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

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Synapse Formation and Function: The Neuromuscular Junction and Central Nervous System II

Growth Cones and Neurite Guidance

B5-001 INTRACELLULAR CALCIUM SIGNALS REGULATING GROWTH CONE BEHAVIOR, S. B. Kater, Program in Neuronal Growth and Development and the Department of Anatomy and Neurobiology, Colorado State University, Ft. Collins, CO 80523.

The neuronal growth cone responds to a diverse spectrum of environmental cues. Both diffusible cues, in the form of neurotransmitters, and surface molecules, in the form of both repulsive and permissive substrate molecules, have been analyzed for how they signal changes in the behavior of neuronal growth cones. An antennae-amplifier model has been developed to describe the relationship between filopodia and the body of the growth cone. Filopodia appear to be a unit sensory-motor structure with the body of the growth cone capable of integrating multiple filopodial inputs in both time and space. Many of the molecules we have studied can affect significant rises in intracellular calcium within growth cones that can signal alterations in growth cone behavior. There is a high degree of stimulus-specificity in terms of magnitude and the localization of changes in calcium evoked by a given stimulus. Filopodia can act as independent sensors with local rises in their calcium levels (left-most diagram). Very strong stimuli (right-hand diagram) evoke calcium release from stores in the growth cone resulting in micromolar rises in intracellular calcium. Intermediate stimuli produce intermediate effects. Taken together, a picture is emerging in which a diverse environmental cue can act through calcium second messenger signalling systems to select from amongst the growth cone's repertoire of behaviors employed in neuronal pathfinding.

Target Selection and Synaptic Specificity

B5-002 DEVELOPMENT OF SYNAPTIC SPECIFICITY IN THE SPINAL CORD, Eric Frank, Zeljka Korade, Simon Mears, Kamal Sharma and Peter Wenner, Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Synaptic connections made by sensory neurons within the spinal cord provide a good system for exploring mechanisms responsible for synaptic specificity in the CNS. Different functional classes of sensory neurons project to different target regions. Even within a single class, afferents supplying different peripheral targets choose different synaptic partners in the cord. We are studying how these specific connections are formed during embryonic development.

In the developing mammalian visual system, the initial set of synaptic connections is subsequently refined. Are patterns of synaptic connections between muscle spindle afferents and motoneurons also refined after their initial formation? To address this question we recorded from motoneurons in an isolated preparation of mouse spinal cord and measured the strengths of muscle afferent input to synergistic and antagonistic motoneurons throughout the first postnatal week. The pattern of these connections remained constant during this period, and was similar to that in adult animals, suggesting that these connections are already highly specific shortly after their initial formation and do not undergo substantial subsequent remodeling.

What factors determine the choice of a sensory neuron's synaptic targets in the CNS? The specificity of these connections is controlled, at least in part, by the particular peripheral targets of these neurons. If sensory neurons at thoracic levels are made to innervate leg muscles in a developing tadpole, these afferents develop novel synaptic connections with the now functionally appropriate leg motoneurons. A more precise localization of the source of this peripheral influence comes from experiments in which sensory neurons normally supplying ventral limb muscles in chicken embryos are made to supply a duplicate set of dorsal limb muscles. Sensory afferents supplying the duplicated muscles make synaptic contacts with motoneurons that are appropriate for the new muscle they supply, suggesting that the cues responsible for these effects are located in the peripheral limb tissue itself, not on the motoneurons with which these sensory neurons project.

Studies of the molecular cues used by growing sensory afferents to seek out their targets in the CNS would be facilitated by a preparation in which appropriate sensory projections developed in culture. Segments of spinal cord with attached sensory ganglia provide such a system. Cutaneous afferents pre-labeled with DiI establish projections that are restricted to dorsal laminae of the cord, just as in normal embryos, whereas large diameter muscle afferents project through this region and extended ventrally towards the motoneurons, their normal synaptic targets. To test whether the dorsal cord alone has sufficient cues for guiding these afferents, we removed the ventral half of the cord and replaced it with an inverted dorsal half from an adjacent segment. Both cutaneous and muscle afferents still formed their normal projections, showing that sufficient cues are contained within the dorsal cord at this time to permit the formation of appropriate sensory projections.

B5-003 BRAIN WAVES AND SYNAPTIC TRANSMISSION DURING VISUAL SYSTEM DEVELOPMENT, C.J. Shatz, Howard Hughes Medical Institute and Department of Molecular and Cell Biology, University of California, Berkeley, CA.

Neural connections in the adult central nervous system are highly organized. In the visual system, retinal ganglion cells (RGCs) in the two eyes send their axons to target neurons in the LGN, where they terminate in adjacent but non-overlapping eye-specific layers. During early development, however, inputs from the two eyes are intermixed, and the adult pattern emerges gradually as axons from the two eyes sort out to form the layers. How do these layers form? Evidence does not favor the existence of specific molecular cues that designate left and right eye LGN zones. Rather, experiments suggest that the sorting out process, even though it occurs before vision, requires specific patterns of action potential activity and involves synaptic competition between axons from the two eyes for LGN neurons.

Experiments indicate that the synaptic machinery necessary for an activity-dependent competition is present during the relevant developmental times. RGC axons form synapses even prior to the onset of layer formation; some of these synapses, later eliminated, are initially located in territory that will ultimately belong exclusively to the other eye. Physiological studies demonstrate that about 90% of LGN neurons initially recieve convergent excitation from both optic nerves, whereas after the layers have formed, only about 10% do. Moreover, RCGs can generate action potentials spontaneously even in utero, and blocking their activity by intracranial infusion of tetrodotoxin prevents layer formation. These observations suggest that the segregation of RGC axons from the 2 eyes to form the set of eye-specific LGN layers requires the formation and selective elimination of functioning synaptic connections between RGC and LGN neurons.

Activity per se is not sufficient to promote the formation of eye-specific layers in the LGN; correlations in the firing of neighboring retinal ganglion cells are required (1). Using a multielectrode array, physiological recordings were made from up to 100 ganglion cells simultaneously in fetal cat or neonatal ferret retinas in vitro (2). Results showed that cells can fire spontaneously generated action potentials that are synchronized with each other in time and space: the pattern of RGC firing is wavelike, with nearest neighbors firing in near synchrony. Waves of activity sweep across the retina at a velocity of about 100 μm/sec. By means of optical recording, calcium waves with dynamics similar to those recorded on the multielectrode array have been observed. Not only ganglion cells, but also amacrine cells, appear to undergo calcium bursting. Moreover, intracellular injections of tracers that cross gap junctions indicate that subsets of ganglion and amacrine cells are interconnected (3). Thus, there is an early neuronal circuit that is likely to be responsible for generating the correlations in firing and waves of activity within the retina.

These waves are of the appropriate pattern and at the appropriate developmental times to be useful in providing essential information to postsynaptic LGN neurons concerning the location and eye of origin of the presynaptic ganglion cell axons. However, LGN neurons must be able to detect these correlations and strengthen or weaken these synaptic inputs accordingly. To study such synaptic mechanisms, we (4) made whole cell recordings from slices of neonatal ferret LGN in vitro. Tetanic stimulation of RGC axons produced marked and maintained (up to 2 hours) enhancement of synaptic transmission, indicating that retinogeniculate synapses can indeed undergo activity-dependent changes in strength. Thus, such changes may underlie the process of synaptic rearrangement occurring during the formation of layers within the LGN. Since spontaneously generated activity is present elsewhere in the developing CNS, these observations suggest a general role for neural activity in promoting the formation of precise connections.

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Presynaptic Components and Vesicular Release I

B5-004 A MOLECULAR ANALYSIS OF NEUROTRANSMITTER TRANSPORT INTO SYNAPTIC VESICLES, Yongjian Liu[1], Ali Roghani[1], Doris Peter[2], Andrew Merickel[3] and Robert Edwards[1], Depts. of Neurology[1], Biological Chemistry[1], Molecular Biology Institute[1], Microbiology & Immunology[2] and Neuroscience[3], UCLA School of Medicine, 710 Westwood Blvd., LA, CA 90024-1769.

The regulated exocytotic release of neurotransmitters depends on their efficient packaging into synaptic vesicles. Classical studies have identified four distinct transport activities on synaptic vesicles, including one for monoamines, a second for acetylcholine, a third for GABA/glycine and a fourth for glutamate. Using selection in the neurotoxin MPP^+, we have isolated a cDNA clone for the vesicular amine transporter expressed by adrenal medullary cells, implicating this activity in the form of neural degeneration that occurs in Parkinson's disease. The sequence remotely resembles a class of bacterial antibiotic resistance proteins, further suggesting a role in detoxification that may include protection against the normal neurotransmitter dopamine.

Monoamine populations in the central nervous system express a closely related transporter with several important differences in substrate affinity, preference and pharmacology. In particular, the brain transporter has a higher apparent affinity for most monoamine substrates and especially for histamine. It also has a higher turnover number. The adrenal transporter shows much less sensitivity to tetrabenazine and binding assays show much less binding than to the central transporter. In addition, the brain transporter shows much greater sensitivity to amphetamines than the adrenal transporter. To identify the structural basis for these differences in function, we have constructed a series of chimeric transport proteins, all of which retain function. In addition, we have used site-directed mutagenesis to identify a series of residues essential for transport activity. Classical studies have shown that the vesicular transporters for other transmitters show a variety of differences from the amine transporters. Using the relationship between the vesicular amine transporters and the *C. elegans* gene *unc-17*, we have recently isolated a rat cDNA clone for a vesicular acetylcholine transporter and identified several important functional differences from the monoamine transporters. Further, monoamines appear to differ from other classical transmitters in the mode of release.

The subcellular localization of vesicular transporters largely determines the site of transmitter storage. For acetylcholine, GABA and glutamate, storage occurs in classical synaptic vesicles whereas for monoamines, storage can occur in either dense core vesicles or synaptic vesicles. To understand what determines the site of transmitter storage, we have determined the localization of the vesicular amine transporters. In PC12 cells, the endogenous adrenal transporter sorts preferentially to dense core vesicles and thus constitutes a unique membrane protein marker for the regulated secretory pathway that does not occur in synaptic vesicles. To understand how monoamines can be stored in synaptic vesicles in the central nervous system, we have transfected the transporter expressed in the brain into PC12 cells and found a different pattern of expression.

B5-005 ACTIVITY DEPENDENT PLASTICITY OF SYNAPTIC EFFICACY AT INHIBITING JUNCTIONS. Stephanie Charpier[1], Yoichi-Oda[2], Donald S. Faber[3], and Henri Korn[1], [1]Institut Pasteur, INSERM, Paris, France, [2]Osaka University, Japan, [3]Medical College of Pennsylvania, PA

Up to now, most work on cellular "learning" has been focused on excitatory connections and addressed with *in vitro* preparations. Yet, the optimum function of the nervous system depends upon a balance between excitation and inhibition. Thus, we have questioned whether patterned activity can produce persistent changes in the properties of identified glycinergic connections on the Mauthner (M-) cell *in vivo*. For this purpose, test and conditioning stimuli were applied to the posterior branch of the contralateral eighth nerve (VIIIn), which activates disynaptic inhibitory pathways but not excitatory inputs. Several lines of evidence indicate that LTP-like processes occur not only at the first excitatory relay in the lateral vestibular nucleus, but also at the inhibitory synapses. Brief low intensity VIIIn tetanizations produced a steady-state (up to 1.5 hours) enhancement of the inhibitory conductance of about 100%, half of which could be attributed to each stage of the disynaptic network. The effect on inhibition *per se* was quantified by measuring the relation between the presynaptic volley and the postsynaptic fractional conductance change (i.e. the input-output function). Paired intracellular recordings showed that tetani induced a 50% synapse specific potentiation of inhibition at the activated terminals, for as long as the penetrations remained stable. The mechanism underlying this phenomenon is still unclear. For example, while buffering postsynaptic calcium by injecting BAPTA in the M-cell blocks the induction of this "LTP", it is difficult to envision a pathway by which an inhibitory Cl^--dependent shunt with minimal depolarization could increase $[Ca^{2+}]i$. Conversely, attempts at quantal analysis during the maintenance period (coefficient of variation and constrained deconvolution methods) implicate an increased quantal content, i.e. presynaptic involvement. Along this line, it was previously shown that 5-HT is an endogenous transmitter in this system and enhances the probability of evoked and spontaneous release of glycine. Thus, plasticity *in vivo* may be regulated by both activity and the local environment.

A further degree of complexity is provided by the existence of a substantial reserve pool of "latent" connections. Although their connectivity with the M-cell is similar to that of their potent counterparts, they are not activated by intracellular injections of Ca^{2+} or 4AP, in contrast to the latter; yet they are unmasked after VIIIn tetanization and contribute significantly to the expression and strength of the inhibitory LTP. In the context of neuronal networks, plasticity of inhibition may be a prerequisite for the control of adaptive behaviors and for setting the gain of reflexes, such as the escape reaction triggered by the M-cell.

Presynaptic Components and Vesicular Release II

B5-006 BIOGENESIS OF SYNAPTIC VESICLES. Regis B. Kelly, Eric Grote, Lois Clift-O'Grady, Claire Desnos, Gary Herman & Frank Bonzelius. Dept of Biochemistry & Biophysics and Hormone Research Institute, University of California, San Francisco, CA 94143-0448.

Synaptic vesicle membrane proteins are targeted to synaptic vesicle-like organelles in the neuroendocrine cell line, PC12, but also the plasma membrane and to endosomes. When an epitope-tagged version of synaptobrevin/VAMP is expressed in PC12 cells it is recovered in all three regions. One class of mutant VAMP is concentrated in the plasma membrane, suggesting that it lacks the information necessary for efficient endosome or synaptic vesicle targeting. Another is endocytosed normally but does not get targeted to synaptic vesicles. A third shows enhanced targeting to vesicles, but not to endosomes. Since the mutations are without effect in non-neural cells, accumulation of the mutant VAMPs in subcellular compartments appears to reflect aberrant interactions with neural-specific proteins. The most effective mutations lie in the hydrophobic amino acids of the highly conserved amphipathic helix portion of VAMP, consistent with the formation of homomeric or heteromeric interactions between VAMP and other vesicle proteins. We propose that our screen provides a means to identify proteins and protein domains in the synaptic vesicle life cycle. We can also detect a second class of small vesicles in PC12 cells transfected with the glucose transporter GLUT4. Unlike synaptic vesicles, this class of vesicles can be detected in transfected non-neuronal cells, and in fat cells expressing endogenous GLUT4. We propose that the second class of vesicles is involved in cell surface modification.

B5-007 SYNAPTOTAGMIN AND THE GENETIC ANALYSIS OF SYNAPTIC FUNCTION, Thomas L. Schwarz[1], Aaron DiAntonio[1], Noreen Reist[1], and Kendal Broadie[2], [1]Department of Molecular and Cellular Physiology, Stanford University, Stanford CA 94305, [2] Dept. of Zoology, University of Cambridge, Cambridge UK

The exocytotic pathway in neurons has been specialized to permit the rapid, Ca^{++}-dependent fusion of synaptic vesicles at active zones. Among these specializations is a mechanism for maintaining a subpopulation of these vesicles in a stable, docked, and fusion-ready state at the active zone. Synaptotagmin is a 65kD membrane protein in synaptic vesicles with two Ca^{++}-binding C2 domains in its cytoplasmic portion. Unlike many synaptic proteins for which homologs have been implicated in the constitutive membrane trafficking of all cells, synaptotagmin appears to be restricted to cells that undergo regulated secretion. We have investigated the function of this protein by a genetic analysis in Drosophila, an organism that appears to have only a single synaptotagmin gene (*syt*). We have isolated several mutations in *syt*, including a null allele with a stop codon at amino acid 32, an allele with a stop codon between the two C2 domains, and a point mutation in a highly conserved amino acid in the second C2 domain. Severe alleles of *syt* are lethal but, surprisingly, even null embryos can occassionally hatch and move feebly before dying. Electrophysiologic studies of null embryos confirmed that Ca^{++}-dependent evoked synaptic transmission persists, albeit at greatly reduced levels. Thus synaptotagmin does not appear to be absolutely essential for either Ca^{++}-sensing or fusion to occur. The frequency, but not the amplitude, of spontaneous miniature synaptic currents is increased in *syt* mutants, but the increase in frequency is probably not sufficient to deplete severely the total pool of vesicles. A model in which synaptotagmin stabilizes the docking of synaptic vesicles is consistent with the phenotype of these mutations. Electron microscopy of mutant synapses has revealed that the pool of vesicles immediately adjacent to the active zone membrane was reduced to 24% of wildtype levels. The mutations did not appear to compromise the ability of the terminals to cluster vesicles in the vicinity of the synapse. Thus the mechanism that causes aggregation of vesicles near a release site must be independent of synaptotagmin and distinct from the synaptotagmin-dependent docking mechanism that holds vesicles immediately adjacent to the active zone membrane.

Synapse-Specific Muscle Molecules

B5-008 KNOCK-OUT MUTATIONS AFFECTING THE NEUROMUSCULAR SYNAPSE, John P. Merlie, Medha Gautam, Peter G. Noakes, Jacqueline Mudd, Mia Nichol, and Joshua R. Sanes, Washington University Medical School, St. Louis.

The formation and maintenance of synaptic connections require a complex interplay of signals between pre and postsynaptic partners. At the neuro muscular synapse signals from the motor neuron regulate transcription of AChR genes in muscle as well as the organization and stability of AChR and receptor associated proteins in the postsynaptic membrane. Conversely, signals from the muscle regulate the number and location of nerve terminals and instruct terminals to form active zones for transmitter vesicle release. We have initiated studies involving the targeted disruption of genes thought to encode key components of this model synapse. Here, we report our progress to date.

S-laminin, a laminin B1-like component of the synaptic basal lamina has been shown to have motoneuron-specific adhesive and signaling properties *in vitro*. To assess the function of s-laminin *in vivo* we targeted a gene disruption to the second of 32 coding exons. Mice heterozygous for this null mutation are born with the expected frequency, have no obvious behavioral deficits and are fertile. Homozygous mutants are similar in size and behavior to normal littermates at birth, but begin to show signs of weakness by the second postnatal week, and die before weaning. By light and fluorescence microscopy, the pre and postsynaptic specializations appear normal in size and distribution. However, the ultrastructure of mutant terminals is grossly abnormal: Schwann cell processes invade the space between nerve terminal and basal lamina, the post-synaptic membrane lacks junctional folds, few active zones are present and synaptic vesicles are uniformly distributed within the terminal rather than being concentrated near the terminal membrane. Consistent with these dramatic ultrastructural differences, we have found that the frequency of miniature end-plate potentials is reduced to less than 10% of control values. Thus, both the structure and function of s-laminin mutant synapses indicate a defect in maturation of the synaptic vesicle release mechanism. While further studies will be required to determine if all of the ultrastructural abnormalities are a direct result of s-laminin deficiency, we have already begun to study how the mutation might lead to defective transmitter release. In brief, synapsins, a family of phosphoproteins associated with the cytoplasmic surface of synaptic vesicles are abnormal in conformation and/or association with other components of the nerve terminal.

We are also studying the process of AChR organization in the postsynaptic membrane induced by nerve derived signals. The 43kDa AChR associated protein, rapsyn, is an abundant structural componentt of AChR rich postsynaptic membranes and is associated with AChR clusters on muscle cells in tissue culture. *In vitro* studies in heterologous expression systems have implicated 43k rapsyn in the clustering process, and have led us to test the role of this protein *in vivo*. The phenotype of homozygous 43k rapsyn mutant mice is strikingly different from the s-laminin mutants. 43k rapsyn mutants fail to suckle, and they die within the first day. Although, histological studies have just begun, it is already clear that 43k rapsyn mutants lack detectable AChR clusters, and are also deficient in endplate-associated acetylcholinesterase. It will be interesting to see if failure to organize AChR clusters results in any presynaptic defects. Clearly, 43k rapsyn mediated AChR clustering is critical to postsynaptic specialization. To examine the role of agrin in the induction of AChR clustering, we have begun experiements to create germ line null mutations in the agrin gene.

B5-009 **ACETYLCHOLINE RECEPTOR STABILITY AT VERTEBRATE NEUROMUSCULAR JUNCTIONS.**
Miriam M. Salpeter, Department of Neurobiology and Behavior, Cornell University, Ithaca NY 14853.

Two populations of Acetycholine Receptors, Rr and Rs, can exist at the vertebrate neuromuscular junction depending on the state of innervation of the muscle. The Rr AChRs, which are synthesized in noninnervated or denervated muscle, have a degradation half life of about 1 day, and to date we have not found any conditions which will stabilize them to the adult value (with a $t_{1/2}$ of ~10 days), seen at innervated junctions. Innervation or electrical activity merely down-regulate the Rr so that they disappear and are replaced by Rs. The Rs AChRs, which are synthesized in innervated muscle, have a more complex degradation behavior which can be modulated in the post-synaptic plasma membrane by innervation/denervation and cAMP. The $t_{1/2}$ of the Rs is ~ 8-10 days when the muscle is innervated, accelerates to a half life of 3 days after denervation and restabilizes again upon reinnervation. Experiments using second messengers have found that cAMP via PKA can mimic the nerve in stabilizing the Rs from its accelerated 3 day half life to the stable 8 day half life (Xu and Salpeter). Current experiments in our laboratory are dealing with the questions of how stable AChRs are developed and maintained in the adult nmjs? One question that has been very intriguing and controversial is whether the switch from the embryonic gamma to the adult epsilon subunit of the AChR is in any way involved in stabilizing the AChR. Recent results (O'Malley and Salpeter) suggest that this may be the case. These data will be presented at the meeting. In addition, data will be presented on the complex shift from a 1-day receptor to a 10-day receptor during muscle reinnervation, which could explain how the stability of the nmj is maintained during this critical period. In summary, it now appears that stabilization at the nmj involves the replacement of the embryonic Rr AChR ($t_{1/2}$~1day) by the adult Rs AChR, and the stabilization of Rs (to a $t_{1/2}$ of ~10 d) by innervation, possibly via a cAMP-PKA messenger system. Different degradation values reported in the literature during critical periods of development or denervation and reinnervation often reflect the coexistence, at different ratios, of the Rr and Rs populations at the nmj. Supported by NIH Grant, NS09315

B5-010 RECENT ADVANCES IN UNDERSTANDING TRANSMISSION FAILURE AT HUMAN NEUROMUSCULAR JUNCTIONS,
Clarke R. Slater, Muscular Dystrophy Group Research Laboratories, Division of Neurobiology, University of Newcastle upon Tyne, UK.

Human neuromuscular junctions (NMJs) differ from those in rats, mice and frogs in that the nerve terminals are smaller and release fewer quanta of acetylcholine (ACh) per nerve impulse (quantal content). At the same time the postsynaptic folds, which are known to contain a high density of voltage-dependent sodium channels, are particularly extensive in man. We have recently found that the threshold for action potential generation is lower at the NMJ than elsewhere on the muscle fiber, presumably because of the accumulation of sodium channels in the folds. It is likely that this effect is particularly prominent in man and helps to compensate for the relatively small endplate current. Most diseases in man that cause impairment of neuromuscular transmission are either autoimmune disorders involving antibodies that attack the nerve terminal (Lambert-Eaton myasthenic syndrome) or the AChRs (myasthenia gravis) or congenital conditions, presumably inherited, the so-called congenital myasthenic syndromes. Of the latter, what appears to be the most common form among European patients is characterised by reduced quantal content associated with abnormally small NMJs with reduced postsynaptic folding (intially decribed by Jennekens et al. as a 'congenital paucity of secondary synaptic clefts' (CPSC) syndrome). This reduction of folding is likely to intensify the impact of the low quantal content by reducing the density of postsynaptic sodium channels. Nothing is yet known about the molecular or genetic basis for what appears to be impaired development or maturation of the NMJ in these patients. In a number of other forms of congenital myasthenic syndrome, abnormalities in the properties or abundance of AChRs have been described. In the 'slow-channel' syndrome (SCS) the mean open time of the ACh-gated ion channels is prolonged. In a number of other patients in whom ACh-gated channel behavior is abnormal, mutations of the AChR have recently been described by AG Engel. A further group of patients have been described over the years in whom impaired transmission results from a congenital AChR deficiency. We have recently studied several of these patients and found a striking reduction in the intensity of immunolabelling of the postsynaptic protein utrophin. This autosomally coded homolog of dystrophin is closely associated with AChRs at the NMJ in adults and at all stages of development. Immunolabelling of several other proteins which are normally concentrated at the adult NMJ (including dystrophin, β-dystroglycan, β-spectrin and the 43kD and 58kD AChR-associated proteins) is present at more normal intensity, suggesting that the reduction of utrophin labelling is relatively specific. The genetic defect in these patients is not known; preliminary studies have not revealed large-scale abnormalities of the mRNA for utrophin or AChRs.

B5-011 STRUCTURE AND REGULATION OF EXPRESSION OF THE ACETYLCHOLINESTERASE GENE. Palmer Taylor, Shelley Camp, Annick Mutero, Damon K. Getman, Zhigang Luo, and Barbara A. Coleman. Dept. of Pharmacology, Univ. of California, San Diego, La Jolla, CA 92093-0636.

Acetylcholinesterase (AChE) is the product of a single gene in mammals and this has led to distinctive characteristics in the regulation of its expression. In contrast to nicotinic acetylcholine receptor subtypes which are products of polygenic systems, sequence differences in AChE arise from alternative mRNA splicing of a single gene. Splice preferences depend on the tissue and its differentiation. Alternative splicing leads to 3 distinct carboxyl-termini which allow for: (a) disulfide linkages between catalytic subunits or catalytic and structural subunits, (b) cleavage of a hydrophobic peptide and addition of glycophospholipid to tether the enzyme to the membrane outer face, and (c) formation of a soluble monomeric form of AChE. Enhanced expression of the nicotinic receptor during development in muscle arises largely from transcriptional activation, while AChE expression is increased upon muscle and nerve differentiation by virtue of stabilization of a labile mRNA. Superinduction with protein synthesis inhibitors can be uniquely demonstrated for *AChE* gene expression. mRNA stabilization appears to be controlled by intracellular Ca^{2+} concentrations through a signalling system that has several unique features.

Post-Synaptic Cytostructures

B5-012 THE SUBMEMBRANE MACHINERY FOR ACETYLCHOLINE RECEPTOR CLUSTERING.
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Clustering of nicotinic acetylcholine receptors (AChRs) is one of the earliest signs of membrane differentiation at newly-formed neuromuscular synapses. Small aggregates of AChR begin to form within hours of the initial contact between nerve and muscle, and continue to grow in size and density during prenatal and postnatal development. At the adult rat neuromuscular junction, AChR are present at densities of 8000-10,000 receptors per μ^2 of postsynaptic membrane surface. Since AChR are transmembrane proteins that are inherently capable of diffusion within the plane of the membrane, they must be anchored at synaptic sites, presumably via interactions with the postsynaptic cytoskeleton and other proteins. Signals from the nerve that regulate AChR clustering, such as agrin, may act on the cytoskeleton to stimulate its assembly or its interaction with the receptor. The 43K protein, a peripheral membrane protein associated with cytoplasmic sites on the AChR, appears to be a key element of this complex. Recombinant AChRs expressed in oocytes or fibroblasts are diffusely-distributed on the cell surface. When coexpressed with the 43K protein, AChRs become organized into clusters. Clustering activity is inherent to the 43K protein, since clusters of 43K protein form on the membrane in the absence of the AChR.

In addition to the 43K protein, several other postsynaptic cytoskeletal proteins may play an important role in AChR clustering. These include actin, spectrin, and certain members of the dystrophin family, including utrophin and the 87K protein. Another protein associated with the dystrophin family of proteins is syntrophin (also known as 58K protein and DAP-59). Syntrophin is associated with all members of the dystrophin family, including utrophin, and the short forms (71K, 87K) which contain only the cysteine-rich, carboxy terminal (CRCT) superdomain. Three isoforms of syntrophin with extensive sequence homology have been identified by molecular cloning. These can be divided into two groups, based on their isoelectric points: α-syntrophins (acidic) and β-syntrophins (basic). Two forms of β-syntrophin (β1 and β2) were found. α1-syntrophin is expressed at highest levels in skeletal muscle. Both β-syntrophins have a broad tissue expression pattern, although the levels in skeletal muscle are low. The expression patterns of α and β-syntrophins are similar to dystrophin and utrophin, respectively. Immunostaining for β2-syntrophin is localized to the neuromuscular junction; no extrasynaptic staining was found. Thus, at this synapse, β2-syntrophin may be associated with utrophin. Given its location, this syntrophin isoform may be important in AChR clustering, especially since it is associated with the complex of proteins known to contain a major binding site for agrin.

B5-013 DENDRITIC SPINES: CELLULAR SPECIALIZATIONS FOR SYNAPTIC FLEXIBILITY AND STABILITY
Kristen M. Harris, Dept. Neurology, Children's Hospital and Harvard Medical School, Boston, MA.

Dendritic spines are the tiny protrusions that stud the surface of many neurons and they are the location of over 90% of all excitatory synapses that occur in the central nervous system. Their small size and variable shapes has made detailed study of their structure by conventional approaches difficult. Yet their widespread occurrence and likely involvement in learning and memory has motivated extensive efforts to obtain quantitative descriptions of spines in both steady state and dynamic conditions. At this symposium we will discuss evidence from serial electron microscopy and modeling studies for 4 key effects of dendritic spine structure on synaptic function. First, dendritic spines allow for more synapses to interdigitate within a restricted brain volume. Second, a constricted spine neck transiently enhances the potential that can be achieved in the spine head thereby facilitating the opening of voltage-dependent channels at spine synapses. Third, spine necks are sufficiently short and wide such that the potential achieved at one spine head can ultimately be shared amongst neighboring spines, thereby facilitating co-activation and association of synaptic input over time. Fourth, spine heads are restricted biochemical compartments wherein the concentration of activity-dependent molecules are elevated following synaptic transmission, thereby facilitating the specificity and endurance of synaptic plasticity while reducing the likelihood of cytotoxicity. In the context of these hypotheses, we will describe differences in the three-dimensional structure and subcellular composition of dendritic spines from three brain regions (hippocampal areas CA1 and CA3, and cerebellar Purkinje cells). Finally, preliminary results will be presented from our ongoing research into the effects of long-term potentiation on the structure of dendritic spines and their presynaptic axons in hippocampal area CA1. Supported by NIH-#NS21184 and MR Center Grant #P30-HD18655.

Structure and Function of Neuronal Receptors

B5-014 STRUCTURE AND SYNAPTIC LOCALIZATION OF INHIBITORY GLYCINE RECEPTORS, Heinrich Betz, Jochen Kuhse, Bodo Laube, Volker Schmieden, Maria Fischer, Dieter Langosch, Guido Meyer, Joachim Kirsch and Markus Ramming, Department of Neurochemistry, Max-Planck-Institute for Brain Research, D-60528 Frankfurt, FRG

The inhibitory glycine receptor (GlyR) is a pentameric chloride channel protein, which exists in several developmentally regulated isoforms in the CNS. These result from the differential expression of four genes encoding different variants of the ligand-binding α subunit of the GlyR. The stoichiometric assembly of these α subunits with the β subunit is governed by "assembly cassettes" within the extracellular domains of these proteins and creates chloride channels of distinct conductance properties. Upon affinity chromatography, the GlyR copurifies with the tubulin-binding peripheral membrane protein, gephyrin. During development, the GlyR and gephyrin colocalize in the postsynaptic membrane. This colocalization involves: i) accumulation of different alternative splicing variants of gephyrin; ii) formation of gephyrin clusters at presumptive postsynaptic sites; and iii) accumulation of GlyRs at these gephyrin-rich membrane domains. The molecular mechanisms underlying these processes will be discussed.

Regulation of Neuronal Receptors

B5-015 CO-EXPRESSION OF MULTIPLE ACETYLCHOLINE RECEPTORS DEFINED BY DISTINCTIVE SUBUNIT COMPOSITION IN NEURONS AND A COMPARISON OF FUNCTIONAL PROPERTIES. Darwin K. Berg, William G. Conroy, Phyllis C. Pugh, Margaret M. Rathouz, Suzanne J. Romano, Sukumar Vijayaraghavan, and Zhong-wei Zhang, Department of Biology, University of California, San Diego.
The muscle acetylcholine receptor (AChR) represents the best characterized ligand-gated ion channel with its pentameric structure containing two α1 subunits, a β1, a δ and either a γ or ε subunit. Subsequent gene cloning efforts have yielded gene families encoding subunits of receptors for each of the common neurotransmitters. Despite this progress, relatively little is known about the combinations of subunits making up native ligand-gated ion channels or the functional and regulatory features of individual receptor subtypes that might account for the maintenance of so many genes within a single family. Putative neuronal AChRs genes include ten: seven α-type genes (α2-α8) and three β-type genes (β2-β4). Two of these alone (α7, α8) and others in combination (α2, α3, or α4 together with either β2 or β4) produce functional AChRs when expressed in Xenopus oocytes. Combining α4 and β2 cRNA in oocytes yields receptors having two α and three β subunits. More complex patterns emerge in vivo. Ciliary ganglion neurons express five of the ten known neuronal AChR genes (α3, α5, α7, β2, and β4) both at early and late developmental times. Previous work has shown that the neurons have two major classes of AChRs. One class, identified by binding the monoclonal antibody (mAb) 35, is largely synaptic in location, mediates synaptic transmission through the ganglion, and collectively contains the α3, β4, and α5 gene products (mAb 35-AChRs). The other class, identified by binding α-bungarotoxin (αBgt) but not mAb 35, is largely non-synaptic in location, functions as a ligand-gated ion channel though its role in ganglionic function has yet to be determined, and contains only α7 of the known neuronal AChR gene products (αBgt-AChRs). Using subunit-specific mAbs and a two-site solid phase immunoprecipitation assay, we now find that a fifth of the mAb 35-AChRs contain the β2 gene product and that it is associated with α3, β4, and α5 subunits, suggesting that a class of native AChRs on neurons can be as complex in composition as muscle receptors. Since essentially all ciliary ganglion neurons produce β2 subunits, the heterogeneity found in mAb 35-AChRs is unlikely to result from differences in gene expression among the neurons. In addition to αBgt-AChRs and mAb 35-AChRs both with and without β2 subunits, subunit-specific mAbs identify a fourth and minor class of AChRs in the ganglion, one that binds both mAb 35 and αBgt. It appears to lack all of the known neuronal AChR gene products including muscle α1 subunits though, surprisingly, trace amounts of α1 transcript are present and the α1 subunit could, in principle, bind both mAb 35 and αBgt. The results suggest that the neurons produce at least four types of neuronal AChRs and that one class is composed of subunits encoded by genes yet to be identified. Multiple AChR subtypes are not a unique feature of neurons; we find that embryonic muscle expresses α7-containing AChRs in addition to the conventional α1-containing AChRs, raising questions about the traditional division between neuronal and muscle AChR genes. Potentially unique functions for the AChR subtypes on ciliary ganglion neurons have yet to be identified. Both mAb 35-AChRs and αBgt-AChRs function as ligand-gated ion channels and are also efficient at elevating intracellular calcium levels. They do so both by being permeable to calcium and by depolarizing the cell sufficiently to activate voltage-gated calcium channels which then permit additional calcium influx. The neurons also have muscarinic receptors that rely in part on intracellular stores to elevate intracellular free calcium in response to ACh. The possibly unique contributions of individual receptor subtypes could reside in their locations, distinctive regulation, or conditions of activation.

B5-016 SYNAPTOGENIC SIGNALS IN IDENTIFIED NEURONS. Pierre Drapeau, Centre for Research in Neuroscience, McGill University and Montreal General Hospital Research Institute, Montreal, Quebec, H3G 1A4, Canada.

Although much is being revealed about the cellular and molecular mechanisms of synapse formation at the neuromuscular junction (NMJ), relatively little is known about these events in the CNS. In particular, we are interested in understanding how individual neurons recognize and innervate appropriate targets once they come into contact during development. In order to study these synaptogenic interactions, we have been examining the signals between identified leech neurons which reform specific synapses in culture.

At an inhibitory serotonergic synapse between two well-studied neurons, the postsynaptic cell has an additional (extrasynaptic) excitatory response to 5-HT which underlies a form of activity-dependent modulation. Thus, the presynaptic neuron must select which 5-HT response will be activated and which will be excluded at its synapses. The selection of these responses preceded synapse formation and was specifically induced at sites of contact with the presynaptic neuron, this not being observed for other cell pairings.

The mechanism underlying the selective loss of the extrasynaptic response has been examined by single channel recording. Cation channels in the postsynaptic neuron were modulated by protein kinase C (PKC) upon binding of 5-HT to a 5-HT_2 receptor. However, at sites of contact with the presynaptic neuron, the channels were no longer sensitive to PKC. Furthermore, when cation channels from uncontacted neurons were inserted or "crammed" into contacted neurons, they were rapidly rendered insensitive to PKC, demonstrating a cytoplasmic signal for the uncoupling of channel modulation. Interestingly, the cytoplasm of contacted postsynaptic neurons showed immunoreactivity for tyrosine phosphorylation; exposure of the neurons to specific inhibitors of tyrosine kinases prevented tyrosine phosphorylation, the selective loss of cation channel modulation and synapse formation.

These results suggest that tyrosine phosphorylation is an important signal during the formation of this synapse, as has been found for AchR clustering and synthesis at the NMJ. Tyrosine phosphorylation appears to trigger the selection of transmitter responses at sites of contact, which is followed by the establishment of functionally appropriate synapses. Local signalling could thus be a means to initiate synapse formation at regions remote from the neuronal cell body.

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Sensory Signal Transduction

B5-017 ION-CHANNEL CLUSTERS AND MICRODOMAINS OF ELEVATED Ca^{2+} CONCENTRATION AT PRESYNAPTIC ACTIVE ZONES OF HAIR CELLS. A. J. Hudspeth and N. P. Issa, Howard Hughes Medical Institute and Center for Basic Neuroscience Research, University of Texas Southwestern Medical Center, Dallas, TX 75235-9117.

Ca^{2+} channels subserve two important processes in hair cells, the mechanoreceptors of the auditory and vestibular systems. First, synaptic signaling from such a cell to afferent nerve fibers depends upon the influx of Ca^{2+} at presynaptic active zones. Second, the responsiveness of a hair cell may be tuned to a specific frequency of stimulation by an electrical resonance that involves an interplay between currents borne by Ca^{2+} channels and Ca^{2+}-activated K^+ channels. In a hair cell from the frog's sacculus, these Ca^{2+} and K^+ channels congregate in approximately 20 patches on the basolateral cellular surface[1]. Because each channel cluster contains about 90 Ca^{2+} channels, the entry of Ca^{2+} at individual active zones can be studied with exceptional spatial and temporal resolution in hair cells. Moreover, the Ca^{2+} indicator fluo-3 specifically labels a hair cell's presynaptic densities, and thus provides a highly sensitive measurement of the local Ca^{2+} concentration at active zones[2]. By simultaneously monitoring the whole-cell Ca^{2+} current and the rise and fall of fluo-3 fluorescence intensity, we measured the time course of Ca^{2+} entry, accumulation, and diffusion in response to depolarizing pulses. During large depolarizations, the Ca^{2+} concentration approximately 200 nm from the presynaptic membrane approached a steady state with a time constant of less than 10 ms. Because the space constant of fluo-3 fluorescence at steady state was less than the sectioning depth of the confocal imaging system, we developed a numerical algorithm to compensate for the effects of out-of-focus fluorescence. Application of this algorithm to fluorescence profiles obtained by rapidly scanning across maximally stimulated active zones indicated that the presynaptic Ca^{2+} concentration reached hundreds of micromolar. Inclusion in the whole-cell pipette of an exogenous Ca^{2+} buffer, such as BAPTA or EGTA, slowed the approach to steady-state fluorescence and restricted the spread of fluorescence from the presynaptic membrane. Both the concentration and the *on* rate of this exogenous Ca^{2+} buffer affected the steady-state fluorescence and Ca^{2+} concentration. The observed dynamics of the presynaptic Ca^{2+} concentration is consistent with models of Ca^{2+} entry at and diffusion from a point source.

This research was supported by NIH grant DC00241.

[1] Roberts, W. M., R. A. Jacobs, and A. J. Hudspeth (1990) Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. *J. Neurosci.* **10:** 3664-3684.

[2] Issa, N. P. and A. J. Hudspeth (1994) Clustering of Ca^{2+} channels and Ca^{2+}-activated K^+ channels at fluorescently labelled presynaptic active zones of hair cells. *Proc. Natl. Acad. Sci. USA* **91:** 7578-7582.

Modulation of Synaptic Function

B5-018 SYNAPTIC MODULATION IN THE CONTEXT OF A DEFINED NEURAL NETWORK. Ronald M. Harris-Warrick[1], Bruce R. Johnson[1], and Jack H. Peck[2], [1]Cornell University, Ithaca, NY 14853, [2]Ithaca College, Ithaca, NY 14850.

In order to understand the functional role of synaptic modulation in shaping the output from a neural network, it is important to determine how all the components in the network are modified rather than focusing on only one or a few synapses. The small, well-defined central pattern generator networks that drive simple rhythmic motor patterns are excellent model systems to study how neuromodulators alter neural network output. We are studying the roles of the amines dopamine, serotonin and octopamine as extrinsic modulators of the 14-neuron pyloric network in the crustacean stomatogastric ganglion. These modulators evoke unique variants of the pyloric rhythm by two major mechanisms. First, they alter the strength of graded synaptic transmission between identified neurons. Each amine affects nearly every synapse in the network. A single amine typically has a variety of different effects on different synapses within the same network, including enhancement and diminution of synaptic strength, activation of silent synapses, modulation of electrical coupling and sign reversal at mixed electrical/chemical synapses. When rigorous attempts are made to eliminate all modulatory input to the network, many synapses become functionally weak or even silent; these synapses appear to require modulatory input in order to be functional. The effects of an amine are often subtle (10-50% changes in strength) but are distributed across all the synapses within the network. This fits with theoretical models showing that small changes in synaptic efficacy can evoke large changes in network output when distributed throughout a network. A second major mechanism for modulation of network output is to alter the intrinsic response properties of neurons so that their interpretation of synaptic input (which itself could be unaffected) is qualitatively changed. In one neuron, serotonin can evoke bistability, where a brief excitatory input can evoke a prolonged bursting response rather than a time-locked excitatory response. This is accomplished by a complex mechanism including diminution of $I_{K(Ca)}$ and enhancement of I_h and I_{Ca} (which in turn enhances I_{CAN}). In other neurons dopamine evokes changes in the strength of postinhibitory rebound by modulation of subthreshold currents including I_A and I_h. This leads to a marked change in the phasing and intensity of firing of these neurons in the pyloric motor pattern. Thus, modulation of synaptic strength and the intrinsic response properties of component neurons can qualitatively change the network, such that a single anatomically defined circuit can generate a large family of related behaviors. Supported by NIH NS17323.

B5-019 SIGNAL TRANSDUCTION MOLECULES IN THE POSTSYNAPTIC DENSITY, Mary B. Kennedy, R.V. Omkumar, Il Soo Moon, Jeff Rawlings, Michelle Apperson, Division of Biology, California Institute of Technology, Pasadena, CA 91125.

The postsynaptic density (PSD) is a junctional protein complex located on the postsynaptic side of central nervous system synapses, in association with the presumed site of postsynaptic receptors. The PSD is particularly large and easy to visualize at excitatory glutamatergic synapses which are known to undergo a variety of plastic changes in their synaptic strength. Thus, it seems likely that some of the molecules critically involved in postsynaptic plasticity are associated with the PSD. To gain insight into the organization and function of the PSD, we have undertaken the identification of PSD-associated proteins using the following paradigm: 1. Major proteins from the PSD fraction (prepared from forebrain as described by Cohen et al.[1]) are solubilized and purified by SDS gel electrophoresis. 2. Tryptic fragments from each protein are purified and sequenced by High Pressure Liquid Chromatography. 3. Based upon these sequences, oligonucleotides are designed and full length cDNAs are isolated and sequenced. 4. The localization of the encoded protein is confirmed by immunocytochemistry. Our findings, together with earlier work, support the general hypothesis that the PSD is a specialization of the submembranous cytoskeleton that contains complexes of transmitter receptors and signal transduction molecules that are activated by the receptors or that modulate them. In addition, the PSD may contain specific synaptic adhesion molecules.

We have found four different types of molecules associated with the PSD fraction from the forebrain. The α and β subunits of calcium/calmodulin-dependent protein kinase II (CaMKII) are prominent PSD components, with the α subunit comprising 20 to 40% of the total protein in the fraction. We have verified the presence of the α-subunit in the PSD by immunocytochemistry at the electron microscope level. Genetic[2] and pharmacological[3] studies have shown that CaMKII is an important postsynaptic link in the induction of both long-term potentiation and long-term depression. Influx of Ca^{2+} through the NMDA-type glutamate receptor is believed to be the trigger that activates CaMKII along with other Ca^{2+}-dependent processes. The 2B subunit of the NMDA-receptor is a second molecule that we have found tightly associated with the PSD fraction. This subunit is phosphorylated both by CaMKII and by an unidentified tyrosine kinase. A third class of PSD molecule that we have identified is PSD-95, a homologue of two other junction-associated proteins, discs-large and ZO-1. The homology among these proteins implies an evolutionary relationship that was previously not appreciated among septate junctions in insects, tight junctions, and synaptic junctions. Furthermore, wild type discs-large is essential for formation of septate junctions during fly development. Hence, PSD-95 is likely to play a critical role in assembly of synaptic junctions. All three homologues contain three 90 amino acid repeats, termed GLGF repeats, at their amino terminus; an SH3 domain; and a partially disrupted guanylate kinase domain that is essential for wild type function. Finally, we have recently identified a 195 kDal presumptive adhesion molecule that is highly enriched in the PSD fraction and highly localized to synapses by immunocytochemistry. It is a leucine-rich repeat protein with a domain structure similar to that of the platelet adhesion molecule GP-Ibα and it contains a single GLGF domain at the carboxyl terminus. Each of these molecules reveals new possibilities for the functional roles of the PSD in synaptic plasticity.

[1] Cohen et al. 1977. J. Cell Biol., 74: 181-203. [2] Silva et al., 1992. Science, 257: 201-206. [3] Malinow et al., 1989. Science, 245: 862-866.

Late Abstract

MOLECULAR CONTROL OF NEURONAL LIGAND GATED ION CHANNEL EXPRESSION DURING SYNAPTOGENESIS

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Neural development and synaptogenesis require co-ordinate expression of components of transmitter release and the appropriate transmitter receptors. In specific CNS and PNS neurons we and others have found that both innervation and target contact are accompanied by increases in the rate of nAChR synthesis and insertion, alterations in the biophysical and pharmacological profile of the nAChR channel subtypes and spatial segregation into high density, subtype specific clusters. Since innervation and target contact are nearly concurrent during in vivo development in embryonic chick, we are attempting to analyze the role of presynaptic input vs. target contact in regulating nAChR channel expression with primary co-cultures of either: (a) septal (cholinergic) neurons (SN), medial habenula (MHN) and neurons of the interpeduncular nucleus (IPN) or (b) visceral motoneurons (VMN), lumbar sympathetic neurons (LSN) and various sympathetic targets.

VMN explants or medium conditioned by either VMN or somatic motoneurons increase the expression of nAChR subunit genes as well as increasing the number of surface nAChR channels. Since previous studies of Fischbach and colleagues revealed that ARIA, a splice variant of the Heregulin, neu differentiation factor, and glial growth factor family, increases transcription of muscle type nAChR subunits[1] we searched for related molecules specifically expressed in CNS neurons projecting to cholinoceptive targets. We have cloned a novel splice variant ("nARIA"), unique in the N-terminal sequence, that is expressed by E3-4 in specific CNS nuclei, including the SN and VMN. Transfection of COS1 cells with either ARIA or nARIA cDNA results in the release of recombinant proteins that activate a protein tyrosine kinase and may differentially regulate both nAChR and GABA-R expression in primary LSNs. Treatment of LSNs with recombinant nARIA mimics the effects of VMN innervation, increasing the levels of α3, α5, α7 and β4 subunit mRNAs, significantly increasing macroscopic currents gated by applied ACh, but having little effect (either increasing or decreasing) GABA-evoked macroscopic currents. In contrast, ARIA increases the levels of α5 and α7 yet appears to depress α3 and β4 expression, has little effect on ACh-evoked current amplitude and enhances GABA sensitivity. Thus, our data suggest that nARIA may be an important molecular signal in the control of nAChR expression in the CNS and PNS.

1. Falls et al., Cell 72, 801-815, 1993.

Growth Cones and Neurite Guidance;
Target Selection and Synaptic Specificity
Presynaptic Components and Vesicular Release

B5-100 GENETIC DISSECTION OF PRESYNAPTIC PROTEINS IN DROSOPHILA. Kendal Broadie, Department of Zoology University of Cambridge, Cambridge CB2 3EJ, United Kingdom.
I have characterized the phenotype of genetic mutations in Drosophila that remove four proteins implicated in excitation-secretion coupling at the synapse: synaptotagmin, syntaxin, Rop (Drosophila member of the sec-1 family) and, indirectly, neural synaptobrevin (n-syb). All of these proteins show strong homology with their vertebrate counterparts and all are highly enriched in Drosophila synapses. Null mutations in each of these proteins leads to early lethality. However, the availability of the embryonic NMJ has allowed me to assay the physiology of synapses lacking these proteins. Two of these proteins, Rop and syntaxin, play essential roles in the general cellular exocytosis machinery as well as synaptic transmission. Null mutations in both genes block secretion from multiple cell types and null mutants of syntaxin, at least, completely lack evoked vesicle release. In contrast, the other two proteins, synaptotagmin and n-syb, play neural-specific roles in synaptic transmission. Null mutations in synaptotagmin do not block transmission but rather dramatically reduce the efficiency and fidelity of excitation-secretion coupling. The role of synaptobrevin was assayed in genetically-transformed flies expressing the tetanus neurotoxin under gal4 control; tetanus cleaves synaptobrevin specifically. In the absence of functional protein, evoked vesicle release is completely lost, though spontaneous vesicle release continues. Thus, both synaptotagmin and n-syb appear to be specialized neuronal regulators of exocytosis in synaptic transmission.

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B5-101 **SINAPTOSOMAL MICROTUBULAR PROTEIN ANALYSIS IN EXPERIMENTAL HYPOTIROIDIAN RATS L. V. Chamas, H. Araujo,C. Chagas, V. Moura Neto** Instituto de Biofisica Carlos Chagas Filho-UFRJ-BRASIL

Experimental neonatal hypothyroidism in rats produces an impairment of proliferation and migration of nerve cells, an hypoplasia of the arborization of neuronal processes and retardation synapse formation in several brain areas. Furthermore, thyroid hormones have been shown to play a crucial role in tubulin assembly and stability, and to regulate the expression of two specific Maps, Tau and MAP_2. These microtubular proteins show an increased polymorphism during brain development and contribute to synapse formation in adult brain. We have prepared synaptosomal fractions from normal and hypothyroid adult brain and analyzed the proteins by 2D gel electrophoresis and imunoblotting procedures. Synaptosomal preparations were firstly examined by electron microscopy and then, total proteins, were extracted and analyzed by gel electrophoresis. Several synaptosomal 50-70 KDa proteins from hypothyroid animals migrating on the pH 5-6 region in 2D gel show a lower expression than in normal Synaptosoma. Brain tubulin synthesis seems to be affected in hypothyroid animals. Tau proteins, which are involved in tubulin assembly, show different patterns in these conditions. such changes in components of the cytoskeleton could cause the observed retardation in synapse formation in several brain areas.

B5-102 FLUORESCENT LABELING OF PRESYNAPTIC DENSE BODIES IN HAIR CELLS, Naoum P. Issa and A. J. Hudspeth, Howard Hughes Medical Institute and the Center for Basic Neuroscience Research, University of Texas Southwestern Medical Center, Dallas, TX 75235.
Cells that release neurotransmitter in response to graded changes in membrane potential have presynaptic active zones with three prominent structural components in transmission electron micrographs: a large cytoplasmic density known in hair cells as the presynaptic dense body and in photoreceptors as the synaptic ribbon, a complement of clear-cored synaptic vesicles, and an osmiophilic presynaptic density. Neither the chemical constitution nor the function of the dense body is known. We have found that the Ca^{2+} indicator fluo-3 specifically labels the presynaptic active zones of the frog's saccular hair cell (Issa and Hudspeth, 1994 *Proc. Natl. Acad. Sci. USA,* **91:** 7578-7582). To determine whether fluo-3 binds to dense bodies or accumulates in synaptic vesicles, we isolated from the axolotl (*Ambystoma mexicanum*) lateral line hair cells with exceptionally large presynaptic dense bodies (greater than 1 μm in diameter). Confocal images of fluo-3-loaded cells showed homogenous fluorescence throughout the dense bodies, suggesting that the components of the dense body itself bind fluo-3. Fluorescent probes that displayed similar staining patterns include both the pentapotassium and pentaammonium salts of fluo-3, Calcium Greens 1 and 2, and Lucifer Yellow CH. Because these dyes conjugated to dextrans also labeled dense bodies, there is relatively free molecular access to the binding sites. Labeling is not purely a function of anionic nature of the fluorophores, for anionic forms of tetramethylrhodamine dextran and fluorescein dextran do not label the dense body. Further characterization of the binding properties of dense bodies will give insight into their molecular nature and may lead to a technique for their isolation and biochemical characterization.

B5-103 INHIBITION OF CLATHRIN ASSEMBLY BY HIGH-AFFINITY BINDING OF SPECIFIC INOSITOL POLYPHOSPHATES TO THE SYNAPSE-SPECIFIC CLATHRIN ASSEMBLY PROTEIN AP-3, Eileen M. Lafer*, Weilan Ye+, Stephen B. Shears#, Nawab Ali#, and Michael E. Bembenek@, *Center for Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center, 15355 Lambda Drive, San Antonio, TX, 78245; +Department of Biological Sciences, University of Pittsburgh, Pittsburgh PA, 15260; #Inositol Lipid Section, Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709; and @ E.I. Du Pont Nemours & Co. (Inc.) 549 Albany St., Boston, MA 02118
Recent reports have indicated a role for inositol polyphosphates in vesicular traffic. Consequently, we set out to determine whether the synapse-specific clathrin assembly protein AP-3 (F1-20/AP180/NP185/pp155) could bind to inositol polyphosphates. We found that bacterially expressed AP-3 bound with high affinity both inositol hexakisphosphate ($InsP_6$) (K_D =239 nM) and diphosphoinositol pentakisphosphate (PP-$InsP_5$) (K_D =22 nM). The specificity of this ligand binding was demonstrated by competitive displacement of bound [^{3}H]$InsP_6$. IC_{50} values were as follows: PP-$InsP_5$ = 50 nM, $InsP_6$ = 240 nM, Ins(1,2,4,5,6)P_5 = 2.2 μM, Ins(1,3,4,5,6)P_5 = 5 μM, Ins(1,3,4,5)P_4 > 10 μM, Ins(1,4,5)P_3 > 10 μM. Moreover, 10 μM inositol hexasulfate ($InsS_6$) displaced only 15% of [^{3}H]$InsP_6$. The physiological significance of this binding is the ligand-specific inhibition of clathrin assembly (PP-$InsP_5$ > $InsP_6$ > Ins(1,2,4,5,6)P_5); Ins(1,3,4,5,6)P_5 and $InsS_6$ did not inhibit clathrin assembly. We also observed high affinity binding of $InsP_6$ to purified bovine brain AP-3. We separately expressed the 33 kD amino terminus and the 58 kD carboxy terminus, and it was the former that contained the high affinity inositol polyphosphate binding site. These studies suggest that specific inositol polyphosphates may play a role in the regulation of synaptic function by interacting with the synapse-specific clathrin assembly protein AP-3.

B5-104 CALCIUM LEVELS AFFECT THE STABILITY OF PROTEIN COMPLEXES IN CHOLINERGIC SYNAPSES FROM TORPEDO ELECTRIC ORGAN, Michal Linial, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel

Protein complexes at the nerve terminal are crucial for accurate and timely neurotransmitter release. Interference with the stability of such complexes results in blockage of release. Here we present a biochemical study on*Torpedo* synapses where the stability of protein complexes is shown to be modulated by calcium levels.
VAT-1 is a major 41 kDa protein of the synaptic vesicle membrane. Its hydrodynamic characterization suggests that it is composed of 4 subunits which form a protein complex of 170 kDa. The binding of these subunits is partially disrupted by chelating calcium ions. Recombinant VAT-1 is, in itself, a low-affinity calcium binding protein.
The effect of calcium on the stability of protein complexes is also evidenced by our study on *Torpedo* syntaxin. Syntaxin is a key component in a protein complex which was implicated in vesicular targeting as well as in docking and priming of synaptic vesicles. Following gel electrophoresis,*Torpedo* syntaxin was resolved into 2-3 discrete bands of 35-36 kDa which correspond to different modified forms of a single protein. Changes in calcium concentration during synaptosomes solubilization altered the number of distinct syntaxin variants as well as their relative quantitaties. These modified forms of syntaxin represent different levels of phosphorylation. *Torpedo* syntaxin is found primarily in two high molecular weight protein complexes of 8S and 4-5 S. The distribution of syntaxin variants in these complexes is also calcium dependent. Specifically, we observed that the distribution of the unmodified variant is calcium independent, while one of the modified variants was detected in the 8S protein complex only at the presence of calcium Nevertheless, the syntaxin-VAMP/synaptobrevin interaction was shown to be calcium independent. Our findings demonstrate how calcium levels modulate and consequently affect the interaction within key components of the docking-release apparatus. This suggests that structural changes by calcium might serve to modulate synapse activity.
This work was supported by the Israeli Academy of Science.

B5-105 PRESYNAPTIC COMPONENT OF LONG TERM POTENTIATION VISUALIZED AT INDIVIDUAL CA3-CA1 SYNAPSES
A.Malgaroli#, T.Ting*, B.Wendland, A.Bergamaschi#, A.Villa#, RW.Tsien*, R.Scheller* (#) Dibit, H.S.Raffaele, Via Olgettina 58, Milano 20132, Italy, (*)MCP,Beckman Cnt, Stanford University, Stanford 94305 CA, USA.
LTP has been extensively investigated with electrophysiological techniques which allow great resolution of synaptic events but do not easily separate presynaptic from postsynaptic contributions. Thus we have directly visualized changes in exo-endocytic cycling at individual CA3-CA1 hippocampal synapses by monitoring differential synaptic uptake of antibodies raised against the intraluminal domain of synaptic vesicle protein synaptotagmin. We found that LTP induction leads to a clear enhancement of synaptic exo-endocytic cycling. Although we cannot exclude the possibility of a concomitant postsynaptic change, we can say that the increase in vesicular turnover is clearly free of many interpretational caveats including the possible contribution of silent receptor clusters. Importantly the weight changes are heterogeneously distributed in the synaptic population. A potent determinant of the plasticity potential is the basal synaptic activity, as low p or semi-quiescent synapses are more enhanced then high p or highly active synapses. As APV was present during the pre-induction period, we can exclude that high p/low p synapses translate directly to high/low NMDA receptor activation levels, thus explaining the differential facilitation. We speculate that this property reflects a quasi-digital behavior with presynaptic weights fluctuating between two different levels. In future experiments this method can be used to elucidate spread of LTP to nearby synapses and to visualize engrams or memory traces coded as presynaptic weight changes in synaptic matrixes.

B5-106 **A RADIOIMMUNOASSAY TO MONITOR SYNAPTIC ACTIVITY IN HIPPOCAMPAL NEURONS IN VITRO**
Olaf Mundigl[a], Claudia Verderio[b], Kajetan Kraszewski[a], Pietro De Camilli[a] and Michela Matteoli[b], [a]Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, Boyer Center for Molecular Medicine, New Haven, CT, USA, and [b]Department of Pharmacology, CNR Center of Cytopharmacology/ "Bruno Ceccarelli" Center of Peripheral Neuropathies, University of Milan, Italy

Exocytosis of synaptic vesicles (SV) results in the surface exposure of lumenal epitopes of SV proteins. We have recently described the use of antibodies directed against the lumenal N-terminus of synaptotagmin I (Syt_{lum}-Abs) to morphologically monitor exo-endocytic recycling of SVs. We report here that a radioimmunoassay based on these antibodies can be used to quantify levels of synaptic activity in primary neuronal cultures. High density cultures of hippocampal neurons grown in the absence of glia were used for these studies. A significant cell surface pool of synaptotagmin I immunoreactivity was detectable by Syt_{lum}-Abs at steady sate. The increase in the amount of Syt_{lum}-Abs which became cell bound during a 3 min incubation at 37°C over the Ab binding due to this cell surface pool, was estimated to be 18-fold higher in depolarizing media containing extracellular Ca^{2+} than in Ca^{2+}-free media. Incubation of the cultures with Syt_{lum}-Abs for longer time periods indicated a sustained increase in the rate of SV exocytosis in depolarizing media which lasted for at least 1hr. This increase was completely abolished by pretreating the neurons with tetanus toxin and this block correlated with a disappearance of synaptobrevin immunoreactivity. This radioimmunoassay offers therefore a new way to monitor SV exocytosis of large populations of neurons in vitro irrespective of the type of neurotransmitter secreted and of postsynaptic effects.

B5-107 P19 EMBRYONAL CARCINOMA CELLS DIFFERENTIATE INTO FUNCTIONAL CHOLINERGIC NEURONS, Parnas D. and Linial M., Department of Biological Chemistry, The Hebrew University, Jerusalem 91904, Israel

Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, represent good model systems for the study of cellular commitment, differentiation and development. P19 is a mouse-derived EC cell-line capable of differentiation toward ectodermal, mesodermal and endodermal lineages. Thus, P19 cells represent an early stage of development. Following treatment with retinoic acid these cells differentiate into neurons, astrocytes and fibroblast-like cells.
We induce P19 differentiation under conditions which provide a homogenous, neuronal culture (>98% neurons). We show that P19 neurons express a battery of neuronal genes that are not expressed before differentiation. These genes include cytoskeletal proteins (MAP-2, NF-M and tau), synaptic vesicle proteins (synaptophysin, SV2 and VAMP/synaptobrevin) and terminal specific proteins (SNAP-25 and Rab3A). Furthermore, the proteins encoded by these genes are also expressed in P19 neurons, and show a characteristic pattern of staining by immunofluorescence.
The P19 neurons reach a mature state which is indicated by the segregation of neurites into axons and dendrites, and by the ability to release neurotransmitter (acetylcholine) following depolarization with KCl. The release is Ca^{2+} dependent, and drops to baseline levels at 0.5 mM Ca^{2+}. The cells also respond to α-latrotoxin (0.5 nM) by an intense release of ACh.
The cholinergic phenotype of the P19 neurons is further demonstrated by immunostaining for AChE and ChAT. Finally, we show that P19 cells are also able to form synapses with a muscle cell line - C2.
P19 neurons have been shown by others to be primarily GABAergic, when using culturing conditions differing from our own. The major difference is the existence or elimination of the fibroblasts and glial cells which are derived from the P19 cells.
We suggest that the phenotype of P19 neurons is governed by cell-cell interactions between neuronal and non-neuronal cells (fibroblasts and glial cells). Thus, the P19 cell line can serve as a model system for the study of neuronal differentiation and of phenotypic determination.

B5-108 Identification of the phosphorylation sites in MAP kinase kinase 1 in nerve growth factor-stimulated PC12 cells

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Stimulation with nerve growth factor(NGF) causes neurite formation in PC12 cells,probably through the activation of the mitogen-activated protein(MAP) kinase cascade.The activation of MAP kinase requires its phosphorylation on a threonine and a tyrosine residue,catalysed by MAP kinase kinae(MAPKK) which is itself activated by phosphorylation.raf-1 activates and phosphorylates MAPKK-1 at Ser-217 and Ser-221,in vitro.MAP kinase phosphorylates MAPKK-1 at Thr-291 and Thr-385,which do not appear to affect the activity,in vitro.To elucidate the mechanism of activation of MAPKK in vivo,^{32}P-labelled PC 12 cells were stimulated with NGF and chromatographed on Mono Q to seperate active MAPKK from inactive MAPKK,followed by immunoprecipitation.^{32}P-radioactivity associated with the immunoprecipitated inactive MAPKK-1 was 2.3 fold higher than that with active MAPKK-1,indicating that less than 30% of MAPKK-1 is converted to the active form after stimulation with NGF.The immunoprecipitated MAPKK-1 was digested with thermolysin and chromatograghed on a C_{18} column.The digests of active and inactive MAPKK-1 each contained two major ^{32}P-labelled peptides(peak1 and 2) while active MAPKK-1 contained an additionl peptide(peak3).Solid phase sequencing and isoelectric focusing experiments revealed that peak 1 and 2 were peptides containing Thr-291 and Thr-385,respectively.Peak 3 was a phosphoserine-containing peptide which was also generated by thermolytic digestion of MAPKK-1 that had been phosphorylated by raf-1 in vitro.These results indicated that MAPKK-1 is a physiological substrate for MAPkinase and that MAPKK-1 is activated through phosphorylation at Ser-217 and Ser-221,in vivo.

B5-109 EXPRESSION OF *WNT-7A* IN CEREBELLAR GRANULAR CELLS DEPENDS ON INTERACTIONS WITH THEIR PRESYNAPTIC PARTNERS.

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Wnt genes encode secreted proteins implicated in cell fate changes during development. To begin to define the specific cell populations in which *Wnt* genes act, I chose to examine *Wnt* expression in the cerebellum which has a relatively simple structure and contains well characterized cell populations. I found that *Wnt-7A* is expressed in the adult granular cell layer. *Wnt-7A* starts to be expressed from postnatal day 6 to 22 (P6 to P22) to then declines to low levels in adult cerebellum. From P22, *Wnt-7A* expression becomes restricted to the cerebellar lobules I to VIII, showing very low levels of expression in lobules IX and X . In this posterior region, called vestibularcerebellum, granule cells receive inputs from mossy fibers of vestibular origin. These results suggest that *Wnt-7A* expression may be down regulated by vestibular mossy fibers.
To determine the factors that control *Wnt-7A* expression, I analysed the agranular cerebellum of *weaver* mutant mice in which granule cells are present in the EGL at early stages but fail to migrate and then die in the EGL. *Wnt-7A* is undetectable in the EGL of *weaver* animals even though granule cells are still present at these stages as determined by the expression of the granule cell marker, *En-2*. Thus, granule cells from *weaver* fail to express *Wnt-7A* before they die. Moreover, *Wnt-7A* is ectopically expressed in the Purkinje cell layer of P6 and P15 animals and subsequently disappears. It is of interest that in *weaver* animals, mossy fibers, which normally will make synaptic contact with granule cells, form heterologous synapses with Purkinje cells. These results suggest that*Wnt-7A* may play a role during maturation and/or synapse formation of the cerebellum. The role of *Wnt-7A* in cerebellar maturation is currently being investigated.

B5-110 CHARACTERIZATION OF STRUCTURAL PROTEINS OF PHOTORECEPTOR SYNAPSES.

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Photoreceptor synaptic complexes consist of presynaptic terminals that contact a large number of postsynaptic dendrites from bipolar and horizontal cells. This large number of postsynaptic dendrites is accomodated within an invagination of the presynaptic terminal in a complex structural arrangement. At this bell-shaped photoreceptor synapse exocytosis of synaptic vesicles does not occur along the whole presynaptic membrane but is concentrated at distinct "active zones". A key component of these active zones in photoreceptors are conspicuous presynaptic densities, so called synaptic ribbons. We present evidence that a 70kDa-protein is a component of synaptic ribbons. This assumption is mainly based on an autoantibody that specifically recognizes synaptic ribbons if analyzed by immunogold electron microscopy. The molecular characterization of this protein is in progress. With the help of this autoantibody we were able to establish a protocol for the purification of a photoreceptor synapses-enriched membrane fraction that also contains presynaptic densities and its associated structures. First analysis of this retinal membrane fraction by immunoblotting and immunofluorescence on sections of the rat retina revealed the presence of two proteins already known as components of the neuromuscular junction: (i) the actin-binding, peripheral membrane protein dystrophin, the gene product defective in Duchenne's muscular dystrophy, and (ii) one of its membrane attachment proteins: dystroglycan. These data might complement the finding that actin is a main cytoskeletal component of the postsynaptic dendrites. Actin extends from the base to the tips of the finger-like postsynaptic dendrites. Actin was not detected in significant amounts at the presynaptic plasma membrane. In the photoreceptor synapse dystrophin might possess an adhesive function in maintaining the complex structural arrangements of pre- and postsynaptic components. This idea of a structural role of dystrophin and dystrophin-associated proteins such as dystroglycan is supported by the finding that these proteins are not present in significant amounts in structurally less complex synapses as e.g. in the inner plexiform layer of the retina. The functional importance of dystrophin-mediated membrane associations with the photoreceptor synaptic complex can be concluded from night blindness found in cases of Duchenne's muscular dystrophy.

B5-111 NEUROTRANSMITTER RELEASE: MODELING THE EFFECT OF VESICLE PORE DIMENSIONS AND OPENING RATE.

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The mechanism and time course of vesicular neurotransmitter release are unknown. Present hypotheses encompass narrow fusion pores with gap junction dimensions, as suggested for mast cell exocytosis (Almers & Tse, 1990, Neuron 4:813-818; Monck & Fernandez, 1994, Neuron 12:707-716), or wider pores which open to a significant fraction of the vesicle radius, as suggested by "omega figures" in electron micrographs of the neuromuscular junction (Torri-Tarelli et al., 1985, J. Cell Biol. 101:1386-1399). Prolonged release of neurotransmitter should lower quantal efficiency and increase quantal variability both at endplates and central synapses. We have explored this range of possible pore dimensions and opening rates using computer simulations of vesicular acetylcholine (ACh) release and subsequent miniature endplate current (mEPC) generation. We utilized Monte Carlo algorithms (Bartol et al., 1991, Biophys. J. 59:1290-1307) and sub-nanosecond time-step resolution to obtain highly accurate 3D realism. For any cylindrical pore which opens instantaneously to a fixed height and radius, the intravesicular [ACh] decays exponentially. When the pore dimensions are not fixed but change with time, ACh release is no longer exponential. For example, a linearly increasing radius (fixed pore height) yields a smooth sigmoidal release, where the approximate time for half-emptying may be predicted from the results obtained with fixed dimensions. Our results indicate that narrow pores with dimensions and opening kinetics similar to those suggested for mast cell vesicles will significantly reduce the amplitude and prolong the rise time of very fast currents such as the mEPC. On the other hand, opening rates consistent with the time required for the appearance of omega figures are in the range where mEPC efficiency would be little affected. Therefore, we suggest that the mechanism underlying transmitter exocytosis from nerve terminals may be quite different from that underlying exocytosis in an endocrine setting. At the endplate, a reasonable opening rate would be on the order of 10 nm per 100 μs. Rates significantly slower might result in a detectable sigmoidal foot at the onset of the mEPC. Funded by F32NS09126 (JRS), NS09315 (MMS), and the Howard Hughes Medical Institute (TMB). Simulations were conducted at the Cornell National Supercomputer Facility, Ithaca, NY.

B5-112 SYNAPSE ELIMINATION VIA HEBBIAN COMPETITION CAN BRING ABOUT ERROR CORRECTION AND THE SIZE PRINCIPLE . Jes Stollberg. Békésy Laboratory of Neurobiology, University of Hawaii, Honolulu, HI 96812

Synapse elimination is a ubiquitous phenomenon in developing vertebrates. The utility (if any) of "over producing" synapses and then eliminating many of them has not been established. The present work documents a theoretical analysis of Hebbian (correlation) rules connecting pre- and post-synaptic activity and synaptic strength at the neuromuscular junction. The analysis suggests a novel function for synapse elimination in this system: it may help to establish the size principle of recruitment.

The following points are demonstrated. 1. Correlational competition leads to the reduction of polyinnervation to a stable monoinnervated state. 2. The competition gives rise to the size principle over a wide range of the plausible parameter space. 3. Over a significant subrange the competition selectively eliminates topographically incorrect synapses. 4. In cases where topographic projection errors overwhelm the system, both error correction and the development of the size principle are disrupted.

Correlational competition may explain apparently contradictory experimental results in which subpopulations of motor neurons are stimulated or poisoned; some have reported that these manipulations lead to expansion of active motor units, and others that inactive motor units are expanded. The present analysis demonstrates that subtle changes in the correlation rule, or in the developmental timing of the experiments, can shift the response from one finding to the other. Correlational competition may also explain an otherwise puzzling instance of a breakdown in the size principle seen in humans undergoing neural regeneration. Patients with largely correct (or synergistic) reinnervation display the size principle, while those with significant levels of non-synergist reinnervation do not. These findings support the novel hypothesis that synapse elimination in neuromuscular systems serves to help establish the size principle.

B5-113 DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNELS ARE INVOLVED IN EVOKED TRANSMITTER RELEASE AT DEVELOPING AND REGENERATING NEUROMUSCULAR JUNCTIONS. Yoshie Sugiura, Yasushi Hayashi, Chuck Li and Chien-Ping Ko. Department of Biological Sciences, Section of Neurobiology, University of Southern California, Los Angeles, CA 90089.

At adult mammalian neuromuscular junctions (NMJs), evoked transmitter release is believed to be mediated by P/Q-type voltage-sensitive calcium channels (VSCCs) which are inhibited by omega-conotoxin MVIIC. To characterize the ontogeny of VSCC subtypes, we examined the effects of several Ca^{2+} channel ligands on synaptic transmission at developing NMJs. Phrenic nerve/diaphragm muscle preparaions from embryonic day 17 (E17) to 3-month old rats were studied. Nerve-evoked end-plate potentials (epps) were recorded in the presence of curare. As at adult NMJs, omega-conotoxin MVIIC blocked evoked transmitter release at developing NMJs. However, nifedipine, a dihydropyridine (DHP) that blocks L-type VSCCs, *increased* evoked transmitter release at developing NMJs. At E17, one, five, and 10 μM nifedipine increased epp amplitude 1.6-, 2.3- and 8.3-fold, respectively. At postnatal day 4, 10 μM nifedipine also increased epp amplitude 1.8-fold. In contrast, 10 μM nifedipine showed no effect at the adult rat NMJs. Bay K 8644, an L-type VSCC activator, showed opposite effect (epp amplitude reduced to 30%) in newborn muscles, consistent with the involvement of DHP-sensitive calcium channels in synaptic transmission. Similar effects of nifedipine were also observed at reinnervated NMJs in low Ca^{2+}/high Mg^{2+} saline. Ten μM nifedipine increased epp amplitude 1.7-fold 7 days after nerve crush in mouse sternomastoid, and 2.8-fold 14 days after nerve crush in frog cutaneous pectoris muscles. Nifedipine had little effect on the amplitude and frequency of spontaneous miniature end-plate potentials. The present results indicate the existence of DHP-sensitive calcium channels that may modulate transmitter release at developing and regenerating motor nerve terminals. We hypothesize that DHP-sensitive calcium channels are colocalized with Ca^{2+}-activated K^+ channels in newly formed NMJs, and distant from the VSCCs that mediate transmitter release. Blocking DHP-sensitive Ca^{2+} channels may prevent the activation of K^+ channels which allows broadening of the presynaptic action potential and an increase of transmitter release from immature nerve terminals. This is in contrast to adult NMJs where Ca^{2+}-activated K^+ channels are colocalized with the VSCCs that mediate transmitter release.

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B5-114 MECHANISMS OF SYNAPTOGENESIS IN CULTURED HIPPOCAMPAL NEURONS, Claudia Verderio, Silvia Coco, Olaf Mundigl, Pietro De Camilli, Guido Fumagalli and M. Matteoli, Department. of Medical Pharmacology, University of Milano, Italy and HHMI and Department. of Cell Biology, Yale University, CT 06510.

A regional redistribution of presynaptic and postsynaptic components takes place during the establishment of neuronal polarity and synaptogenesis in hippocampal neurons maintained in primary cultures (Fletcher et al., 1991; Craig et al., 1993). To verify whether this regional redistribution has implications for the functional maturation of pre- and post-synaptic compartments, we have used immunocytochemical and calcium imaging techniques to assess whether 1) the ability of synaptic vesicles to undergo exo-endocytotic recycling and 2) the functionality of glutamate receptors, are differently regulated in cultured hippocampal neurons during maturation and synaptogenesis. Using an immunocytochemical assay, we have recently demonstrated that synaptic vesicles undergo recycling even before the formation of synaptic contacts. We show here that this property temporally correlates with the ability of neurons to release glutamate in the extracellular space.We also demonstrate that mechanisms for calcium- and depolarization-dependent exocytosis are at least partially activated even before synaptogenesis. Similarly, glutamate receptors appear to be already functional from the very early stages of development in culture. The differentiation and the establishment of synaptic contacts are accompanied by a redistribution of glutamate-induced calcium transients which show a homogeneous distribution in all neuronal compartments at early stages of development, then become restricted in the somato dendritic region and eventually acquire a focal distribution in synapse-enriched portions of dendrites. These results indicates that the functionality of both pre- and post-synaptic compartment is already established well before synaptogenesis. The formation of synaptic contacts may coincide with some changes in the fine regulation of synaptic vescile exocytosis and glutamate receptors transducing activity.

B5-115 BACTERIALLY EXPRESSED F1-20/AP-3 ASSEMBLES CLATHRIN INTO CAGES WITH A NARROW SIZE DISTRIBUTION: IMPLICATIONS FOR THE REGULATION OF QUANTAL SIZE DURING NEUROTRANSMISSION, Weilan Ye+ and Eileen M. Lafer*, *Program in Molecular Medicine, Institute of Biotechnology; University of Texas Health Science Center, San Antonio, TX, 78245; and +Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, 15260

F1-20/AP-3 is a synapse-specific phosphoprotein. In this study we characterized the ability of bacterially expressed F1-20/AP-3 to bind and assemble clathrin cages. We found that both of two bacterially expressed alternatively spliced isoforms of F1-20/AP-3 bound and assembled clathrin as efficiently as preparations of F1-20/AP-3 from bovine brain. This establishes that the clathrin assembly activity found in F1-20/AP-3 preparations from brain extracts is indeed encoded by the cloned gene for F1-20/AP-3. It also demonstrates that post-translational modification is not required for activation of the clathrin binding or assembly function of F1-20/AP-3. Ultrastructural analyses of the clathrin cages assembled by bacterially expressed F1-20/AP-3 revealed a strikingly narrow size distribution. This may be important for the regulation of quantal size during neurotransmission. It has been suggested that the 33 kD NH_2-terminus of F1-20/AP-3 constitutes a clathrin binding domain. We expressed the 33 kD NH_2-terminus of F1-20/AP-3 in *E. coli*, and measured its ability to bind to clathrin triskelia, to bind to clathrin cages, and to assemble clathrin triskelia into clathrin cages. We found that the bacterially expressed 33 kD NH_2-terminus of F1-20/AP-3 binds to clathrin triskelia, fails to bind to preassembled clathrin cages, and is not sufficient for clathrin assembly. We expressed the 58 kD COOH-terminus of F1-20/AP-3, and found that it bound to clathrin triskelia, and assembled them into regular clathrin cages. It is clear from these experiments that while the 33 kD NH_2-terminus of F1-20/AP-3 is sufficient to carry out some aspects of clathrin binding, the 58 kD COOH-terminus of F1-20/AP-3 is sufficient to bind to clathrin triskelia and assemble them into regular cages.

B5-116 MAKING SENSE OF THE RELATIONS BETWEEN CYSTEINE-STRING PROTEINS, CALCIUM CHANNELS, AND NEUROTRANSMITTER RELEASE. Konrad E. Zinsmaier, Joy A. Umbach[1], Cameron B. Gundersen[1], and Seymour Benzer, California Institute of Technology, Division of Biology 156-29, Pasadena, CA 91125. [1]University of California, Los Angeles, School of Medicine, Dept. of Molecular and Medical Pharmacology, Los Angeles, CA 90024.

Cysteine-string (Csp) proteins are synaptic vesicle proteins that have been isolated in *Drosophila*[1,2] and *Torpedo*[3]. Phenotypic analysis of *csp* deficient *Drosophila* mutants demonstrates a crucial role for Csp in synaptic transmission[2,5]. Expression studies in frog oocytes suggest that Csp may modulate presynaptic Ca^{2+} channels[2].

The deletion of the *csp* gene is essentially lethal, but there are few escapers at low temperature. Among these, one observes temperature-sensitive paralysis and premature death. This ts-behavior correlates with ts-defects in the ERG and in neuromuscular synaptic transmission. Ejps are impaired at room temperature, and completely blocked at high temperature. This effect is exclusively presynaptic; it occurs without elimination of spontaneous release events.

A model has been suggested where Csp serves as a link by which docked synaptic vesicles modulate Ca^{2+} channel activity[4]. Alternatively, Csp action may involve chaperone activity, or the regulation thereof, in the assembly of components of the exocytotic neurotransmitter release machinery at the release site.

Whether the biology of Csp proteins involves either or both possibilities is the topic of our current research and will be discussed.

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Synapse-Specific Muscle Molecules; Post-Synaptic Cytostructures

B5-117 DYSTROGLYCAN INTERACTS WITH 43K/ RAPSYN, THE 43 kD ACETYLCHOLINE-RECEPTOR ASSOCIATED PROTEIN. Elizabeth D. Apel[1], Steven L. Roberds[2], Kevin P. Campbell[2] and John P. Merlie[1]. [1]Dept. of Molecular Biology and Pharmacology, Washington University, St. Louis, MO 63110 and [2]Howard Hughes Medical Institute, University of Iowa, Iowa City, Iowa 52242.

During synapse formation highly specialized structures develop both pre and postsynaptically. At the neuromuscular junction, a particularly striking example of molecular specialization is the accumulation of nicotinic acetylcholine receptors (AChR) at an extremely high density in the postsynaptic membrane. A number of other molecules, including the 43 kD receptor-associated protein (rapsyn), the dystrophin-associated glycoprotein (DAG) complex, and a 59K protein are found colocalized with AChR at these clusters. Our laboratory has used a fibroblast expression system (QT-6 cells) to study the mechanisms underlying AChR cluster formation. These fibroblasts do not contain any of the known proteins normally found in AChR clusters, yet recombinant rapsyn and AChR readily form clusters when coexpressed by transfection in QT-6 cells. Rapsyn expressed in the absence of AChR also forms clusters, thus suggesting a direct role for rapsyn in receptor aggregation. In this study, we have used QT-6 cells to examine the interaction of one component of the DAG complex, dystroglycan, with rapsyn and AChR. When expressed alone without rapsyn and AChR, dystroglycan is uniformly distributed throughout the surface membrane. However, dystroglycan colocalizes with rapsyn-AChR clusters when all three proteins are present. Furthermore, dystroglycan is colocalized with rapsyn clusters even in the absence of AChR. These results suggest that rapsyn and dystroglycan interact directly, independent of AChR and other components of the postsynaptic apparatus. Rapsyn may therefore be the molecular link between the AChR and the DAG complex.

B5-118 A GLYCOPROTEIN LOCALIZED TO FROG NEUROMUSCULAR JUNCTIONS AND PERIPHERAL NERVES, Stephanie H. Astrow, Minh Thuoc T. Nguyen, and Chien-Ping Ko, Dept. of Biol. Sci., Univ. of Southern California, Los Angeles, CA 90089.

Molecules embedded in the extracellular matrix (ECM) play important roles in the formation and maintainence of the neuromuscular junction (NMJ). To further characterize the composition of the synaptic ECM, we generated monoclonal antibodies against ECM derived from the synapse-rich electric organ of *Torpedo*. One of these antibodies, designated 6D7, recognizes the perisynaptic region of NMJs in *Rana pipiens*, as identified with fluorescently labeled alpha-bungarotoxin. When observed in wholemount preparations, the indirect immunofluorescent labeling of NMJs obtained with 6D7 appears to label the perijunctional basal lamina and is continuous with labeling of intramuscular axons and nerve branches. Cryostat cross-sections support the notion that the 6D7 immunolabeling lies outside the muscle fibers at the NMJ. Denervated sartorius muscles showed no change in localization or intensity of 6D7 immunolabeling at NMJs indicating that the 6D7 epitope is not an intracellular component of axons. To test the hypothesis that the 6D7 epitope is a component of the extracellular matrix, we are preparing basal lamina "ghosts" from cutaneous pectoralis muscles. Two weeks after damage to the nerve and muscle, the preparations will be examined for 6D7 immunoreactivity. Teased sciatic nerve preparations are also immunopositive for 6D7. Labeling of nerve fibers appears on/near both small and large caliber axons as well as concentrated near nodes of Ranvier. 6D7 immunolabeling is also observed around axons in cryostat cross-sections of dorsal root ganglia. On immunoblots of a detergent-soluble extract derived from frog sciatic nerve, 6D7 recognizes a major band of approximately 110 kD, as well as two minor bands of 125 kD and 153 kD. Treatment of this extract with recombinant N-Glycanase results in greater mobility of the major immunoreactive band, thus we believe that the 6D7 antigen is a glycoprotein. Further molecular characterization and ultrastructural localization are underway to determine the identity of the 6D7 antigen.

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B5-119 TARGETED DISRUPTION OF THE CYSTEINE RICH DOMAIN OF THE MURINE UTROPHIN GENE.
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Utrophin is a widely expressed protein in both foetal and adult tissues, and in the nervous system is found in the brain and peripheral nerves. However, in adult skeletal muscle it is exclusively localised to the neuromuscular junction.

Utrophin is an autosomal homologue of dystrophin. The 13kb transcript encodes a 395 kDa protein which, like dystrophin, exhibits four distinct domains: the N-terminus, the rod-like triple helical repeat domain, the cysteine-rich and the c-terminus. The cysteine rich and c-terminal domains are highly conserved between utrophin and the different protein products of the dystrophin locus. It has been shown that in muscle, the cysteine rich/c-terminal domains of dystrophin bind to the dystrophin-glycoprotein complex at the sarcolemma, thus linking the underlying cytoskeleton to the extracellular matrix. A similar utrophin-glycoprotein complex has been reported at the neuromuscular junction suggesting similar function for utrophin.

To analyse the function of the cysteine rich and c-terminal domains of utrophin we have used homologous recombination in embryonic stem (ES) cells to direct a mutation within the putative glycoprotein binding domain. Our data will be presented.

B5-120 ANALYSIS OF NEUROMUSCULAR JUNCTIONS IN A MUTANT MOUSE DEFICIENT IN THE 43KDA ACETYLCHOLINE RECEPTOR ASSOCIATED PROTEIN.
Medha Gautam[1], Peter G. Noakes[2], Jacquelene Mudd[1], Mia Nichol[1], Joshua R. Sanes[2], and John P. Merlie[1], Departments of [1]Molecular Biology and Pharmacology and of [2]Anatomy and Neurobiology, Washington University Medical School, St. Louis, MO 63110.
We are analysing the development and maturation of the vertebrate neuromuscular junction (NMJ) by making mutant mice that are deficient in one or more synaptic proteins. One of the post synaptic components of the NMJ is a 43KDa acetylcholine receptor (AChR) associated protein, frequently referred to as 43K/rapsyn. In response to nerve-derived agrin, 43K and ACh receptors reorganise into large clusters in the underlying muscle membrane. Colocalisation of 43K and AChRs occurs in mature as well as developing synapses suggesting that 43K is important in cluster formation. Co-expression of recombinant 43K and AChRs in heterologous systems support such a role. To analyse the function of 43K in vivo, we have generated mice deficient in 43K. The knockout mice were derived using the technique of targeted mutagenesis in ES cells. Mutant mice die within 12 hours after birth. There are no receptor aggregates in mutant muscle when examined by rhodamine-labelled bungarotoxin. A characterisation of the defects in the NMJs of these mice will be presented.

B5-121 IDENTIFICATION OF THE SYNAPTIC LOCALIZATION DOMAIN IN S-LAMININ, A SYNAPTIC LAMININ HOMOLOGUE,
Paul T. Martin and Joshua R. Sanes, Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110.
The basal lamina (BL) in the synaptic cleft of the neuromuscular junction is chemically and functionally specialized. For example, the extrasynaptic basal lamina of the muscle fiber contains B1-laminin, while its homologue, S-laminin, is present only in the synaptic BL. This mutually exclusive expression occurs despite the fact that these two proteins are 50% identical at the amino acid level. We have used the C2 muscle cell line to test which regions of S-laminin are responsible for its synaptic localization. C2 cells are a mouse muscle cell line which, when fused into myotubes, form spontaneous clusters of acetylcholine receptors (AChRs). S-laminin is concentrated at these clusters, while B1-laminin is present throughout the extracellular matrix. C2 cells were transfected with a plasmid containing the cDNA for rat S-laminin. Expression was assayed using monoclonal antibodies which could recognize the transfected rat S-laminin but not the endogenous mouse protein. Transfected cells expressed rat S-laminin protein all along the myotube intracellularly, while all of the secreted protein was found to be localized exclusively at AChR clusters. By making full-length chimeric molecules containing portions of S- and B1-laminin, we show that the extreme C-terminus of S-laminin is required for its localization to AChRs. Furthermore, the N-terminus of the molecule defines an aggregation domain which differentiates the expression pattern of S- and B1-laminin in the extracellular matrix.

B5-122 DEVELOPMENTAL REGULATION OF A PROTEIN KINASE C ISOFORM LOCALIZED TO THE NEUROMUSCULAR JUNCTION,
Kathryn Miles and Lutz Hilgenberg, Department of Anatomy and Cell Biology, SUNY at Brooklyn, Brooklyn NY 11203
Protein kinase C (PKC) is a family of protein serine/threonine kinases consisting of multiple isoforms whose distinct physiological roles within cells are unknown. The message encoding the nPKC θ isoform, a member of the novel calcium-independent class of PKCs, has recently been shown to be enriched in mouse skeletal muscle. The message for cPKC α, a calcium-dependent isoform, was also found to be highly expressed in this tissue. In an effort to distinguish the physiological roles of these two isoforms of PKC in rat skeletal muscle, we examined their subcellular distribution, developmental expression and intracellular localization. We generated an isotype-specific antiserum directed against a peptide sequence unique to nPKC θ. This antiserum recognized a 79 kD protein highly enriched in rat skeletal muscle which is likely to be nPKC θ. cPKC α was also readily detectable in skeletal muscle, using another isotype-specific antibody, but it appeared to be ubiquitously expressed in all of the tissues we examined. Together these results suggest that nPKC θ, rather than cPKC α, is involved in physiological functions that are specific for skeletal muscle. Immunoreactivity for nPKC θ was highest in the membrane subcellular fraction compared to the cytosolic fraction of skeletal muscle. In contrast, cPKC α was found to be predominantly distributed in the cytosolic rather than membrane fraction. nPKC θ appeared to be developmentally regulated postnatally in rat skeletal muscle with a 4-fold increase in expression occurring exclusively in the membrane fraction during postnatal days 3 through 21. This time course coincides with the period in rat development associated with maturation of neuromuscular junctions. Expression of nPKC θ in rat spleen, another tissue expressing detectable levels of this isoform, was not found to be developmentally regulated during this time. cPKC α expression was found to increase slightly from postnatal days 3 through 11 and no developmental increase in expression of this isoform was observed in skeletal muscle during postnatal days 11 through 21. The intracellular localization of the PKC θ and α isoforms in rat skeletal muscle was examined by immunocytochemistry. nPKC θ was detected in association with the sarcolemma of skeletal muscle and was found to be localized in the neuromuscular junction. Enhanced staining for nPKC θ in the neuromuscular junction appeared as early as postnatal day 4 during development. Staining for nPKC θ in the neuromuscular junction persisted after prolonged denervation suggesting that the enzyme is distributed postsynaptically. In contrast, in adult rats, the most intense cPKC α immunoreactivity appeared as a punctate stain in the cytosol as well as associated with the sarcolemma. While cPKC α was also detected in the neuromuscular junction, the strongest staining signal was not found to be localized in this synapse. Taken together, these data suggest that nPKC θ may play a specific role in skeletal muscle signal transduction in both the developing and mature neuromuscular synapse.

B5-123 STUDIES ON THE STRUCTURE AND FUNCTION OF NATURALLY OCCURRING MUTATIONS IN HUMAN SEROTONIN$_{1A}$ RECEPTOR GENE. Bita Nakhai, David Goldman, Gary Jenkins and David A. Nielsen, Section of Molecular Genetics, LN, NIAAA, Bethesda, MD 20892.

The serotonin 1A (5-hydroxytryptamine) receptor gene codes for the 5-HT$_{1A}$ receptor. It is expressed both presynaptically in serotonergic cell bodies and in dendrites of the dorsal raphe nucleus and postsynaptically in hippocampus. Serotonin plays a key role in many physiological and behavioral functions including aggressive behavior, intolerance to delay, impulsivity, appetite and control of temperature and sleep. Serotonergic activity is regulated, in part, by binding of serotonin to the 5-HT$_{1A}$ receptor.

Two rare polymorphisms were identified for 5-HT$_{1A}$ gene by SSCP analysis in human. These polymorphisms changed the protein sequence of the 5-HT$_{1A}$ receptor. To study the biological function and biochemical properties of these allelic variants of the 5HT$_{1A}$ gene we subcloned them in a eukaryotic expression vector, under the control of CMV promoter. They are being transfected into mammalian COS-7 cells to characterize the 5HT$_{1A}$ receptor function by various methods including receptor binding assay. Expression of these mutated 5-HT$_{1A}$ receptors will allow us to study the effect of the polymorphisms on function. Each amino acid substitution may be an important tool in the study of serotonergically-influenced traits in humans. We are studying the relationship of these polymorphisms to impulsive behavior in alcoholic Finns violent offenders and in several other human populations.

B5-124 THE ROLE OF *XENOPUS* FREQUENIN IN THE DEVELOPMENT OF THE NEUROMUSCULAR SYNAPSE. Olafsson, P. and Lu, B., Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA.

A recent study on Drosophila frequenin suggests that it is a neuro-specific, Ca^{2+}-binding protein potentially interacting with guanylyl cyclase. Overexpression of the protein facilitated transmitter release at neuromuscular junctions in the adult fly. However, it is difficult to study the function of frequenin during synapse development in Drosophila using electrophysiological and biochemical approaches. To determine the role of frequenin during vertebrate synapse formation, we cloned the *Xenopus* homolog (X. frequenin) using degenerated primers combined with low stringency hybridization. The deduced protein has 70% identity with Drosophila frequenin and about 40%-50% with other Ca^{2+}-binding proteins. The most prominent feature of the protein is that it contains four EF-hand motifs. X. frequenin mRNA is very abundant in the brain and virtually absent in the muscle of the adult *Xenopus*. During early development the message was detected by a sensitive RT-PCR method as early as stage 18 (neurulation stage) and was consistently expressed thereafter. We are currently investigating the expression and physiological function of X. frequenin in *Xenopus* nerve-muscle co-cultures. We have also purified the recombinant X. frequenin protein and generated specific antibodies. Western blot analysis of different tissues showed the presence of the protein in the adult brain and traces in muscle. X.frequenin was absent in the other tissues examined, heart and kidney. Preliminary electrophysiological results obtained from cells containing exogenous recombinant frequenin indicated that frequenin elicits an increase of the frequency of spontaneous synaptic currents (SSC) as well as an increase of the amplitude of evoked synaptic currents (ESC).

B5-125 ISOLATION OF DYSTROGLYCAN AND ASSOCIATED PROTEINS FROM BOVINE SYNAPTOSOMES, Tamara C. Petrucci, Stefano Barca, Michaela Cavaldesi and Maurizio Zini, Laboratory of Cell Biology, Istituto Superiore di Sanità, Rome, Italy

Dystrophin, a protein whose deficiency leads to Duchenne muscular dystrophy, is thought to play an important role in the membrane physiology of muscle cells. It is also present in brain. Although dystrophin's functional role is still unknown, its localization and homology of sequence with spectrin suggest that it is an important structural component of membrane skeleton. In the murine central nervous system dystrophin has been localized by immunocytochemistry in neurons of the cerebral cortex and cerebellum at postsynaptic sites. At the synapse, dystrophin has been found as part of the postsynaptic apparatus, thus suggesting that it plays a role in the formation and maintenance of specialized regions of neuronal plasma membrane, possibly as an anchor for the postsynaptic apparatus. In this study we examined the expression of spectrin isoforms in control and dystrophic mdx mice during neuronal development and investigated the subcellular localization of dystrophin in rat brain. No differences were detected in the expression of spectrin isoforms, α- and β-fodrin (αIIΣ2* and βIIΣ1) and β-spectrin (βIΣ2), between control and mdx mice. However, proteolysis of spectrin was observed in 2- and 3-week-old mdx mice. Dystrophin and βIΣ2 spectrin were enriched in postsynaptic density (PSD) structures of rat brain. To investigate the presence of the neuronal dystrophin-glycoprotein complex, bovine brain synaptosomes were fractionated on a laminin-Sepharose column. Proteins bound to the laminin-Sepharose in a calcium dependent fashion, and eluted with EDTA, were Western blotted with anti-dystrophin, anti-spectrin antibodies and ^{125}I-laminin. Our results suggest that unique isoforms of spectrin together with dystrophin and dystrophin-associated protein (α-dystroglycan), may play a pivotal role in organizing topographically defined clusters of receptors or cytoplasmic protein complexes that are functionally important in nerve cells.

The financial support of Telethon-Italy to the project "Role of spectrin isoforms in the central nervous system of normal and dystrophic mdx mice" is gratefully acknowledged.

B5-126 ELECTRIC FIELDS AND AGRIN INDUCE ACETYLCHOLINE RECEPTOR CLUSTERING VIA COMMON MOLECULAR MECHANISMS. Farial Sabrina*, Justin Fallon**, and Jes Stollberg*. *Békésy Laboratory of Neurobiology, University of Hawaii, Honolulu, HI 96812; **Worcester Fnd Exper Biology, Shrewsbury, MA 01545

The aggregation of acetylcholine receptors (AChRs) at the developing neuromuscular junction is critical to the development and function of this synapse. In vitro studies have shown that AChR aggregation can be induced by the binding of agrin to the muscle cell surface or by the electric field-induced concentration of some (non-AChR) molecule at cathodal cell pole. We report here on the fluorescence visualization of the agrin binding sites on living muscle cells, and the interaction between agrin binding and electric fields with respect to the distribution of agrin binding sites and AChRs.

1. Pre-treatment of cells with agrin completely blocks the development of field-induced AChR clusters. 2. When cells are exposed to 4 V/cm for 40 minutes and immediately labeled, the majority finding is that the agrin sites are not concentrated at the field-induced AChR aggregate. 3. If instead the cells are labeled 30 minutes after field termination, the majority of cells do show co-localization of agrin binding sites with the field-induced AChR aggregate. 4. Either manipulation blocks agrin-induced AChR patching over the majority of the cell surface (everywhere except within the cathodal AChR aggregate), despite the fact that the non-cathodal region has a significant density of AChRs.

Taken together these results suggest that the agrin/binding site complex must interact with (and presumably concentrate) another molecular species in order to induce AChR clustering. Electric fields appear to concentrate this other molecule at the cathode, leading to AChR trapping at the cathode and the depletion of the unidentified molecule over the non-cathodal cell membrane. Thus the two stimuli (agrin, electric fields) appear to have at least one mechanistic component in common.

B5-127 EPSILON SUBUNIT DOES NOT CONFER METABOLIC STABILITY TO ACETYLCHOLINE RECEPTOR (ACHR) IN MYOTUBES, Carlo Sala and *Guido Fumagalli, Department of Medical Pharmacology, University of Milano and *Institute of Pharmacology, University of Verona, Italy.

In cultured muscle cells, two types of AChR can be distinguished by their degradation rates (O'Malley et al., Exp Cell Res 208, 44-47, 1993): most of the receptor degrades rapidly (called R_f, t1/2=1 d) and approximately 10% of the molecules degrade slowly (R_s,t1/2=~3 d). R_s degradation rate is reversibly modulated between 3 and 10 d by cAMP. A similar situation exists at neuromuscular junctions (nmj): R_s is predominant in innervated nmj and R_f appears after denervation and replaces R_s. They differ both in degradation rate and in their response to reinnervation and to pharmacological treatments (Shyng et al., Neuron 6, 1-20, 1991). The coexistence at a same endplate and thedifferent sensitivity to modulatory events suggest that R_f and R_s are two distinct molecular entities. Muscle AChRs contain either γ or ε subunits: the second is present in innervated nmj, the first appears after denervation. With this study we are testing the hypothesis that the differences in degradation rates are due to the presence of ε subunit in the R_s molecule.

After labelling superficial AChR molecules of new-born rat myotubes in culture with ^{125}I-α-bungarotoxin (^{125}I-BGT), ε subunit was detectable by immunoprecipitation with subunit specific mabs (kind gift of Dr. Tzartos) starting from day 5 of culture and reached a maximum level of ~10% of total superficial AChR at day 9. To measure the contribution of the ε subunit-containing AChR to the R_s population, myotubes were labelled at day 9 of culture with ^{125}I-BGT. Degradation rates were then calculated from the release of radioactivity in the medium; using ε subunit-specific mabs and polyclonal anti-AChR antibodies, we calculated the fraction of ε subunit-containing AChR remaining on myotubes surface at various time points after labelling. T1/2 were 1d in most and 3.5 d in 10.5±0.8% of AChRs. On the other hand, ε subunit-containing receptors were ~10% of total AChR at all the time point tested (0, 4 and 6 days after labelling with ^{125}I-BGT) indicating that this subunit is present in both R_s and R_f. Incubation of cells with 1 mM db-cAMP shifted R_s degradation rate to 9 d but did not change the ε/total AChR ratio measured 0 and 4 d after toxin labelling. These results and the late appearance of ε subunit strongly suggest that this subunit is not responsible for the metabolic differences between R_f and R_s.

B5-128 ACTION POTENTIAL GENERATION AT THE MAMMALIAN NEUROMUSCULAR JUNCTION, Sarah J. Wood and Clarke R. Slater. Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Newcastle upon Tyne NE4 6BE, UK.

Voltage-gated sodium channels are concentrated at the neuromuscular junction (NMJ) and localized to the depths of the postsynaptic folds. By analogy to myelinated neurones and mammalian central neurones, where high densities of sodium channels occur at sites of electrogenesis, it is predicted that the threshold for action potential (AP) generation would be lower at the NMJ than elsewhere on the muscle fibre.

To assess this effect we have used two different methods of generating APs at the NMJ and in extrajunctional regions of rat skeletal muscle. Firstly, using two microelectrodes for intracellular recording, currents resembling endplate currents were injected into muscle fibres. Small step increases in current amplitude were made until an AP was triggered. Muscles were incubated in d-tubocurarine (d-TC) to allow NMJs to be identified by the presence of endplate potentials (EPPs) with fast rise times. Threshold values at junctional and extrajunctional regions differed by 3-5mV. At a membrane potential of -75mV, the AP threshold at the endplate was -55mv.

The second approach was to examine APs generated by transmitter release following nerve stimulation. Partial neuromuscular transmission block was achieved using d-TC such that, on occasion, stimulation of the nerve elicited EPPs and APs in the same fibre. The threshold of these nerve-evoked APs was -63mV.

These results are consistent with the idea that a high concentration of sodium channels at the NMJ lowers the threshold for AP generation. The two different threshold values obtained at the NMJ suggest that the nerve-evoked AP has a lower threshold than previously supposed and this has implications for deriving estimates of safety factor.

Neuronal Receptors; Sensory Signal Transduction; Modulation of Synaptic Function

B5-129 THE INVOLVEMENT OF $GABA_B$-LIKE RECEPTORS IN PRESYNAPTIC INHIBITION AT THE CRAYFISH NEUROMUSCULAR JUNCTION, Yacov Fischer, Itzchak Parnas, The Otto Loewi Center for Cellular & Molecular Neurobiology, Dept. of Neurobiology, Hebrew University, Jerusalem, Israel 91904

Presynaptic inhibition has been demonstrated at the crayfish opener neuromuscular junction. At the synaptic level, a single action potential (AP) evoked in the inhibitory axon 2-3 msec prior to producing a single test AP in the excitatory axon, produces a marked decrease (ca. 30%) in quantal content without affecting quantal size, indicating the effect to be presynaptic. This presynaptic inhibitory effect depends on the precise timing of arrival of the two action potentials at the nerve terminal, and is believed to involve an increase in Cl- conductance because it is blocked by picrotoxin (35 μM; Takeuchi, 1969).

However, we found an additional form of presynaptic inhibition--in the crayfish opener neuromuscular junction--that is picrotoxin insensitive (50 μM). This effect is produced by train activation (as few as 2 pulses @ 100Hz) of the inhibitory axon prior to a single excitatory test simulation. This novel effect is different from the single paired axons stimulation in its timing dependency, its level of inhibition, and sensitivity to picrotoxin. For optimal inhibition, the last inhibitory AP should arrive ca. 10 msec before the excitatory test AP. The average level of inhibition is ca.60%, and picrotoxin is ineffective in blocking this effect.

We suggest that this new presynaptic inhibition mechanism involves the combined action of $GABA_A$ and $GABA_B$-like receptors, since it is blocked only by the combined action of picrotoxin and 2OH-saclofen (50 μM and 100 μM, respectively).

B5-130 THE FYN-DEPENDENT SIGNALING PATHWAY IN THE BRAIN: FYN IS A CRITICAL REGULATOR OF FOCAL ADHESION KINASE

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The induction of LTP in the CA1 region of the hippocampus requires signal transduction cascades involving tyrosine and serine-threonine kinases in the postsynaptic neuron. To identify a specific tyrosine kinase required for LTP we examined mice carrying mutations in four nonreceptor tyrosine kinase genes, Fyn, Src, Yes and Abl, and found that only Fyn mutants had a detectable neural phenotype. Fyn mutants showed a blunting in the induction of LTP, although they had normal synaptic transmission, paired-pulse facilitation, and post-tetanic potentiation. The *fyn* mutants also showed abnormal hippocampal architecture and defects in spatial learning.

We found that nine proteins were hypophosphorylated specifically in *fyn* mutants and one of these was Focal Adhesion Kinase (FAK). FAK was expressed at highest levels in axons, dendrites and in the intermediate filament cytoskeleton of astrocytes. This is in distinction to fibroblasts where it is restricted to focal contacts. Hypophosphorylated FAK from *fyn* mutants showed reduced activity in immunecomplex kinase assays.

Since Src can regulate FAK and neuronal specific isoforms of Src have been implicated in neuronal signaling, the lack of a neural phenotype in Src mutants is puzzling. We found, in each of the *fyn, src* and *yes* mutants, compensatory increases in the activity of the remaining kinases as well as changes in Csk. Thus a neural phenotype in Src and Yes mutants may be masked by compensatory changes in Fyn and other kinases.

B5-131 NMDA-INDUCED PROTEOLYTIC CONVERSION OF THE LONG FORM OF THE CLASS C L-TYPE CALCIUM CHANNEL α1 SUBUNIT INTO ITS MORE ACTIVE SHORT FORM

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Calcium channels control a variety of neuronal functions such as neurotransmitter release or integration of electrical signals in dendrites. We used class-specific anti-peptide antibodies against the class B N-type, class C L-type, and class E calcium channels to determine the subcellular localization of these channels. Whereas N-type channels are localized presynaptically, electron microscopy after immunohistochemical staining reveald that the class C L-type channel exists mainly in postsynaptic spines in the CA2/CA3 region of the hippocasmpus and the dentate gyrus. Class E channels are detectable on pyramidal cell bodies. The differential subcellular distribution of these calcium channels suggests their involvement in different cellular functions. Calcium channels consist of different subunits. The α1 subunits constitute the ion-conducting pores. Biochemical analysis demonstrates the existence of two size forms of the α1 subunits for each of the three channles. The two size forms differ at their C-terminal ends as shown for the class B and C α1 subunits.

Activation of the postsynaptic NMDA-type glutamate receptor and subsequent calcium influx is necessary for several forms of synaptic plasticity in the hippocampus including long term potentiation and long term depression. Using a hippocampal slice preparation we discovered that activation of the NMDA-receptor induces the conversion of the 210 kD form of the class C L-type channel α1 subunit into the 190 kD form, which is fourtimes to sixtimes more active than the former one. There was no decrease in the total amount of class C L-type channels upon NMDA treatment. NMDA did not cause proteolytic conversion of the class B or class E α1 subunits. The NMDA induced proteolytic processing is therefore specific for the postsynaptic class C L-type channel. It requires calcium and can be prevented by several NMDA-receptor blockers.

The class C L-type calcium channel is permanently activated by NMDA-induced proteolytic processing and may, therefore, contribute to an increase of synaptic strength in the CA2/CA3 area of the hippocampus or in the dentate gyrus.

B5-132 AGRIN IS LOCALIZED AT SYNAPSES BETWEEN CNS NEURONS

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Agrin is an extracellular matrix protein which, at the neuromuscular junction (NMJ), is associated with the basal lamina of the synaptic cleft where it induces the formation and maintenance of postsynaptic specializations including aggregates of the acetylcholine receptor (AChR). Alternative splicing of the agrin mRNA yields several agrin isoforms which differ in their distribution and their AChR-aggregating activity. Because of the presence of agrin isoforms in the central nervous system (CNS), it has been hypothesized that agrin might also induce postsynaptic specializations at neuron-neuron synapses. We have begun to test this hypothesis using the developing avian retina as a model system. Retinal cells prepared at E8 and cultured for 6 days form synapses and secrete several different agrin isoforms. Staining of these cultures with anti-agrin antibodies shows a punctate labelling along the cell bodies and the neurites as well as a general staining of the substrate. Examination of cultures double-labelled with antibodies against agrin and against either gephyrin (a protein associated with the glycine- and the GABA receptor in the retina) or synaptotagmin with a confocal laser scanning microscope, shows a clear colocalization of the punctate agrin staining with both synapse-specific proteins. To determine if agrin is similarly localized at synapse-like structures in vivo, we injected anti-agrin Fab fragments into the vitreous humour of embryonic (E3 to E20) chick eyes, a method which labels only extracellular agrin. Staining is seen in the optic fiber layer and in the synapse-containing inner- and outer plexiform layers. The inner limiting membrane (the basal lamina separating the vitreous humour from the neural retina) is also stained. No staining was observed in the nuclear retinal layers, which contain few if any synapses. The appearance of agrin immunoreactivity in the inner plexiform layer (IPL) changes from diffuse to punctate, starting in the central part of the retina at E 13, the time and location where the first synapses are formed. The punctate appearance of the agrin immunoreacivity in the IPL was very similar in size and morphology to the punctate staining observed in retinal cell cultures. That agrin immunoreactivity is localized to areas of synaptic specializations between CNS neurons in vivo and in vitro is very similar to the distribution of agrin at the NMJ. Thus, our results are consistent with the idea that agrin isoforms might play a role in synapse formation in the CNS similar to that at the NMJ.

B5-133 MOLECULAR DISSECTION OF LONG-TERM POTENTIATION BY SUBTRACTIVE HYBRIDIZATION.

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Several studies have demonstrated that the late stages of long-term potentiation (LTP) are sensitive to inhibitors of transcription thus suggesting that gene expression is required for the maintenance of LTP. We have utilized a subtractive hybridization method to isolate genes that are induced at different time points after tetanic stimulation of the perforant path. Several directional cDNA libraries from hippocampi of rats that had been stimulated "in vivo" at high frequency and sacrificed after 1, 3 and 24 hours of verified LTP were made. Also, control cDNA libraries from the contralateral unstimulated hemispheres were constructed and used as drivers for the subtractive hybridization.

After subtraction, 95 % of the labeled target cDNA was removed, which represents the population of mRNAs that are present in both stimulated and unstimulated tissues. Thus, clones from the subtracted cDNA libraries have been identified as being upregulated at different stages of LTP. These cDNA clones will allow us to further characterize the molecular mechanisms that are responsible for the maintenance of long-term potentiation in the dentate gyrus.

B5-134 CHRONIC AND ACUTE EFFECTS OF NEUROTROPHINS ON SYNAPSE DEVELOPMENT AND FUNCTION. Lu, B., Kim, H. G., Xie, K., Olafsson, P., and Wang, T. Roche Institute of Molecular Biology, Nutley, NJ, 07110 USA

Neurotrophins (including NGF, BDNF, NT-3, NT-4/5) have traditionally been regarded as slowly acting signals essential for neuronal survival and differentiation. However, little is known about their role in synaptic development and function. We have investigated both acute and long-term effects of neurotrophins on the physiological properties of synapses during development. Using *Xenopus* nerve-muscle co-cultures, we found that BDNF and NT-3, but not NGF, significantly potentiated spontaneous and evoked synaptic activities in neuromuscular synapses. Most of the of synapses treated by the neurotrophins exhibited a bell-shaped amplitude distribution of spontaneous synaptic currents, which reflects mature quantal secretion. Impulse-evoked synaptic currents also showed higher and more consistent amplitudes, indicative of more mature and reliable synapses. Moreover, the neurotrophins enhanced the expression of synapsin I, a synaptic vesicle associated protein involved in synaptic maturation. The neurotrophin effects appear to be mediated by the Trk family of receptor tyrosine kinases, primarily through a presynaptic mechanism. These results suggest that BDNF and NT-3 promote functional maturation of synapses.

To study the acute effects of neurotrophins on the functional properties of synapses, we used cortical neurons in culture. We found that NT-3 and NGF rapidly increased the frequency of spontaneous action potentials, and synchronized synaptic activities in developing cortical neurons. In addition, the inhibitory synaptic transmission mediated by $GABA_A$ receptors was reduced by NT-3. Thus, the excitatory effects of neurotrophins on spontaneous action potentials were attributable to a reduction of GABAergic transmission. Our finding, together with previous report of the rapid regulation of CNS neurotrophin expression by neuronal activity, suggest a new mechanism for modulation of synaptic transmission and activity-dependent synaptic plasticity.

B5-135 MUTATION OF A SINGLE AMINO ACID RESIDUE IN THE HUMAN GLYCINE RECEPTOR TRANSFORMS B-ALANINE AND TAURINE FROM AGONISTS INTO COMPETITIVE ANTAGONISTS. *P R Schofield, °S Ragendra, *J W Lynch, *K D Pierce, °C R French and °P H Barry. *The Garvan Institute of Medical Research, 384 Victoria Street, Sydney, 2010, Australia; °School of Physiology and Pharmacology, University of New South Wales, Sydney, 2052, Australia.

Missense mutations in the α1 subunit of the glycine receptor (GlyR) which result in the substitution of Leu or Gln for Arg 271 have been identified in individuals affected by the autosomal dominant neurological disorder, familial startle disease (hyperekplexia). When expressed as homomeric α1 subunit GlyRs, these mutations produce dramatic decreases in both glycine-binding affinity and the sensitivity of the glycine-activated chloride currents. Although the glycinergic agonists β-alanine and taurine bind to the mutant GlyRs with higher apparent affinities than glycine, their application fails to elicit a detectable current in the mutant GlyRs, however, does result in a dose-dependent inhibition of glycine-activated currents. Comparison of glycine dose-response curves generated in the absence and presence of either β-alanine or taurine reveals this antagonism is competitive in nature. Moreover, in contrast to glycine, prior application of either β-alanine or taurine does not alter the size of glycine-activated currents in the mutant receptors, indicating that the binding of these to compounds does not result in desensitisation of the receptor. The transformation of β-alanine and taurine from agonists into competitive antagonists is consistent with a decoupling of agonist binding and channel activation processes. The startle disease mutations have thus identified Arg 271, located at the extracellular border of the channel region, as a critical element in the mechanism that transduces these two processes in the GlyR.

B5-136 CONTACTIN/F11 CO-LOCALISES WITH TENASCIN IN RETINAL SYNAPSE LAYERS AND COMPLEXES WITH NON-RECEPTOR TYROSINE KINASE FYN. Lloyd Vaughan, Andreas H. Zisch, Luca D'Alessandri. Laboratorium für Biochemie I, ETH-Zentrum, 8092 Zürich, Switzerland.

The neural CAM contactin/F11 and the extracellular matrix glycoprotein tenascin-C are prominent molecules in the developing nervous system which interact in *in vitro* assays (Vaughan et al., 1994. Perspectives in Dev. Neurobiol. 2:43-52). To determine the potential role of this interaction in neural development, the distribution of tenascin-C and contactin/F11 was examined in the developing chick retina. The receptor-ligand pair is expressed in an overlapping pattern in the major synaptic inner (IPL) and outer plexiform (OPL) layers. In situ hybridization reveals that tenascin-C and contactin/F11 mRNAs are synthesized by different neuron types building the IPL and OPL. The mechanism whereby GPI-linked contactin/F11 modulates signal transduction was investigated. Analysis of detergent-resistant immune-complexes from neuronal cells shows contactin/F11 specifically complexes with cytoplasmic Fyn. Additionally, contactin/F11 transfected into HeLa cells is induced by capping antibodies to form co-clusters with Fyn, thus confirming their physical association. This indicates that contactin/F11-mediated signalling requires Fyn and opens up new possibilities for synapse modulation mediated by contactin/F11 and tenascin-C.

B5-137 THE ROLE OF NO IN ACTIVITY-DEPENDENT SYNAPTIC DEPRESSION AT DEVELOPING NEUROMUSCULAR SYNAPSES. T. Wang and B. Lu. Roche Institute of Molecular Biology, Nutley, NJ 07110

Temporal correlation between pre- and postsynaptic activities serves as an important mechanism that regulates synaptic connectivity during development and synaptic plasticity in the adult. In developing neuromuscular junctions, postsynaptic activity plays a critical role in functional suppression, and ultimately elimination of the synapses. While repetitive postsynaptic firing asynchronous to the presynaptic activity results in a persistent synaptic suppression, the underlying molecular mechanism remains unknown. Here we provide evidence that nitric oxide (NO), a free radical implicated in several forms of synaptic plasticity, may serve as a retrograde signal for the activity-dependent suppression in the neuromuscular synapse. NO donors and activators of the cGMP pathway suppressed spontaneous and evoked synaptic currents. Moreover, the synaptic suppression induced by postsynaptic depolarization was prevented by the NO binding protein hemoglobin and by inhibitors of NO synthase. Thus, synaptic suppression may be triggered by NO released from a postsynaptic myocyte that fires asynchronously to the presynaptic terminal.

Late Abstract

UPREGULATION OF ACETYLCHOLINE RELEASE AT THE MYASTHENIC ENDPLATE: A STUDY ON THE ROLE OF NITRIC OXIDE, ARACHIDONIC ACID METABOLITES AND PROTEIN KINASES. *J.J. Plomp and P.C. Molenaar, Div. of Membrane Electrophysiology and Pharmacology, Dept. of Physiology, University of Leiden, P.O. Box 9604, 2300 RC Leiden, The Netherlands*

Treatment of rats with α-bungarotoxin reduces endplate acetylcholine (ACh) receptors (AChRs). This state of Toxin-Induced Myasthenia Gravis' (TIMG) leads to Ca^{2+}-dependent upregulation of ACh release from motor nerve endings[1,2]. We considered involvement of nitric oxide (NO), arachidonic acid (AA) metabolites and protein kinases in this synaptic adaptation mechanism.

We recorded endplate potentials and miniature endplate potentials in diaphragms in which the muscle action potentials were blocked by μ-conotoxin and calculated quantal contents from their amplitudes. The *in vitro* effects of the following compounds were tested: hemoglobin (binds NO), DEDA (inhibitor of part of the arachidonic metabolic cascade), H7 and polymyxin B (protein kinase C (PKC) inhibitors), K252a (inhibitor of PKC, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and other kinases) and KN62 (CaMKII inhibitor).

Hemoglobin (100 μM) had no effect on the mean quantal content of TIMG muscles, while DEDA (100 μM) decreased it insignificantly by about 10%. Both H7 (120 μM) and polymyxin B (7-30 μM) had no effect on the quantal contents of TIMG muscles. K252a (200 nM) caused a 30% decrease in the quantal content (P_2<0.01, *t*-test). It had no effect on the quantal content of control muscles. Incubation of TIMG muscles with 10 μM KN62 resulted in an 18% decrease of quantal content (P_2<0.02, *t*-test). At TIMG endplates with the highest quantal contents, K252a and KN62 had the largest effect.

We conclude that it is unlikely that NO or PKC plays a role in the upregulation of ACh release at endplates from TIMG rats but that it could well be that CaMKII is involved.

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2. Plomp, J.J., Van Kempen, G.Th.H. & Molenaar, P.C. (1994) J Physiol (Lond) 478, 125-136.