms and expressed the proteins in CHO and COS-7 cells. Immunostaining of transfected cells with different fusion protein antibodies demonstated that agrin is secreted and concentrated on the extracellular surface of the cells. Intracellular staining of a perinuclear reticular network most likely represents newly synthesized agrin in the ER and Golgi on its way to the surface.

To investigate the effects of agrin on the distribution of acetylcholine receptors (AChRs), agrin transfected cells were cocultured with rat myotubes. Staining of the AChRs with α-bungarotoxin revealed that clusters of AChRs were frequently found in areas of contact between myotubes and agrin expressing cells. In contrast, no increase in the number of AChR clusters was observed outside the sides of contact. Furthermore, all four agrin isoforms cocultured with myotubes induced AChR clusters at contact sites with similar efficacy.

T 310 A SEARCH FOR NOVEL GENE PRODUCTS INVOLVED IN NEUROMUSCULAR SYNAPSE FORMATION.
Charles Jennings, Xuejun Zhu, Stephen M. Dyer and Steven Burden, Dept. of Biology, MIT, Cambridge, MA 02139.

The vertebrate neuromuscular junction is a highly specialized structure, and a number of muscle gene products are localized to the junctional region of the muscle fiber. The best-studied of these is the acetylcholine receptor, and recent work from our lab (Simon et al., *Development*, in press) indicates that the AChR δ subunit gene is specifically transcribed by synaptic nuclei. We are interested in how synapses are formed in response to inductive signals from the nerve, and to this end we have begun a search for muscle gene-products that may be involved in this process. We have taken two approaches. First, we have developed a subtractive hybridization procedure which we hope will enable us to identify novel mRNAs that, like the AChR subunit mRNAs, are localized to the synaptic region of the muscle fiber. Second, we have used PCR to look for receptors that may be involved in the response to innervation. By analogy with inductive events in invertebrate development, we speculated that a receptor tyrosine kinase might be involved in the induction of synapses, and this speculation was strengthened by the observation (Qu et al., *Neuron* 2, 367) that phosphotyrosine is concentrated at neuromuscular junctions. We have therefore used PCR to isolate tyrosine kinases from *Torpedo* electric organ, a tissue homologous to muscle. Preliminary results suggest that we have identified a novel receptor tyrosine kinase that is highly expressed in the electric organ.

T 311 CHARACTERIZATION OF A SYNAPSE SPECIFIC PHOSPHOPROTEIN WHICH IS A SUBSTRATE FOR NEURONAL CALPAIN: I. DEVELOPMENTAL EXPRESSION AND CELLULAR LOCALIZATION OF THE F1-20 PROTEIN AND mRNA, Eileen M. Lafer, Shibin Zhou, Nancy Hrinya Tannery, and Rui Sousa, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

Our long-range goals are to contribute to an understanding of the regulation of synaptic transmission. That is to say, we want to understand how changes in synaptic strength are modulated during the development of an organism, and as a consequence of learning and experience. These changes in synaptic strength are believed to depend, at least in part, on changes in the levels and activities of synaptic proteins. Therefore, there is a great deal of interest in characterizing the proteins found at neuronal synapses.

A monoclonal antibody library generated against synaptosomes was immunohistochemically screened to identify proteins localizing to synapse rich regions. The antibodies were then used to isolate cDNA clones by expression screening. Here we report the detailed characterization of the protein reactive with monoclonal antibody F1-20. Immunohistochemistry and western blot analyses reveal that the F1-20 protein is a synapse associated, brain specific protein with an apparent molecular weight by SDS-PAGE of 190,000 daltons. Northern blot analysis indicates that probes generated from a cDNA clone hybridize to a single brain specific mRNA of approximately 4.8 kb. F1-20 mRNA levels increase abruptly at postnatal day 4 and protein levels increase abruptly at postnatal day 7. *In situ* hybridization experiments demonstrate that F1-20 mRNA expression is neuronal specific. Variation in the expression of the F1-20 protein is complex and reveals patterns also exhibited by probes directed against other synapse associated molecules.

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T 312 SYNAPSIN I PROMOTES FUNCTIONAL MATURATION OF DEVELOPING NEUROMUSCULAR SYNAPSES, Bai Lu, Paul Greengard and Mu-ming Poo, Lab. Mol. & Cell. Neurosci., Rockefeller Univ., New York, N. Y. 10021 and Dept. Biol. Sciences, Columbia Univ., New York, N. Y. 10027

We have investigated the possible function(s) of synapsin I, a nerve terminal-specific protein, during the formation of neuromuscular synapses in *Xenopus* cell cultures. Purified synapsin I was loaded into embryonic spinal neurons by injection of the protein into one of the early blastomeres of *Xenopus* embryos. At synapses made by synapsin I-loaded neurons, spontaneous synaptic currents occurred with higher frequency and their amplitudes exhibited an earlier appearance of bell-shaped distribution, both of which are indicative of more mature quantal secretion. Functional synaptic efficacy was also enhanced, as impulse-evoked synaptic currents exhibited a significant increase in amplitude. Using cell manipulation techniques, we demonstrated that the enhancement of synaptic activities by exogenous synapsin I occured at the onset of synapse formation, suggesting a predominantly presynaptic mechanism. These results suggest a potential role of synapsin I in the functional maturation of synapses.

T 313 ON THE TRANSCRIPTIONAL BASIS OF BUTYRATE- AND DIBUTYRYL CYCLIC AMP-INDUCED DOWNREGULATION OF NICOTINIC ACETYLCHOLINE RECEPTOR EXPRESSION BY BC_3H-1 CELLS, Ronald J. Lukas and Linda Lucero, Division of Neurobiol., Barrow Neurological Inst., Phoenix, AZ 85013

Innervation of developing muscle fibers is known to induce a net reduction in levels of muscle nicotinic acetylcholine receptors (nAChR) and a subunit switch that underlies the transformation of embryonic nAChR to the adult isoform composed of alphal, betal, delta and epsilon (instead of gamma) subunits. The nature of the presumably motor neuron-derived factor(s) that induces these changes in nAChR expression is unknown. We investigated the effects of different agents that are known to alter second messenger signaling and/or gene transcription on numbers of nAChR and nAChR subunit-encoding transcripts in the BC_3H-1 mouse muscle cell line. The most dramatic effects are observed in cells treated with sodium butyrate or with dibutyryl cyclic AMP (dbcAMP), both of which produce about a 90% reduction in levels of nAChR expression as assessed by radioligand binding assays using alpha-bungarotoxin. Levels of mRNA corresponding to nAChR alphal, betal, or delta subunits are reduced between 2- and 10-fold by butyrate or dbcAMP treatments, but the most dramatic effect is an enormous reduction (estimated to be about 250-fold) in levels of gamma subunit-encoding transcripts. Butyrate and dbcAMP effects have distinct dose- and time-dependencies, but maximum effects are observed at 5 days of treatment with 1mM drug. Effects of butyrate are approximated by effects of valerate or propionate treatments, and dbcAMP effects are mimicked following forskolin and/or isobutyl methylxanthine treatments. These results suggest that effects of butyrate or dbcAMP treatments on nAChR numbers involve inhibition of nAChR subunit gene transcription and the virtual elimination of gamma-encoding transcripts. They also suggest, for example, that stimulation of cyclic AMP production (perhaps via motor neuronal release of calcitonin gene-related peptide?) in newly innervated muscle cells might be the signal that naturally downregulates gamma subunit expression and induces a net reduction in nAChR numbers in developing muscle cells *in vivo*.

T 314 FODRIN POSTTRANSLATIONAL MODIFICATIONS INDUCED BY NMDA RECEPTORS ACTIVATION, Tamara C. Petrucci, A. Maria M. Di Stasi and Gianfranco Macchia, Cell Biology, Istituto Superiore di Sanità, Roma, Italy

Activation of N-Methyl-D-aspartate (NMDA) receptors has been implicated in several neuronal process including neuronal development, long-term potentiation and excitotoxic cell death. Cultured cerebellar granule cells, prepared from postnatal rats, express all three subtypes of excitatory amino acid receptors and are susceptible to the toxic action of glutamate and its analogs. We studied the expression of proteins of the cortical cytoskeleton and of synaptic vesicles and we started to investigate the link between excitatory amino acid receptor activation and posttranslational modifications of fodrin, the major component of neuronal membrane skeleton. We have already showed that exposure of neuronal cell cultures to NMDA induces the appearance of 150 KDa fragments of alpha-fodrin subunits, as detected by immunoblot analysis. Proteolytic cleavage of alpha- fodrin is calcium-/calpain I-dependent and is selectively associated to the functional activation of NMDA receptors. The cleavage of fodrin occurs also in the absence of NMDA-mediated neurotoxicity (DiStasi et al.,1991). Recent studies performed in rat hippocampus have showed that activation of excitatory amino acid receptors induces activation of protein kinases and of calcium-/calmodulin-dependent phosphatases as well. In cerebellar granule cell cultures, translocation of protein kinase C from the cytosol to the membrane is linked to glutamate neurotoxicity (Favaron et al.,1990) and in hippocampal slices the rapid dephoshorylation of the cytoskeletal protein MAP2, induced by NMDA, may play a role in dendritic plasticity (Halpain and Greengard, 1991) We are currently investigating protein phosphorylation induced by excitatory amino acid in cerebellar granule cell cultures. Preliminary experiments have showed that phosphorylation of 55-60 KDa/ pH 6.8-7 components is induced by NMDA and that the beta subunits of fodrin are also phosphorylated. Experiments to characterize the 55-60 kDa phosphoproteins and to understand the relationship between the phosphorylation of the beta subunits and the cleavage of alpha subunits of fodrin induced by NMDA are in progress.

T 315 JUNCTIONAL AND EXTRA-JUNCTIONAL GOLGI IN MUSCLE, Evelyn Ralston, Laboratory of Neurobiology, NINDS, NIH, Bethesda, MD 20892.

The restriction of certain proteins, such as the acetylcholine receptor, to the neuromuscular junction, has been suggested to result from an exclusive localization of the Golgi to the subsynaptic compartment (Jasmin et al., P.N.A.S., *86*, 7218-22, 1989). In order to study the distribution of Golgi and other subcellular organelles between junctional and extra-junctional zones, single muscle fibers were observed as whole-mounts after immunofluorescent staining with a panel of antibodies. Single fibers were prepared from 4-6 week-old rat flexor digitorum brevis by collagenase dissociation. Antibodies to centrosomes, endoplasmic reticulum, and to two transient Golgi proteins, clathrin and β-coat protein (β-COP), recognized their repective antigens both at the junction and elsewhere. The antibodies to clathrin and β-COP gave a discontinuous perinuclear staining, similar to the distribution of Golgi in cultured myotubes. When the muscle was observed by electron microscopy, small, easily identifiable Golgi stacks (<10 μ in length) were found in both transverse and longitudinal sections. In addition, at the neuromuscular junction, the antibodies gave a staining pattern similar to that of α-bungarotoxin, a specific ligand of the acetylcholine receptor, suggesting that their antigens are enriched in the subsynaptic cytoplasm. In contrast, antibodies to α-mannosidase, a resident Golgi enzyme often used as Golgi marker in cultured cells, did not produce any detectable staining anywhere, suggesting that the enzyme is down-regulated in mature innervated muscle. I conclude that Golgi and other subcellular organelles involved in protein biosynthesis persist in both junctional and extra-junctional zones during muscle fiber maturation, although their content appears to change, possibly reflecting different metabolic needs in adult muscle.

This work was initiated at the University of California, San Francisco, with help from H. Gordon and support from Z.W.Hall.

T 316 EXPRESSION OF PURKINJE CELL $GABA_A$ RECEPTOR SUBUNIT mRNAs PRECEDES, AND IS INDEPENDENT OF, SYNAPSE FORMATION, A. Rotter, D. Zdilar, V. Luntz-Leybman and A. Frostholm, Department of Pharmacology, Ohio State University, Columbus, OH 43210

It is of considerable interest to determine the temporal relationship between presynaptic innervation and the appearance of corresponding postsynaptic receptors during synaptogenesis in the CNS. In the present studies, we have utilized subunit specific riboprobes to examine the temporal appearance of GABA-A receptor mRNAs in cerebellar Purkinje cells by in situ hybridization during normal murine development and in the reeler mutant. During normal development, Purkinje cells arise from the ventricular germinal layer, migrate into the cortex and subsequently receive GABAergic input. These cells become sensitive to exogenously added GABA, contain GABA-A receptors and express corresponding subunit mRNAs. In the reeler mutant, the majority of Purkinje cells fail to reach the cerebellar cortex and, as a consequence, do not receive their full complement of inhibitory innervation. Our results indicate that Purkinje cell receptor subunit mRNAs are already expressed at birth, prior to the formation of synaptic inputs from basket and stellate cells. This expression pattern is also observed in reeler Purkinje cells even though these cells do not become innervated by inhibitory interneurons.

T 317 43K PROTEIN INTERACTS WITH THE ALPHA AND/OR BETA SUBUNIT(S) OF THE NICOTINIC ACETYLCHOLINE RECEPTOR, Paula B. Scotland and Stanley C. Froehner, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755-3844.

The nicotinic acetylcholine receptor (nAChR) is highly concentrated at the neuromuscular junction. A cytoplasmic protein of M_r 43,000 (the 43K protein, or 43K) is also localized to this region, and is thought to interact with, and cluster, the receptor. Involvement of 43K protein in nAChR clustering has been demonstrated by coexpression studies. Injection of mRNA encoding the receptor subunits (α, β, γ, δ) into *Xenopus laevis* oocytes results in diffusely distributed surface receptors whereas coinjection with 43K mRNA results in clustering of the receptors. These receptor clusters also contain the 43K protein. Therefore, the 43K protein probably interacts directly with the receptor to cause clustering (Froehner et. al., (1990) Neuron *5*, 403-410). To determine which part of the receptor is essential for 43K-induced receptor clustering, experiments were performed using nAChR with altered subunit composition. Oocytes injected with α, β, δ and 43K mRNA, but not γ mRNA, still exhibited clustering of the receptors. Likewise, injection with α, β, γ and 43K mRNA, but not δ mRNA, also resulted in clustered receptors. While these results demonstrate that both γ and δ in the same receptor complex are not necessary, it remains possible that either γ or δ are required. Because it was not possible to express receptors that contained only α and β subunits we used a chimeric subunit called εβ (kindly provided by Xiao-Mei Yu and Zach Hall). εβ contains the extracellular region of the ε subunit and the intracellular and transmembrane regions of the β subunit. Expression of α, β, εβ and 43K mRNA still results in clustering of the nAChRs. These data demonstrate that only the α or β subunit may be necessary for 43K-induced receptor clustering.

T 318 THE EXPRESSION OF ACHR GENES AT SYNAPTIC SITES ON REGENERATED MUSCLE FIBRES IN THE ABSENCE OF THE NERVE, C.R. Slater* and H.R. Brenner*, + Division of Neurobiology, University of Newcastle upon Tyne NE2 4HH, U.K., * Department of Physiology, University of Basel, Vesalgasse 1, CH-4051, Switzerland.

Innervation of mammalian skeletal muscle fibres induces a local change in the pattern of expression of the genes for the nicotinic acetylcholine receptor (AChR) in the underlying myonuclei. This results in a switch in the subunit composition of the AChRs from $\alpha_2\beta\gamma\partial$ to $\alpha_2\beta\varepsilon\partial$ (1) and in the gating and conductance properties of the ionic channels associated with them (2). In addition, the expression of α-, β- and ∂-subunits at the neuromuscular synapse, but not elsewhere, becomes resistant to the down-regulating effects of muscle activity (3). The nature of the neurally derived signals that control the synapse-specific expression of AChR genes is not known. Here we show that when adult rat soleus muscle fibres are destroyed by injection of the venom of the Australian tiger snake (4) and are then allowed to regenerate in the absence of innervation, but within pre-existing basal lamina sheaths, ε- and α-subunit mRNAs are again expressed in an activity resistant manner in the region of the original synaptic sites. Furthermore, AChR channels with brief open times, characteristic of those containing the ε-subunit, accumulate at these same sites. Thus, factors which are capable of inducing the synapse-specific expression of AChR genes survive at synaptic sites in spite of degeneration of the nerve and the original muscle cells.

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T 319 DIFFUSION-TRAPPING OF ACETYLCHOLINE RECEPTORS: A NUMERICAL MODEL.

Jes Stollberg and Herman Gordon. Békésy Laboratory of Neurobiology, University of Hawaii at Manoa; Department of Anatomy, University of Arizona, Tucson.

The early aggregation of acetylcholine receptors at the developing neuromuscular junction proceeds in good measure by the lateral migration of receptors. Several lines of evidence are consistent with the aggregation taking place by way of a diffusion-trap - a region into which receptors diffuse freely , but within which diffusion is hindered.

While the diffusion-trap model is intuitively appealing, there has been little quantitative analysis of the model to determine whether such a mechanism could account for the observed rate and geometry of receptor aggregation. Here we present several such analyses based on numerical modeling of the diffusion-trapping process. Trap efficiency as well as realistic cell geometries, receptor densities, and diffusion constants are incorporated into the model so that we can present information concerning the density of receptors in and adjacent to the trap as a function of real (physiological) time.

Of particular interest is a study based on a trap region of 7 x 2 μm on a 300 μm long, 10 μm diameter cylinder. Results show that with only a 5% trap efficiency (5% of receptors in the trap are bound per second) receptors accumulate to more than 100 times their initial density in 4 hours. Receptor density immediately adjacent to the trap region is depressed to approximately 20% of the initial density. These results are similar to the observed formation of isolated receptor aggregates on the surface of cultured myotubes. Our findings support the view that receptor aggregation on cultured and embryonic myotubes proceeds by way of a diffusion-trap mechanism.

T 320 **A CONTINUOUS BASEMENT MEMBRANE WITH SPECIFIC SYNAPTIC ELEMENTS IS ASSOCIATED WITH ANEURALLY CULTURED HUMAN SKELETAL MUSCLE CELLS**

Toin v. Kuppevelt, Ad Benders, Jacques Veerkamp University of Nijmegen, Dept. Biochemistry, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Human skeletal muscle cells are completely encompassed by a basement membrane (BM), which is specialised at the site of neuromuscular junctions (the synaptic BM). BM-components were studied on human skeletal muscle cells in vivo and in vitro by immunofluorescence and electron microscopy. In vivo, muscle cells are surrounded by a continuous layer of heparan sulfate proteoglycans (HSPGs), laminin, type IV collagen and fibronectin. HSPGs are distinctively concentrated at the synaptic BM and so are molecules reacting with isolectin B4 from Vicia villosa.

Myotubes cultured on a combination of the serum substitute Ultroser G and brain extract show a continuous layer of HSPGs, laminin and type IV collagen. At sites of acetylcholine receptor clusters, HSPGs and lectin-positive molecules are distinctively concentrated, resembling the in vivo situation. In contrast, myotubes cultured on serum-containing media are not ensheated by a continuous layer of BM components, nor do they show acetylcholine receptor, HSPG, or lectin clustering. Electron microscopy reveals that myotubes cultered on Ultroser/brain extract are surrounded by a continuous BM consisting of a lamina lucida, a lamina densa and a lamina fibroreticularis. Proteoglycans are present on the external site of the lamina densa, and associated in a regular fashion with collagen fibrils.

In conclusion, BM associated with myotubes cultured on Ultroser G/brain extract resemble in many ways the in vivo situation, including synaptic specializations. The cultures of myotubes may serve as a model system for studies on the structure and function of human muscular (synaptic) BM.

T 321 PKC-PHOSPHORYLATED RC3 PROTEIN INCREASES CALCIUM LEVELS IN XENOPUS OOCYTES.

Joseph B. Watson, Randy W. Cohen and Jody E. Margulies. Mental Retardation Research Center and Department of Psychiatry and Biobehavioral Sciences, UCLA, Los Angeles, CA 90024. RC3 is a forebrain-specific postsynaptic protein first identified as a cDNA clone of a rat cortex-enriched mRNA by subtractive hybridization [Watson et al (1990) J Neurosci Res 26:397-408]. RC3 resembles GAP-43, a presynaptic axonal protein, in a small domain of amino acid sequence containing a serine residue phosphorylated by protein kinase C (PKC) and a calmodulin-binding site. It would seem that a small domain has been conserved throughout evolution to provide a common site of interaction with PKC and calmodulin in either presynaptic or postsynaptic terminals. To assess the function of RC3 in PKC-mediated signal transduction pathways, assays of RC3 heterologously expressed in Xenopus oocytes were performed using the two electrode voltage-clamp technique. Phorbol ester activation of endogenous oocyte PKC produced large calcium-activated Cl^- currents ($\geq$500 nA) in oocytes injected with wild type RC3 cRNA and not in control oocytes injected with mutant RC3 cRNA (glycine substituted for PKC-serine) or in oocytes injected with water. Experiments are underway to test whether PKC-phosphorylated RC3 modulates either IP3- or calcium-sensitive intracellular calcium stores. RC3's ability to amplify calcium levels may be relevant to its postsynaptic function and may have important implications for synaptic plasticity during development and molecular cascades that underlie memory storage.

T 322 REGULATION OF AChR γ AND ε SUBUNIT GENE TRANSCRIPTION. V. Witzemann, M. Numberger, I. Dürr, W. Kues, and M. Koenen, Abteilung Zellphysiologie, Max-Planck-Institut für medizinische Forschung, Jahnstr. 29, 6900 Heidelberg, FRG

The five subunits, α, β, γ, δ and ε, of the nicotinic acetylcholine receptor (AChR) are regulated differently during development and synaptogenesis. In order to identify regulatory DNA sequences which are involved in gene transcription we have isolated and characterized 5'-flanking sequences of the γ and ε subunit genes that confer muscle-specific expression upon transient transfection of primary cultures of rat muscle cells. Although these cells may have an incomplete differentiation program lacking especially factors required for the synapse-specific expression of AChR subunits in normal innervated muscle they are suitable to analyse regulatory signals involved in the muscle-specific expression of the AChR genes. Myogenic factors such as MyoD, myogenin and MRF4 as putative transcription activating factors are probably not involved in synapse-specific events but may be linked to signalling pathways arising from the electrical activity of the muscle which is known to regulate the biosynthesis of the extrasynaptic AChRs. The results demonstrate that different mechanisms are involved in the regulation of of the muscle-specific expression of AChR γ and ε subunit genes.

T 323 THE CYTOPLASMIC LOOPS OF THE δ AND ε SUBUNITS OF THE MOUSE MUSCLE ACETYLCHOLINE RECEPTOR ARE NOT REQUIRED FOR CLUSTER FORMATION IN COS CELLS TRANSIENTLY EXPRESSING THE RECEPTOR AND THE 43 kD PROTEIN. Xiao Mei Yu and Zach W. Hall, Department of Physiology, University of California, San Francisco, CA 94143.
To investigate mechanisms of formation of acetylcholine receptor clusters, we have transiently expressed subunits of the adult form of the mouse muscle nicotinic acetylcholine receptor (AChR) and the 43 kD protein in COS cells. In COS cells transfected only with cDNA for the four receptor subunits (α, β, ε and δ), the AChR is diffusely distributed on the surface of the cells. When the 43 kD protein is also expressed in COS cells, small clusters of the AChR appear on their surface. We have made chimeric subunits to determine which cytoplasmic domains are required for cluster formation. When the ε or δ subunit cDNAs are replaced by cDNAs encoding subunits in which the N- and C-terminal regions come from the ε or δ subunits, respectively, and the intervening portion from the β subunit, a toxin-binding complex is expressed on the surface whose size and toxin-binding characteristics resemble those of the AChR. When co-expressed with the 43 kD protein, these complexes appear in clusters on the cell surface. We conclude that the cytoplasmic domains of neither the δ or ε subunits are required for cluster formation. (We thank Paula Scotland and Stan Froehner for generously providing advice and mouse muscle 43 kD cDNA; this work was supported by grants from the NIH and the Muscular Dystrophy Association).

T 324 CHARACTERIZATION OF A SYNAPSE SPECIFIC PHOSPHOPROTEIN WHICH IS A SUBSTRATE FOR NEURONAL CALPAIN: II. cDNA CLONING AND SEQUENCE ANALYSIS, Shibin Zhou, Rui Sousa, Nancy Hrinya Tannery, and Eileen M. Lafer, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

This paper reports the cloning of the cDNA sequence for the F1-20 protein, a novel neuronal specific, developmentally regulated, synapse specific protein. The complete protein sequence has been deduced from this cDNA sequence and the C-terminal portion of the deduced amino acid sequence of the F1-20 protein was found to share homology with sequences from rabbit calpastatin (calpain protease inhibitor). Here we demonstrate that the F1-20 protein is a substrate for neuronal calpain, as well as being heterogeneously phosphorylated. Protein phosphorylation is involved in signal transduction in many systems including the nervous system and is affected by changes in calcium concentration either directly, through the action of calcium dependent kinases, or indirectly through complex signal transduction cascades. The phosphorylation and calpain sensitivity of F1-20 may therefore provide mechanisms through which its activity is regulated by changing calcium concentrations.

Utilizing RNase protection analyses as well as PCR, the F1-20 mRNA is found to undergo alternative splicing with the consequent removal of a potential casein kinase II phosphorylation site.

This work was supported by NIH grant RO1 NS29051 to Eileen M. Lafer, and by a March of Dimes Basal O'Connor Starting Scholar Award to Eileen M. Lafer.

Late Abstracts

ANCHORAGE OF ACETYLCHOLINE RECEPTORS AND EXTRACELLULAR GLYCOPROTEINS BY A SPECTRIN SKELETON. R.J. Bloch, G.M. Dmytrenko* and D.W. Pumplin**, Departments of Physiology, Neurology*, and Anatomy**, University of Maryland School of Medicine, Baltimore, MD 21201.

The clusters of acetylcholine receptors (AChR) of cultured rat myotubes contain equal amounts of AChR and the 43kD and 58kD receptor-associated proteins, and 4-7 fold more ß-spectrin. Selective extractions suggest that these proteins are removed from the membrane in the following order (from easiest to most difficult): ß-spectrin > 58kD > 43kD > AChR. Ultrastructural studies show that ß-spectrin is part of an extensive network of very thin filaments applied to the intracellular face of the membrane, associated with a regular array of AChR in the membrane itself. These results are consistent with a model for AChR clusters in which receptors are bound to a spectrin-rich network by two intermediary proteins, 43kD and 58kD.

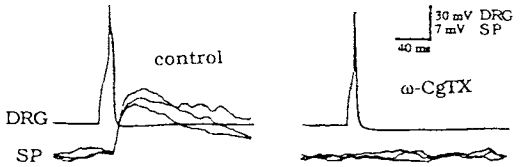
Components of the extracellular matrix (ECM), including fibronectin, laminin, heparan sulfate proteoglycan, and type IV collagen, codistribute with AChR in clusters. Treatment of isolated AChR clusters with insoluble chymotrypsin removes ß-spectrin and 58kD and simultaneously redistributes AChR and ECM, even though insoluble chymotrypsin does not gain direct access to ECM at clusters. This suggests that ECM accumulates by virtue of its ability to anchor, presumably by a transmembrane protein, to a structure on the cytoplasmic face of cluster membrane. We propose that this structure is the same spectrin-rich membrane skeleton that anchors AChR.

These results, consistent with linkage amonge AChR, ECM and the membrane skeleton, can explain the common finding that AChR clusters are always associated with both a submembrane density and elaborations of ECM. They also help to explain the abilities of agrin, ARIA, and physical contact to induce AChR clustering.

ω-CONOTOXIN-SENSITIVE CALCIUM CHANNELS TRIGGER EPSPs AT THE SENSORY NEURON-SPINAL CORD SYNAPSE. W. Gruner, L.R. Silva and K. Dunlap, Dept. of Physiology, Tufts Univ., 136 Harrison Ave., Boston, MA 02111.

In order to examine Ca^{2+} channels that mediate transmitter release, we have studied synapses between single sensory afferent (DRG) neurons and spinal neurons grown together in primary culture. Dorsal root ganglia and spinal cords were dissected from chick embryos, dissociated, and plated onto dishes containing small collagen "islands" (Segal and Furshpan 1990, J. Neurophys. 64:1390). Dual recordings from DRG-spinal cell pairs (R_{in}=0.5-3 GΩ) were made using whole cell patch clamp techniques.

DRG action potentials evoked short-latency EPSPs in spinal neurons. EPSPs had amplitudes up to 20 mV and were rapidly and reversibly blocked by the glutamate receptor antagonist CNQX (1 µM). ω-conotoxin (ω-CgTX; 1 µM), which blocks N-type Ca^{2+} channels in chick DRG neurons (Cox and Dunlap 1992, J. Neurosci. In Press), irreversibly blocked EPSPs in spinal neurons. ω-CgTX did not alter spinal neuron responses to focally applied L-glutamate. DRG action potentials were narrowed by ω-CgTX but no change in input resistance or resting membrane potential was observed.



These experiments provide direct evidence that N-type Ca^{2+} channels mediate transmitter release by DRG neurons. Paired recording experiments are in progress to examine how modulation of presynaptic N-type Ca^{2+} channels alters the spinal neuron EPSP.

MEPP CLASSES AND SUBUNIT COMPOSITIONS ARE DYNAMICALLY FORMED AT MOMENT OF RELEASE AT THE TORPEDO NEUROMUSCULAR AND ELECTROCYTE JUNCTIONS. M.E. Kriebel and G. Fox. Dept. Physiology., SUNY Health Sci. Ctr., Syracuse, N.Y. and M.P.I. Goettingen, F.R.G.

There are two classes of miniature endplate potentials (MEPPs) in the frog, mouse, skate and Torpedo junctions based on amplitude distributions. The larger class forms a bell-shaped distribution (bell-MEPPs) and this class was first described by Fatt and Katz (J. Physiol. 1952). The second class has a mode 1/7th - 1/10th that of bell-MEPPs and it is skewed. Giant MEPPs belong to the skew-class (Kriebel et al 1976, J. Physiol.). Amplitude distributions of MEPPs show that both classes are composed of subunits. The two classes have very different pharmacological and physiological properties. Because there are rapid changes in class percentages as well as size, and there are interdependencies between events, we have proposed that MEPP size is determined at the moment of release by a dynamical process with kinetics similar to those that form water drops from a faucet (Kriebel, et al, 1990, Brain Res. Rev.). In the Torpedo electrocyte terminal, we have shown that synaptic vesicle membrane is not incorporated into the nerve terminal with evoked release, that changes in vesicle classes occur long after both evoked and elevated rates of spontaneous release, that MEPP size can vary 10 fold with the generation of only 100 MEPPs, and that there is a periodic property of the spontaneous process of release. These findings suggest that the release process is very labile in the electric organ compared to Torpedo eye muscle and frog and mouse preparations. Torpedo electrocytes have 3 to 5 classes of vesicles based on diameter whereas muscle fibers have only one class. Yet, MEPP size and class structure are similar in muscle and electrocyte. We have found that vesicle classes are evenly distributed and there is no correlation between vesicles and transmitter release. We propose that there is a dynamic release process in the membrane that meters the release of transmitter in subunit amounts from a presynaptic store and it has two functional states. One state generates bell-MEPPs and the second state generates skew-MEPPs. Thus, the ratio of skew-to-bell-MEPPs is changeable, both classes are composed of the same subunits but the two states have different pharmacological properties. Supported by NIH NS25683.