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MONDAY SESSION

Plenary lecture 1

PL1

CONTROL OF SYNAPTIC JUNCTION DYNAMICS: ROLES OF THE CADHERIN–CATENIN COMPLEX

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Synaptic junctions provide the physical bases for interneuronal communications; therefore, to elucidate the regulatory mechanisms of their formation and dynamics is important. Cadherin is a group of transmembrane homophilic adhesion proteins, essential for general cell–cell adhesion, and their activities are controlled by cytoplasmic molecules including catenins. These molecules are also localized at synaptic contacts. Evidence has been provided that, if cadherin activity is suppressed in hippocampal pyramidal neurons in culture, dendritic spine morphogenesis is severely perturbed, resulting in a transformation of the spines into filopodia-like processes. Genetic deletion of α N-catenin, known to serve as a linker between the cadherin and actin cytoskeleton, results in destabilization of synaptic contacts. On the contrary, overexpression of this catenin causes excess spine formation and reduced spine turnover. Pharmacological suppression of neural activities in hippocampal cultures induces a release of α N-catenin from synapses, whereas elevation of neural activities have opposite effects, i.e., enrichment of this molecule in synapses, suggesting the existence of an activity-dependent mechanism to control the association of α N-catenin with synapses. In addition, it is known that a number of different cadherin subtypes with distinct adhesive specificities, generated due to the sequence diversity of their extracellular domain, are expressed in the nervous system, and each neuron has a unique set of these cadherins. These observations suggest that the cadherin–catenin complex regulates synapse dynamics from the cytoplasmic side, and possibly synaptic specificity from the extracellular side, although the latter idea is awaiting experimental tests.

ISN young scientist lectureship award

YL1

MECHANISMS OF ION CHANNEL LOCALIZATION IN AXONS

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The brain and nervous system process and transmit information as electrical signals. The generation, propagation, and modulation of these signals occurs through the precisely timed actions of ion channels and neurotransmitter receptors in neurons and other cells of the central and peripheral nervous system. These properties require the appropriate complement and localization of ion channels. In recent years it has become clear that the distribution and kinds of channels found in myelinated axons are regulated by neuron–glia interactions. For example, demyelination (as occurs in multiple sclerosis) alters both the location and type of Na^+ channels found in axons. Further, node of Ranvier formation and maintenance depends on axon – glia contact. However, it is also clear that at one location in the axon – the axon initial segment – Na^+ channels are appropriately clustered in very high densities without the influence of myelin or glia. Thus, both intrinsic and extrinsic mechanisms exist to localize ion channels to specific domains in the axon. This lecture will describe recent work to elucidate the extrinsic (glial) and intrinsic (neuronal) mechanisms that underlie ion channel clustering at nodes of Ranvier and the axon initial segment.

S1 Glial cells as therapeutic targets for neurological disorders

S1.A
BRAIN AND SPINAL CORD INJURY – ROLE OF GLIA AND TREATMENT BY ADULT STEM CELLS

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Bone marrow stromal cells (MSC) are pluripotent progenitor cells that have the capacity to migrate towards lesions and induce site-dependent differentiation in response to environmental signals. We examined the behavior of rat and human MSC grafted to injured rat brain and spinal cord and studied whether these cells participate in lesion repair, differentiate into neurons or astrocytes and promote functional recovery. The fate of MSC was further studied using cells labeled with superparamagnetic iron-oxide nanoparticles. The cells were transplanted into rats with a cortical photochemical lesion or a spinal cord compression lesion. In vivo MR imaging was used to track their fate; electron microscopy and Prussian blue staining confirmed the presence of nanoparticles inside the cells. MSC labeled with nanoparticles preferentially migrated into the brain or spinal cord lesion. However, only a few of the cells that entered the lesion expressed the neuronal marker NeuN (<5%), and even fewer GFAP-positive cells were detected. There was no significant difference in the number of MSC entering the lesions between animals directly injected in the cortex or spinal cord and those systemically infused. We found that intravenous injection of MSC 24 hours or 7 days after spinal cord injury improved the behavioral outcome of the animals (BBB score and plantar test), presumably by the production of regeneration-promoting factors as yet unknown. Moreover, the implantation of biocompatible polymer hydrogels reduced scar tissue formation and bridge a lesion, providing a scaffold to reform the tissue structure. Our studies demonstrate the potential of MSC as a therapeutic tool in the treatment of brain and spinal cord injury.

S1.B
ATP RECEPTORS IN MICROGLIA AS A TARGET FOR NEUROPATHIC PAIN

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Pain following nerve damage is an expression of pathological operation of the nervous system, one hallmark of which is tactile allodynia. We already reported that activation of P2X2/3 heteromeric channel/receptor in primary sensory neurons causes acutely tactile allodynia. We report here that tactile allodynia under chronic pain state requires an activation of P2X4 ionotropic ATP receptor and p38 mitogen-activated protein kinase (MAPK) in spinal cord microglia. Two weeks after L5 spinal nerve injury, rats displayed a marked mechanical allodynia. In the rats, activated microglia were detected in the injury side of the dorsal horn where the level of the dually-phosphorylated active form of p38MAPK (phospho-p38MAPK) was increased. We performed the double-immunostaining analysis using cell-type specific markers and found that phospho-p38MAPK-positive cells were microglia. Moreover, intraspinal administration of p38MAPK inhibitor, SB203580, suppressed the allodynia (Tsuda *et al.*, Glia 45, 89–95, 2004). We also found that the expression level of P2X4 was increased strikingly in spinal cord microgila after nerve injury and that pharmacological blockade of P2X4 reversed the allodynia (Tsuda *et al.*, Nature 424, 778–783, 2003). Intraspinal administration of P2X4 antisense oligodeoxynucleotide (ODN) reduced induction of P2X4 and suppressed tactile allodynia. These results demonstrate that activation of P2X4 or p38 MAPK in spinal cord microglia is necessary for tactile allodynia following nerve injury.

S1.C
NOVEL THERAPEUTIC TARGETS IN GLIA–NEURON INTERACTION: G-PROTEIN COUPLED RECEPTORS AND DEUBIQUITINATING ENZYMES

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G-protein coupled receptors (GPCRs) form the largest super-family of receptors. About 60% of medicines are thought to target GPCRs. We recently developed a novel strategy to screen GPCRs that are highly or selectively expressed in particular cells in the brain. Since recent literature suggests causative roles of glial cell dysfunction and/or altered neuro/glio-genesis in many neuropsychiatric disorders, we aimed to characterize GPCRs expressed in matured glia as well as neural progenitor cells by using the method. Among about 300 GPCRs expressed in the adult mouse brain, we found that type 2 neurotensin receptor (Ntsr2) was highly expressed in mouse embryonic neural progenitor cells. In situ hybridization of the adult mouse brain suggested that the molecule was abundantly expressed in astrocytes. Mice lacking Ntsr2 showed altered emotional behaviors. Application of an Ntsr2 agonist also modified the behavior of wild type mice. These results suggest the possible involvement of Ntsr2 in neuropsychiatric disorders. On the other hand, we found possible regulatory role of a deubiquitinating enzyme, UCH-L1, in neuro/glio-genesis. The gracile axonal dystrophy mouse, which lacks endogenous expression of UCH-L1, showed modified gliogenesis and altered emotional behaviors. Since UCH-L1 is expressed in neurons and nestin-positive progenitors but not in astrocytes, our observation suggests a novel link between neurons and glia through UCH-L1. Recently UCH-L1 was reported to regulate the activity of neurotransmitter receptors. Further investigation on UCH-L1 as well as GPCRs should provide important information to develop novel therapies for neuropsychiatric disorders.

S1.D
PURINE RECEPTOR-MEDIATED NEURON–GLIA INTERACTION: PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL RELEVANCE

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Glia cells are known to secrete active factors that modulate neuronal activities. Using hippocampal cultures and slices, we found that activation of glutamatergic neurons causes ATP release from astrocytes,

which induces both homo- and heterosynaptic suppression either directly through activation of presynaptic P2Y receptors or indirectly through adenosine degraded from ATP by ecto-nucleotidase. Thus, through the mediation of astrocytes, neuronal activity can influence not only on the activated neural synapse, but also on other adjacent synapses. Such unique activity-dependent heterosynaptic suppression may have important physiological relevance on neural signaling integration and neural circuit functions. We further found that ATP applied in large dose (1 mM) produced inward current in astrocytes with properties characteristic of P2X7 receptor activation. The current was amplified in low divalent cation medium, more potently activated by P2X7 specific antagonist BzATP, and blocked by P2X receptor antagonist PPADS and by P2X7 receptor specific antagonist oxidized ATP. Analysis of the reversal potentials of BzATP-induced currents under various intra- and extracellular ionic conditions showed that P2X7 channels are permeable to large anions like glutamate and D-aspartate. Furthermore, astrocytes exposed to BzATP also became permeable to Lucifer yellow, indicating a large channel opening. Release of L-glutamate and D-aspartate through P2X7 channels was confirmed using radiolabeled tracers. Efflux through P2X7 channels is a novel route of ligand-stimulated, nonvesicular astrocyte glutamate release that may play important roles in various pathophysiological conditions such as ischemia and neuro-degenerative disease.

S2 New frontiers in functional roles for lipids in the nervous system

S2.A
THE ROLE OF LIPID MICRODOMAINS IN AXON GROWTH

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Axon growth is stimulated by region-specific and coordinated activities of the cytoskeletons and cell adhesion molecules (CAMs) in the growth cone. Furthermore, CAM functions could be modified by its localization to lipid rafts/detergent-resistant membranes, specialized microdomains enriched in signaling molecules. My talk will deal with a question of whether and how lipid rafts are involved in axon growth stimulated by CAMs, such as L1, N-cadherin, and β1 integrin. L1 and N-cadherin are present in lipid rafts while β1 integrin is exclusively detected in non-raft membranes of neurons. I will present a strategy for acute and localized disruption of raft integrity in a living cell, which employs a technique known as micro-scale fluorophore-assisted laser inactivation (micro-FALI). Axon growth stimulated by L1 and N-cadherin but not by β1 integrin is inhibited by micro-FALI-mediated raft disruption in growth cones or by pharmacological treatments that deplete cellular cholesterol or sphingolipids, essential components for lipid rafts. Micro-FALI within the peripheral domain of growth cones, or even within smaller areas such as the filopodia and the lamellipodia, is sufficient to impair their migration. However, micro-FALI within the central domain does not affect growth cone migration. I will present some preliminary results suggesting that lipid rafts in the peripheral domain are implicated in the spatial regulation of L1 trafficking in the growth cone. These results demonstrate the region-specific involvement of lipid rafts in CAM-dependent growth cone motility. I will also introduce our proteomic studies that aim to identify raft-dependent signal transduction pathways downstream of CAM ligation.

S2.B
DEFECTIVE CALCIUM HOMEOSTASIS IN SPHINGOLIPID STORAGE DISEASES

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Work from our laboratory has recently begun to provide clues that may provide molecular descriptions for the neuropathophysiology in three sphingolipid storage diseases, neuronopathic forms of Gaucher disease, Sandhoff disease and Niemann-Pick A (NPD-A) disease. In a mouse model of Gaucher disease (Gba), there is a significant increase in the rate of Ca^{2+}-release from the endoplasmic reticulum (ER) via the ryanodine receptor (RyaR), resulting in elevated cytosolic Ca^{2+}-levels which leads to enhanced sensitivity to agents that induce cell death. Microsomes prepared from human Gaucher type 2 and 3 brain samples show a similar elevation in Ca^{2+}-release via the RyaR. Cytosolic Ca^{2+}-levels are elevated in neurons from a mouse model of Sandhoff disease (Hexb), but in contrast to Gba neurons this is caused by changes in the rate of Ca^{2+}-uptake into the ER via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). Hexb neurons are more sensitive to thapsigargin-induced cell death suggesting that elevated cytosolic Ca^{2+} may contribute towards apoptotic cell death in Hexb neurons. Finally, Ca^{2+}-homeostasis is also altered in a mouse model (ASM) of NPD-A, with reduced rates of Ca^{2+}-uptake via SERCA observed in the cerebellum of 6–7 month-old mice. However, the mechanism responsible for defective Ca^{2+}-homeostasis is different from that observed in the other two diseases. Levels of SERCA expression are significantly reduced in the ASM cerebellum by 6–7 months of age, immediately prior to death of the mice, as are levels of the inositol 1,4,5-triphosphate receptor (IP3R), the major calcium-release channel in the cerebellum. Together with recent studies from another laboratory on the GM1 gangliosidoses, NPD-A becomes the 4th sphingolipid storage disease in which neuronal death and/or dysfunction might be caused by defective Ca^{2+}-homeostasis.

S2.C
HIGH-LEVEL CHOLESTEROL AS A RATE-LIMITING FACTOR OF MYELIN MEMBRANE GROWTH

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In the central nervous system, oligodendrocytes ensheath axonal processes and assemble compact myelin. The largest fraction of myelin membrane lipids is cholesterol, synthesized locally in the brain. Cholesterol limits ion leakage through membranes, potentially relevant to the electrical insulation property of myelin. Conventional genetic disruption of cholesterol biosynthetic enzymes causes early embryonic death in mice. Therefore, we addressed the function of cholesterol biosynthesis *in vivo* using the conditional Cre/loxP system. The squalene synthase/fdft1 gene was targeted, because its encoded protein, Squalene Synthase, is the first enzyme strictly devoted to sterol biosynthesis. We have inactivated the floxed fdft1 gene by Cre recombination in myelinating glial cells, using the Cnp1 regulatory region. The conditional mouse mutants are viable but severely dysmyelinated with a motor defect that included ataxia, tremor, and frequently premature death. Unexpectedly, in surviving older mice myelination continued at a low rate for several months. Moreover, purified myelin has a close to normal biochemical composition, including highly elevated levels of cholesterol. Thus, cholesterol when provided by non-recombinant cells in the brain and taken up by oligodendrocytes, is sufficient to guarantee basic cellular survival. Cholesterol incorporation becomes an essential and rate-limiting step for myelin membrane growth. These data also provide *in vivo* proof for an efficient horizontal transfer of cholesterol between different cell types in the brain.

S2.D
LIPID MICRODOMAIN-MEDIATED SIGNALING CASCADES: IMPLICATIONS FOR DEMYELINATING DISEASE

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Myelin is a dynamic membrane in communication with both the axon and environment. Glycosphingolipid-Cholesterol Microdomains (lipid rafts) are small (nm) liquid ordered membrane entities with few proteins; upon ligand- or Ab-mediated cross-linking they can coalesce into larger, functionally active structures providing 'activation centers' for

signal transduction. The molecular order of rafts is used to isolate raft complexes by detergent extraction (e.g., TX-100, 4°C). The high cholesterol/glycosphingolipid content in oligodendrocytes (OLs) and myelin suggested that rafts contribute functionally to their physiology [Kim & Pfeiffer (99) J Neurocytol 28:281; Taylor *et al.* (02) J Neurochem 81:993]. Indeed, rafts are important in axon–myelin [Marta *et al.* (04) Neuron Glia Biology 1:35; Schafer *et al.* (04) J Neurosci 24:3176] and myelin–environment [Marta *et al.* (03) J Neurosci 23:5461; Marta (05) JBC, in press] signaling. Ab cross-linking of the myelin protein MOG or MAG results in (a) their rapid repartitioning into rafts, followed by (b) raft-dependent phosphorylation–dephosphorylation of specific proteins and downstream events, and in the case of MOG, (c) dramatic, rapid loss of myelin-like membrane [Marta *et al.* (03) J Neurosci 23:5461; Marta (05) JBC, in press]. The end results are highly specific for the target protein (i.e., MOG vs. MAG). We propose that these events contribute to the molecular mechanism for the B-cell component of multiple sclerosis and in the animal model EAE. We predict that an understanding of these mechanisms will be instructive with regard to mechanisms by which these antibodies effect an important component of demyelinating pathophysiology.

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S3 Calcium-induced calcium release and presynaptic function

S3.A
NEURONAL CALCIUM SIGNALING

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Neurons employ two sources of calcium. Entry of external Ca^{2+} through voltage- or receptor-operated channels plays a major role, but there also is an important contribution of Ca^{2+} released from the endoplasmic reticulum (ER). The latter is a continuous membrane network that extends throughout the neuron. This ER may be considered both structurally and functionally as a neuron-within-a-neuron. Indeed the ER has many properties that one normally associates with the plasma membrane. In particular, it maintains a large concentration gradient of Ca^{2+} that can be released in a regenerative manner thus enabling information to spread through Ca^{2+} waves. InsP3 receptors (InsP3Rs) and ryanodine receptors (RYRs), which display a process of calcium-induced calcium release (CICR), carry out this regenerative process. The concentration of Ca^{2+} within the lumen is an important factor in determining the sensitivity of these release channels. During bouts of neuronal activity, the Ca^{2+} that enters from the outside is taken up the ER, which then sensitises these channels such that Ca^{2+} is released under appropriate conditions. In effect, this accumulation of Ca^{2+} provides a short-term memory in that it integrates the brief entry pulses associated with neural activity. When the accumulated Ca^{2+} reaches a critical threshold, there is a large explosive regenerative release of Ca^{2+} that may provide the neuron with information concerning previous levels of neuronal activity. The important point to stress, therefore, is that the ER and the plasma membrane operating as a binary membrane system control a wide range of processes such as excitability, plasticity and gene transcription. Particular attention will be paid to their role in presynaptic transmitter release.

S3.B
PRESYNAPTIC STORE CALCIUM AND MULTIVESICULAR RELEASE OF GLUTAMATE AT MOSSY FIBER CA3 SYNAPSES

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Action potential-independent transmitter release has not been thought to play a significant role in impulse propagation across synapses. Here we show that activation of presynaptic nicotinic acetylcholine receptors at the mossy fiber-CA3 pyramidal cell synapses in the hippocampus can synchronize glutamate release. This effect is mediated by calcium flux through the receptors resulting in calcium-induced calcium release from endoplasmic reticulum stores. The synchronization of glutamate release at these terminals leads to a burst of high frequency release events, portion of which is contributed by large amplitude multivesicular release. This burst of glutamate release, mediated by events occurring locally at the presynaptic terminal, is sufficient to drive the postsynaptic neuron above its firing threshold, thus providing a mechanism for presynaptic action potential-independent form of transmission at a CNS synapse.

S3.C
VICaR IN NEURON TERMINALS SKIRTS CICR TO GENERATE Ca^{2+} SYNTILLAS

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Ca^{2+} syntillas are brief, focal cytosolic Ca^{2+} transients in hypothalamic nerve terminals which resemble sparks (scintilla, L. spark, in a synaptic structure, a nerve terminal). Syntillas were studied in single, freshly dissociated nerve terminals from mouse, using simultaneous high resolution, widefield Ca^{2+} imaging (50–200 Hz) and whole-terminal patch recording. Since syntillas occur spontaneously in the absence of external Ca^{2+} (+200 μM extracellular EGTA) at a membrane potential of –80 mV, they must arise from intracellular stores. Their sensitivity to caffeine stimulation and ryanodine blockade and their insensitivity to heparin indicate that they are mediated by ryanodine receptors (RyRs), a conclusion corroborated by the immunocytochemical identification of both type 1 (RyR1) and type 2 (RyR2) in the terminals. Surprisingly, depolarization of the terminals, in the absence of Ca^{2+} influx, increases the rate of syntillas over a range of –40 to +40 mV. This voltage-induced Ca^{2+} release (VICaR) is blocked by ryanodine but not by heparin. A variety of pharmacological manipulations demonstrate that VICaR is mediated by dihydropyridine receptors. This finding and the presence of RyR1, but not RyR2, close to the plasma membrane suggest that VICaR is mediated by the same signalling molecules as is EC coupling in skeletal muscle (Mouse chromaffin cells also display Ca^{2+} syntillas, but these cells they lack RyR1 and their syntillas are not regulated by VICaR.), It appears that VICaR plays a role in the pathophysiology of acute spinal cord injury, and it may also mediate a variety of physiological processes other than the final step in exocytosis.

S3.D
CALCIUM HOMEOSTASIS IN THE NEURONAL ER: GENERAL PRINCIPLES AND IMPLICATIONS FOR PHYSIOLOGY AND PATHOPHYSIOLOGY

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The neuronal endoplasmic reticulum (ER), represented by a three-dimensional network formed by an endomembrane, extends from the nucleus and the soma to the dendrites and, through the axon, to presynaptic terminals. The ER is intimately involved in neuronal Ca^{2+} signalling via Ca^{2+}-induced Ca^{2+} release or InsP3-induced Ca^{2+} release, controlled by two subsets of Ca^{2+} release channels residing in the ER membrane, the ryanodine receptors (RyRs) and the InsP3-receptors (InsP3Rs). The ER Ca^{2+} store emerges as a single interconnected Ca^{2+} pool, although the RyRs and InsP3Rs show heterogeneous localisation in distinct cellular sub-compartments, conferring thus specificity in local Ca^{2+} signals. The intimate mechanisms of ER integration remain largely unknown, yet a key role for Ca^{2+} is emerging. First, Ca^{2+} is a key input and output signal of the ER as cytosolic Ca^{2+} increases affect

its concentration in the ER, and in turn, the ER Ca^{2+} release and uptake influence the cytosolic Ca^{2+} concentration. Second, the intra-ER chaperones are Ca^{2+} binding proteins, and changes in ER Ca^{2+} content affect their functional activity. Therefore, fluctuations in the ER Ca^{2+} concentration provide the link between rapid signalling and long-lasting adaptive responses. The disruption of intra-ER Ca^{2+} homeostasis can be involved in neurodegenerative disorders such as diabetic peripheral neuropathies and Alzheimer disease.

S4 Dynamics of the neuronal cytoskeleton

S4.A
MOLECULAR MOTOR PROTEINS ORGANIZE MICROTUBULES DURING NEURONAL DEVELOPMENT

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Recent live-cell imaging studies have shown that microtubules move rapidly down the length of the axon, but only if the microtubules are relatively short. During axonal growth, microtubules move in both the anterograde and retrograde directions. The balance of these two directions of transport is presumably a major determinant of axonal growth. We have sought to better understand the underlying mechanisms that regulate these transport events. In one set of studies, we used siRNA to lower the levels of cytoplasmic dynein in cultured neurons, after which we determined the effects on microtubule transport in the axon. We found that the anterograde movements were notably compromised, whereas the retrograde movements were unaffected. Thus cytoplasmic dynein is an important motor for transporting microtubules in one direction, but not both. We are currently seeking to determine whether a kinesin-related protein such as Eg5 fuels the retrograde movements of microtubules. In other studies, we are pursuing the hypothesis that the capacity of a microtubule to be transported is dependent upon its length, and hence that microtubule-severing proteins such as katanin are a key factor in mobilizing microtubules by fracturing them into shorter pieces. Our studies suggest that neuronal microtubules in different regions of the neuron vary in their sensitivity to katanin, and that the relative sensitivity of a microtubule to katanin is determined by particular microtubule-associated proteins (MAPs) such as tau. Thus, a number of different categories of molecules (molecular motors, severing proteins, and traditional MAPs) are essential for regulating microtubule transport in the neuron.

S4.B
NEURONAL POLARITY AND CYTOSKELETON

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Neurons are one of the most highly polarized cells, comprised of two structurally and functionally distinct parts, axon and dendrites. However, it remains largely unknown how neuronal polarity is established. We previously showed that collapsin response mediator protein-2 (CRMP-2) is enriched in growing axon of cultured hippocampal neurons. The overexpression of CRMP-2 induces the formation of multiple axons, whereas truncated fragment of CRMP-2 or knockdown of CRMP-2 by RNAi inhibits the formation of the primary axon. Thus, CRMP-2 appears to play a crucial role in the determination of axon/dendrite fate and axon elongation. We have recently found that CRMP-2 interacts with tubulin dimers to promote microtubule-assembly for axon growth, and that CRMP-2 binds to Sra-1, an effector of Rac1 to regulate WAVE-dependent reorganization of actin filaments. CRMP-2 links kinesin-1 to tubulin dimmers and Sra-1, and participates in the kinesin-1-dependent transport of tubulin dimmers and the Sra-1/WAVE complex to developing axons. We have also found that the PAR-6/PAR-3 complex and the Akt/GSK-3beta pathway are involved in neuronal polarization downstream of PI3-kinase. The PAR-6/PAR-3 complex mediates the Cdc42-induced Rac1 activation through direct interaction with STEF/Tiam1 (Rac GEF) for axon growth. GSK-3beta phosphorylates CRMP-2 and inactivates its tubulin-binding activity. Akt appears to phosphorylate GSK-3beta and inactivates its kinase activity, thereby increasing non-phosphorylated active CRMP-2 which promotes axon growth. This time, we summarize and discuss functions of these 'polarity' molecules in regulation of neuronal polarity and cytoskeleton.

S4.C
NEW ROLES FOR FIBROUS MICRORUBULE-ASSOCIATED PROTEINSDURING AXONAL DEVELOPMENT

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Neurons are highly polarized cells that extend single long axons, and several much shorter but highly branched dendrites. Polarization occurs when one of the multiple neurites that arise from the cell body initiates a phase of rapid elongation, becoming the axon. Current views favor the view that one mechanism for the axon to differentiate from the other neurites relates with differences in cytoskeletal dynamics. Axon formation entails assembly and extension of microtubules within growth cones that contain abundant actin filaments. It has been proposed that actin filaments within axonal growth cones are more dynamic than those in other neurites, thereby allowing microtubule penetration and stabilization when neurons polarize. Unfortunately, the precise nature of the interaction between microtubules and microfilaments during neuronal polarization has remained largely unknown. We now show that the cytoplasmic dynein light chain Tctex-1 plays a key role in multiple steps of hippocampal neuron development, including initial neurite sprouting, axon specification, and later dendritic elaboration. The neuritogenic effects elicited by Tctex-1 are independent from its cargo adaptor role for dynein motor transport. Finally, our data suggest that the selective high level of Tctex-1 at the growth cone of growing axons drives fast neurite extension by modulating actin dynamics. This effect involves recruitment and activation of Tiam1, a guanosine-nucleotide exchange for Rac, to the axonal growth cone.

S4.D
CYTOSKELETAL MECHANISMS IN NEURITE INITIATION

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Process outgrowth in neurons ultimately generates axons, dendrites, and the synaptic connections needed to form brain circuitry. The first step is neurite initiation. Our studies on this preliminary event suggest it shares similarities with other aspects of cell migration and morphogenesis, but exhibits unique features as well. During neurite initiation microtubules align to form a tight bundle and actin filaments reorganize to produce a growth cone. Physical association between actin and microtubules may be involved in this spatial reorganization of the two networks. One protein that could mediate actin–microtubule interaction in neurons is the microtubule-associated protein MAP2. Expression of the small isoform MAP2c induces neurite formation in neuroblastoma cells in the absence of other differentiation signals. Dominant negative forms of MAP2c inhibit neurite initiation. MAP2c not only binds microtubules, but also binds F-actin via its microtubule binding domain (MTBD). Surprisingly, the homologous MTBD in the closely related

molecule tau lacks this F-actin binding activity, and tau is also incapable of inducing neurites. These and other findings suggest that certain MAPs are crucial in remodeling both microtubules and F-actin during neurite initiation. Another key step is the generation of forces that initiate and stabilize process outgrowth. When expressed in heterologous cells, MAP2c induces bundles of microtubules that are subject to rapid anterograde transport. Our data suggest a model in which microtubules, coupled via dynein to a rigid scaffold such as the plasma membrane, promote neurite initiation by generating outward forces at the cell margin.

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C1 Regulation of synaptic plasticity and memory by reactive oxygen species in the normal, aged, and diseased hippocampus

C1.A
INFLAMMATORY CHANGES IN REGULATION OF HIPPOCAMPAL SYNAPTIC PLASTICITY IN THE AGED HIPPOCAMPUS

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Among the several changes which occur in the aged brain is a deterioration in synaptic function and consequently a deficit in synaptic plasticity; thus it has been consistently reported that there is a decrease in the ability of aged rats to sustain long-term potentiation (LTP) in hippocampus. A great number of changes have been catalogued in the aged brain which are likely to contribute to this and these include inflammatory and oxidative stress. One of the hallmarks of inflammatory stress is an increase in concentration of the proinflammatory cytokine IL-1beta, and the evidence has indicated that hippocampal IL-1beta concentration is inversely correlated with LTP. Here evidence will be presented which indicates that the age-related increase in IL-1beta concentration is coupled with age-related decreases in the concentration of two anti-inflammatory cytokines IL-10 and IL-4, and that the signalling pathways induced by these cytokines, which exert neuroprotective effects, are similarly depressed with age. To address the question of whether activated microglia might be the cell source of IL-1beta in the hippocampus of aged rats, several phenotypic and functional markers of microglial activation were assessed. The evidence indicates that there is a marked age-related increase in microglial activation and that this activation results in increased production of IL-1beta and also NO. Significantly, the activation state of microglia can be modulated by a number of treatments and evidence will be presented which indicate that the modulatory effects of these treatment may reside in their ability to induce IL-4.

C1.B
AGE-DEPENDENT REDOX MODULATION OF SYNAPTIC PLASTICITY AND SYNAPTIC TARGETS

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Reactive oxygen species (ROS) have long been associated with oxidative stress relevant to aging and age-related neurodegenerative disorders such as Alzheimer's Disease and Parkinson's Disease. However it is now evident that ROS also play pivotal roles in normal cell signaling. The focus of the present study is to understand how ROS modulate long-term potentiation (LTP)-forms of synaptic plasticity in either a positive or negative mode. Previous results showed that LTP induced in vitro by high frequency stimulation is reduced in the CA1 of mouse hippocampal slices from old (2 years) mice relative to young (2 months) mice. Hippocampal LTP at either age is insensitive to micromolar levels of hydrogen peroxide. However enzymatic removal of hydrogen peroxide by catalase inhibits LTP in young hippocampus, but conversely enhances LTP in old hippocampus. Moreover LTP appears normal in adult hippocampus from both glutathione (GSH) peroxidase-overexpressing GPx1 transgenic mice and catalase Cas1 'knock-out' mice, as well as GSH-treated hippocampus. Overall the results suggest that a threshold level of intracellular hydrogen peroxide in the micromolar range is tolerated and often required to sustain LTP but age-related deficits in LTP due to elevated peroxide are reversible. Ongoing experiments examine the source of hydrogen peroxide used to sustain LTP, protein kinase/phosphatase pathways impacted by fluctuating hydrogen peroxide levels, and redox modulation of striatal synaptic plasticity.

C1.C
CONTRASTING ROLES OF REACTIVE OXYGEN SPECIES IN HIPPOCAMPAL SYNAPTIC PLASTICITY AND MEMORY

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Reactive oxygen species (ROS) have been studied intensely in the central nervous system with respect to their role in oxidative stress associated with aging and neurodegenerative diseases. Studies from several laboratories, including our laboratory, have shown that mice that overexpress various isoforms of superoxide dismutase are protected against age-related impairments in hippocampal synaptic plasticity and memory function. However, a number of studies indicate that ROS play a critical role as signaling molecules during synaptic plasticity and memory formation in young animals. We have begun to investigate this dichotomy of ROS function in the hippocampus by identifying sources of ROS that might be responsible for physiological and pathophysiological processes. Using pharmacological and genetic approaches, we have found that NADPH oxidase is necessary for hippocampal synaptic plasticity and memory in young mice. We are currently conducting studies to identify protein targets that are modified by ROS produced via NADPH oxidase during synaptic plasticity. In addition to its role in normal neuronal function, NADPH oxidase also has been implicated in neurodegenerative diseases such as Alzheimer's disease. Consistent with this notion, we have found that amyloid beta peptide activates NADPH oxidase in the hippocampus. In addition, we have found that the activation of NADPH oxidase by amyloid beta peptide requires alpha7 nicotinic acetylcholine receptors. Taken together, our data suggest ROS produced via NADPH oxidase may be involved in both physiological and pathophysiological processes in the hippocampus.

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C1.D
BIMODAL REGULATION OF SYNAPTIC PLASTICITY BY REACTIVE OXYGEN SPECIES

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Reactive oxygen species (ROS) are implicated in age-related memory impairment suggested to result from excessive chronic oxidative stress to neuronal tissue. However, acute effects of ROS have not been

studied systematically. In earlier experiments, we found that acute exposure to 20 μM of hydrogen peroxide (H_2O_2) reduced synaptic plasticity (LTP) in region CA1 of rat hippocampal slices, while not affecting pre-established LTP. In sharp contrast, a much lower concentration of H_2O_2 (1 μM) caused a two fold increase in magnitude of LTP compared to controls. This enhanced LTP is likely to have been caused by activation of voltage gated calcium channels and by the protein phosphatase calcineurin. Further experiments were conducted with transgenic SOD-1 overexpressing mice, which are likely to produce sustained levels of H_2O_2. These mice failed to express normal LTP Surprisingly, added H_2O_2 actually restored LTP in SOD-1 mice, indicating that abnormal redox regulation in these animals may have a complex effect on synaptic plasticity. Aged wt mice were impaired in LTP in a manner that could be reversed by addition of H_2O_2. Surprisingly, aged tg-SOD mice exhibited larger LTP than that found in aged wt mice or in young SOD-1 mice, but this was now reduced by 50 μM H_2O_2. Both young tg-SOD and aged control mice displayed altered protein phosphatase activity, compared to that of young controls, moreover, FK506 inhibited LTP in old tg-SOD as well as in old wt mice treated with H_2O_2. Finally, old SOD-1 mice performed better than controls in spatial memory tasks, similar to the difference in their ability to express LTP. These data promote a dual role for H_2O_2 in the regulation of LTP, and proposes that it is mediated by the protein phosphatase calcineurin.

C2 Molecular neuroimaging

C2.A
IN VIVO MOLECULAR IMAGING FOR STEM CELL DYNAMICS

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The *in vivo* observation of cell mobility using a noninvasive imaging modality has gained high interest, as it allows the noninvasive true intra-individual longitudinal study of cell migration, homing, of neurogenesis. For this purpose, labeling procedures of the cells are introduced to produce sufficient contrast against the host tissue background. Depending on the chosen imaging technology, different demands on the contrast agent as well as on the labeling strategy must be considered. Selecting magnetic resonance imaging (MRI) as the imaging tool, iron oxide nanoparticles are mostly used to achieve a strong contrast on T2*-weighted images. Incorporation of the label into the cells depends on the various fundamental options of contrast agent application routes: i) in vitro labeling followed by cell implantation, or ii) *in vivo* labeling by specific injection of the contrast agent in combination with selective uptake by the desired cell population. Attention must be given the efficiency of the labeling procedure, sufficient contrast generation, tolerance of the procedure by the cells, and label dilution by proliferation. The technical sensitivity and detectability limits must be considered for a project investigating cell migration *in vivo*. Focus of the presentation will be on the potential to observe *in vivo* migration of stem cells in experimental stroke models using μMRI. Considerations regarding application to different examples of (patho-)physiological studies are also discussed.

C2.B
SMART CONTRAST AGENTS FOR MOLECULAR IMAGING: POTENTIALS OR LIMITS?

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Smart contrast agents should be tissue specific and/or responsive to given physiological parameters (pH, pO2, expression of molecules . . .). Alternatively, they should provide the scientist with other useful information at the cellular level. The only practical limit of smart contrast agents for MR cellular and molecular Imaging is their reflexivity, i.e. their ability to modify the image intensity at sufficiently low local concentration. In this respect, paramagnetic molecules are competing with superparamagnetic nanoparticles. In the context of brain imaging, magnetic labeling of cells allows for a MRI monitoring of their migration after implantation [Jendelova P. *et al.* (2003), Magn Reson Med. 50(4):767–776; Hoehn M. *et al.* (2002), Proc Natl Acad Sci U S A. 99(25): 16267–16272]. To optmize the magnetic labeling, we have studied the non-specific endocytosis of iron oxide nanoparticles on bone marrow mesenchymal stem cells and 3T6 embryonic fibroblasts, and quantified their iron oxide loading as a function of particle size and coating. Conversely, a specific labeling of cells can be obtained when iron oxide nanoparticles are specifically targeted to molecules expressed at the cell surface. In vitro molecular MRI reporters were obtained by grafting a sialyl-LewisX mimetic to a paramagnetic complex and to ultra small particles of iron oxide (USPIO), in order to target E-Selectin respectively expressed in inflammated brain and liver, and at the surface of TNF-a stimulated endothelial cells [Boutry S. *et al.* (2005), Magn Reson Med. 53(4):800–807; Sibson NR. *et al.* (2004), Magn Reson Med. 51(2):248–52; Boutry S. *et al.* unpublished results]. We have successfully evaluated USPIO carrying Wheat Germ Agglutinin (WGA)a neurotropic protein [Kang H.W. *et al.* (2002), Bioconjug Chem. 13(1):122–127; Petropoulos A.E. *et al.* (1995), Acta Otolaryngol. 115(4):512–516], with respect to the magnetic labelling of neurons and neuronal tract tracing [Boutry S. *et al.* unpublished results].

C2.C
GENE EXPRESSION IMAGING: APPLICATION TO NEUROSCIENCE

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Now that the human genome project has come to completion most genes have been identified. However, the big challenge for the future is to try to discover where the genes are expressed, how they are regulated, and what their exact function is in the homeostasis of specific cell types or tissues. This will lead to a better understanding of the molecular, cellular, and biological processes involved in normal physiology and pathology in a variety of diseases. It will also allow us to find new drug targets and, consequently, better management of diseases. In order to study the location and regulation of gene expression in vitro as well as *in vivo*, reporter genes (e.g. b-galactosidase, GFP, luciferase) have been widely used. They are also used for tracking the fate of cells injected systemically in small experimental animals. Until recently, the proteins translated from the reporter gene-constructs were either visualized in histological tissue sections or detected biochemically in tissue extracts after sacrifice of the animal. However, development of highly sensitive CCD camera's and bioluminescent imaging (BLI), in which luciferase is used as a reporter gene, currently allows very sensitive image recording of the topographical expression of this enzyme non-invasively and repetitively in the living animal. In this presentation the following points will be discussed: 1. The basic principles and advantages of bioluminescent imaging. 2. How BLI can be used to image gene expression *in vivo*. 3. The broad application of the technology for other applications like neuronal stem cell migration and fate, tumor progression and metastasis.

C2.D
CLINICAL PERSPECTIVES OF MOLECULAR IMAGING

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Positron emission tomography (PET) allows a non-invasive assessment of physiological, metabolic and molecular processes in humans and animals *in vivo* (molecular imaging). With the developments in tracer technology a variety of endogenously expressed and exogenously introduced genes coding for membrane receptors and transporters or cellular enzymes can be analyzed by PET. The most intriguing advantage of molecular imaging is the analysis of the dynamics of a given molecular event in the same experimental subject. This allows a non-

invasive characterization of animal models of human disease at various disease stages, under certain pathophysiological stimuli and after therapeutic intervention. Apart from direct detection and imaging of a certain gene product, indirect imaging technology is based on proportional coexpression of a PET marker gene with any gene of interest where specific probes are not available, yet. Moreover, certain genes can be used as surrogate markers. Important endogenous enzymes, receptors and membrane transporters which are non-invasively assessed in clinical neuroscience by PET include cellular hexokinase and thymidine kinase, aromatic amino acid decarboxylase, acetylcholine esterase, dopamine D2 and benzodiazepine receptors as well as amino acid transporters. With the advent of animal PET these tracers can be applied in animal models for human disease especially in the development of experimental therpeutics, such as growth factor administration, gene therapy and cell transplantation.

C3 Molecular mechanisms of neurodegenerative disease

C3.A
INVESTIGATIONS INTO THE PROPAGATION AND SPREAD OF INFECTIOUS PRIONS IN CULTURED CELLS

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Prion diseases are transmissible neurodegenerative disorders including Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. According to the protein only hypothesis, an abnormal isoform of the host encoded prion protein (PrP^{C}), referred to as PrP^{Sc}, is the sole or major component of the infectious agent (the "prion"). The molecular mechanisms underlying the conversion of PrP^{C} to PrP^{Sc} and the acquisition of infectivity are not well understood at the molecular level. Using a mouse adapted strain of human prions we have developed a cell based model of prion disease which produces infectious prions, providing a means to investigate cellular factors involved in prion propagation. Previous studies have demonstrated that infected cells release prions in association with exosomes. Consistent with this, the culture medium of our infected cell line contains exosome associated PrP^{Sc} which is capable of transmitting infection to further cells. Due to the unique protein composition of exosomes we are investigating the profile of expressed proteins of infected versus un-infected exosomes to identify cellular factors implicated in the propagation and spread of infectious prions. The infected cell model will prove useful in elucidating factors associated with prion infection, screening potential therapeutic compounds, and investigating the transmissible and neurotoxic properties of altered conformations of PrP.

C3.B
METALLOTHIONEINS AS A MODEL FOR INVESTIGATING NEURON–ASTROCYTE INTERACTIONS FOLLOWING CNS INJURY

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In previous studies, we have demonstrated that exogenous administration of the astrocytic protein metallothionein (MT) to injured neurons both in vitro and in animal models of CNS injury confers powerful neuroprotection by a direct, extracellular interaction with neurons (Chung *et al.*, 2003). As well as having clinical importance, our work suggests that the normal physiological role of metallothionein in the CNS involves a significant extracellular component, triggered by the initial neuronal injury (Chung *et al.*, 2004). In this regard, we observed that while MT immunoreactivity was not detectable in the un-injured rat neocortex, by four days following a focal cortical brain injury MT was found in astrocytes aligned along the injury site. At later time points, astrocytes, at a distance up to several hundred microns from the original injury tract, were MT immunoreactive. Using a cortical neuron/astrocyte co-culture model, we observed a similar MT response following in vitro injury. Intriguingly, scratch wound injury in pure astrocyte cultures resulted in no change in MT expression (Chung *et al.*, 2004). This suggests that MT induction was specifically elicited by neuronal injury. In summary, we propose that physical or chemical injury to neurons causes release of a signal that induces MT expression in astrocytes. Significant amounts of MT are then released, either actively or passively by astrocytes, into the extracellular environment of the lesion, where it is available to interact with neurons. Specifically, MT enhances neuronal survival following injury and promotes neurite outgrowth as we have demonstrated previously (Chung *et al.*, 2003). Thus, this model represents a specific example of the general principle of neuron–astrocyte interactions within the injured CNS.

C3.C
MODULATION OF ALPHA-SYNUCLEIN AGGREGATION

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Dopamine and α-synuclein are two key molecules associated with Parkinson's disease. We have identified a novel action of dopamine in the initial phase of α-synuclein aggregation and demonstrate that dopamine induces α-synuclein to form soluble, SDS-resistant oligomers. The dopamine: α-synuclein oligomeric species are not amyloidogenic as they do not react with Thioflavin T and lack the typical amyloid fibril structures as visualised with electron microscopy. Circular dichroism studies indicate that in the presence of lipid membranes dopamine interacts with α-synuclein causing an alteration to the structure of the protein. Furthermore, dopamine inhibited the formation of iron-induced α-synuclein amyloidogenic aggregates. Therefore, dopamine acts as a dominant modulator of α-synuclein aggregation along an off-pathway route from fibrillization. These observations support the paradigm emerging for other neurodegenerative diseases that the toxic species involves a soluble oligomer and not the insoluble fibril.

C3.D
PROBING FOR TRP CHANNEL FUNCTIONS ON CORTICAL NEURONS

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Neurons prominently express most members of the TRP family. We explored the hypothesis that TRP channels contribute to $[Ca^{2+}]i$

homeostasis in cortical neurons by single cell fluorescence imaging. Cortical neurons do not exhibit store-operated Ca^{2+} entry (Chinopoulos *et al.*, 2004, J Neurochem 91:471–83); however, when they are pretreated with a Ca^{2+}-free medium in the absence of intracellular store depletion, reintroduction of a divalent cation to the perfusate causes a sharp rise of $[Me^{2+}]i$. This basal Me^{2+} entry (BME) is inversely correlated to $[Ca^{2+}]e$ and $[Mg^{2+}]e$, and is inhibited by Gd^{3+}, spermine, SK&F 96365, KB-R7943 and FK506 when Ca^{2+} or Sr^{2+} is the $[Me^{2+}]e$, but not for Ba^{2+} or Mn^{2+}. BME is unaffected by removal of $[Na^{+}]e$ and only moderately reduced by a concomitant inhibition of VDCCs and NMDARs. Upon removal of $[Ca^{2+}]e$ and $[Mg^{2+}]e$, there is a slow rise in $[Na^{+}]i$, which is unaltered by TTX, Gd^{3+} and spermine, but is abolished by KB-R7943, probably reflecting reversal of the plasma membrane Na^{+}/Ca^{2+} exchanger. Whole-cell and perforated patch studies showed that removal of $[Ca^{2+}]e$ and $[Mg^{2+}]e$ induces a non-inactivating cation conductance with a reversal potential around 0 mV that is dependent on $[Na^{+}]e$. Currently, the only putative ligand of a subset of TRP channels (TRPC3/6/7) identified is diacylglycerol. Addition of the diacylglycerol analogue OAG, causes $[Ca^{2+}]i$ oscillations, that are abolished by La3+ but not by inhibition of PKC. In addition, inhibition of PKC reverts the response to OAG on $[Ca^{2+}]i$ from oscillatory to sustained. These results together with attempts to silence the expression of individual members of the TRP family will elucidate their contribution in neuronal $[Ca^{2+}]i$ homeostasis.

W1 The human brain proteome project

W1.A
THE HUMAN BRAIN PROTEOME PROJECT (HBPP), AN HUPO INITIATIVE

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HUPO Brain Proteome Project – HBPP is an open international project under the patronage of the Human Proteome Organisation (HUPO) that aims: to analyze the brain proteome of human as well as mouse models in healthy, neurodiseased and aged status with focus on Alzheimer's and Parkinson's Disease; to perform quantitative proteomics as well as complementary gene expression profiling on disease-related brain areas and bodily fluids; to advance knowledge of neurodiseases and aging in order to push new diagnostic approaches and medications; to exchange knowledge and data with other HUPO projects and national and international initiatives in the neuroproteomic field and to make neuroproteomic research and its results available in the scientific community and society. This initiative was launched in 2003 by Helmut Meyer, head of the Medical Proteome Center at Ruhr University, Bochum and Joachim Klose of Charité's Virchow Clinics of Humboldt University, Berlin and followed by more than 100 brain scientists around the world. The first meeting of HBPP was held in Duesseldorf in September 2003, the second was held in April 2004 in Paris at ESPCI and the third in December 2004 at Castle Rauischholzhausen, located in the state of Hessen, Germany. HBPP participants agreed to initiate two pilot studies: 1) Quantitative proteome analysis of normal mice brain (C57/Bl6) for assessment of the quality of 2D- and non 2D-gel based quantitative proteome analysis and comparing proteome and transcriptome by performing mRNA profiling; 2) Quantitative proteome analysis of human brain from biopsies and autopsies for assessment of protein stability in post mortem tissue and feeding the human brain proteome database with reliable data. These studies started in January 2004 and results will be presented at the 4th International HUPO meeting (München August 28–September 1 2005).

W1.B
NEUROPATHOLOGY AND PROTEOME

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BrainNet Europe II is a network of 19 established brain banks in Europe. The main objectives of BrainNet Europe II are: 1. To acquire and distribute well-characterised and high-quality CNS tissues for basic research in neuroscience. 2. To provide a basis and quality control system for research projects dealing with clinical or epidemiological aspects of neurological and psychiatric diseases. 3. To standardise and harmonise neuropathological diagnosis. 4. To develop gold standards for tissue handling, safety aspects, quality control and ethics. These standards will be the basis for using human post mortem brain tissue in new investigative techniques such as expression profiling and proteomics. 5. To contribute to training and exchange of neuroscientists. Diseases of high frequency and outstanding medical and social importance such as Alzheimer, Parkinson, motoneuron disease, prion diseases, multiple sclerosis, schizophrenia and affective disorders will be the focus of the network. In addition we will contribute to research in rare diseases, a research branch, which can only be worked on successfully on an international level. BNE II will thus be able to contribute samples for proteome research from a large number of well characterized CNS tissues from important and rare diseases.

W1.C
CEREBROSPINAL FLUID PROTEIN COMPOSITION

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Our objective is to identify proteins in human CSF. Study participants gave informed consent. Neurological and psychiatric (SCID 1–5) diagnostic classification was determined from structured interviews. Diagnoses included Alzheimer's disease, multiple sclerosis and migraine. CSF total proteins were reduced and alkylated and subjected to protease digestion. Peptides were separated either by 1D (reverse phase) or 2D (cationic exchange and reverse phase) microchromatography, and analyzed via nanospray interface to an ion trap mass spectrometer (either LCQ, LTQ or FTMS). MS/MS spectra were searched against Swiss Prot database, release 7459 (BioWorks™ 3.1, Thermo, San Jose, CA). Identifications were confirmed either by immunostaining or by LC/MS (TSQ), with quantities based on standard curves and spiking of CSF with specific stable-isotope peptides. From 25 assays of the same sample (dementia), 2475 different proteins were identified repeatedly and with good spectra. These represent many biochemical classes, including proteins involved in signaling (639), glycoproteins (733), phosphoproteins (360), oxidoreduction (97), hydrolases (439), transmembrane (576), cell adhesion (128, extracellular matrix (55), ATP-binding (261), G protein coupled receptors (72), ion transport (46), ion channels (73) and tight junctions (7). Identities of 45 of 2475 proteins examined thusfar were confirmed by immunostaining and/or stable-isotope peptide LC/MS. Known functions of these proteins suggest enzymatic and trafficking processes occur within CSF, although the regulation and significance of such activities are not known. Studies to define CSF proteins from multiple persons and further improve understanding of physiology and pathophysiology are underway.

W1.D
CLINICAL NEUROPROTEOMCIS OF DEMENTIAS: NOVEL MULTIPLEX ASSAYS IN CSF AND BLOOD

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In the recently established Human Brain Proteomics Project (HBPP), our group focuses on clinical neuroproteomics of human body fluids to cross-bridge large clinically-oriented networks and a strong proteomic and bioinformatic platform. These clinical requirements are met by the German Federal Ministry of Education and Research (BMBF)-funded COMPETENCE NETWORK DEMENTIA and the CREUTZFELDT-JAKOB DISEASE NETWORK, which guarantee expert clinical dementia diagnosis, highly standardized multicenter pre-analytical sample handling and large-scale sample banking. Recently, in our lab we have established a multiplexing-based technique to simultaneously measure the concentrations of the three crucial biomarkers of demen-

tia disorders (namely: amyloid β peptide 1–42, total Tau, and phospho-Tau181) in a minute volume of cerebrospinal fluid. Furthermore, we have most recently established attomolar sensitive analysis of the Aβ peptide expression profile in human blood plasma using two-dimensional urea-based Aβ SDS-PAGE/immunoblot. Both diagnostic techniques are promising to provide an improved early and differential diagnosis of Alzheimer's dementia (AD). Moreover, our data indicate that neurochemical dementia diagnostic (NDD) can already identify patients at risk for incipient AD during the prodromal clinical stage of mild cognitive impairment.

W1.E

ON THE WAY TO DIRECT DETECTION OF NEUROPEPTIDES RELEASED BY NEURON BY MASS SPECTROMETRY

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Nervous tissue distinguishes from other tissues because of the high diversity of the constitutive cell types. It is interesting to characterize the biochemical diversity of neurons to find a correlation with their functional diversity. In our department, this study has been realized by using electrophysiology and transcriptomics (single cell multiplex RT-PCR). The study of mRNA in one single cell is however an indirect analysis of neurotransmitters and their precursors. The direct analysis the proteic or peptidic content of the cell would allow us to check if the peptides are actually expressed in the cytoplasm of the cells of interest. Our aim is the detection of polypeptides (neuropeptides) involved in neuronal signaling by mass spectrometry, either in the cytoplasm of the neuron or in the extracellular matrix after the induction of their release by electrophysiology. Neuropeptides are presently detected either by immunocytochemistry in the cell or by immunotitration outside the cell. The study of neuropeptides by mass spectrometry involves the similar analytical toolbox as proteomic studies. The detection of very low amounts of peptides in a biological matrix suffers from the following limitations: loss of the compounds by non specific interaction on surfaces or by degradation of the molecules, signal suppression by competition with co-purified molecules, inefficient detection specificity. Here we present our first results for the detection of neuropeptides at the subfemtomol level by mass spectrometry.

C4 Proopiomelanocortin signaling and control of body weight

C4.A
PROOPIOMELANOCORTIN DEFICIENCY IN HUMAN OBESITY

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Within the last 10 years a detailed picture of the molecular components of hypothalamic body weight regulation has evolved. In a critical gene dosage sensitive position of this circuits the POMC could be located as the precursor for the neurotransmitter MSH peptides, which binds to the brain MC3R and MC4R. This central role of the POMC gene in the anorectic leptin-pathway of weight reduction stimulated the search for genetic changes in the POMC gene as important contribution to the genetic predisposition for human obesity. So far three different strategies were performed to discover genetic alterations in the POMC gene in human obesity. 1. In a focused study based on the predicted phenotype of a complete defect of the POMC gene product five children could be identified with the clinical trials of early onset obesity, ACTH deficiency and red hair which are affected by complete loss of function mutations of the POMC gene. This new disease turned out to be very rare but recapitulates the functional roles of POMC derived peptides in human physiology. 2. Several candidate gene mutation studies were performed in non-syndromic "common" obese individuals, which clearly excluded coding region mutations of the POMC gene as a major contribution to the genetics of obesity. 3. In genome screens conducted to identify genes linked to obese phenotypes, the POMC gene was identified independently in two studies to be linked to increased levels of leptin and in one American-Mexican study to increased body fat mass but no mutation in the coding region could subsequently identified. Taken together, in the last few years human genetic studies have shown that POMC derived peptides seem to play the same physiological roles in humans as described in rodents but if the POMC gene contributes significantly to the genetics of obesity remains an open question.

C4.B
MELANOCORTIN RECEPTOR GENETICS AND ALTERED SIGNAL PROCESSING

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Through the study of humans with extreme early-onset obesity, we have identified a number of individuals with mutations in elements of the melanocortin signalling pathway. These include a growing number of patients with null and missense mutations in POMC itself, patients with compound heterozygous mutations in the prohormone convertase 1 and a large number of subjects with the loss of function mutations in the melanocortin 4 receptor. The genotype/phenotype relationships with these mutations will be discussed as well as the lessons for the understanding of the molecular controls of human energy balance that are provided by such experiments of nature. We have also used a POMC-null mouse to further explore aspects of energy balance and intermediary metabolism in relation to melanocortins. New observations from this mouse will be discussed.

C4.C
MOUSE MODELS OF POMC MUTATIONS AND THE INTEGRATED ROLE OF POMC PEPTIDES AND GLUCOCORTICOIDS IN ENERGY BALANCE

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Mice lacking all proopiomelanocortin (POMC) peptides (–/–) in brain and pituitary are obese and glucocorticoid (GC) deficient. We therefore generated a strain of neural specific (NS) POMC–/– mice to differentiate between the roles played by central POMC peptides and GCs in the control of energy balance. To further characterize the early mechanisms of altered energy homeostasis we replaced global POMC–/– mice with physiological doses of corticosterone in their drinking water and performed pair-feeding (PF) experiments in NS-POMC–/– mice starting at age 5 wk. 9 wk-old POMC–/– mice consumed 25% more food than the control group while the NS-POMC–/– mice ate 70% more. Body weight in this group increased by 60% between 6–13 wks, in contrast to less than 20% increase in the PF mice, which were similar to the POMC–/– mice. At 10 wk, VO_2 was decreased in POMC–/– and particularly in NS-POMC–/– mice. In contrast to POMC–/– mice, which were not diabetic and displayed low leptin levels, NS-POMC–/– mice were hyperglycemic and hyperleptinemic. The correction of body weight in the PF group was associated with normalized glucose levels and a decrease in leptin concentrations that correlated with intermediate levels of fat stores. POMC–/– mice treated for 12 weeks with corticosterone displayed increased body weight, fat deposits, and leptin levels and decreased VO_2 similar to the NS-POMC–/– mice. In conclusion, these data show that both hyperphagia and low metabolism occur early in POMC–/– mice and NS-POMC–/– mice to increase body weight. The presence of GCs in the absence of central melanocortin control may account for the accentuated obesity phenotype observed in NS-POMC–/– mice.

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C4.D
NEURAL-SPECIFIC EXPRESSION AND MODULAR ENHANCER STRUCTURE OF THE POMC GENE

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The proopiomelanocortin (POMC) gene is expressed in the pituitary and arcuate neurons of the hypothalamus. The aim of the present study was to identify functional cis-acting elements that control neuronal POMC-expression in the hypothalamus. Using a combination of phylogenetic footprinting and transgenic mouse analysis we identified two conserved neuronal POMC enhancers designated nPE1 (600 bp) and nPE2 (150 bp). Our data show that (1) a distal genomic region con-

taining nPE1 and nPE2 is necessary and sufficient to direct authentic neuron-specific expression of reporter genes to POMC arcuate neurons; (2) either nPE1 or nPE2 assures proper reporter expression in POMC arcuate neurons whereas simultaneous deletion of these two enhancers completely eliminates expression in POMC neurons; (3) nPE1 and nPE2 nucleotide sequences and genomic organization are both highly conserved among mammals but not between mammals and birds, amphibians or fish; (4) the enhancer activity of mouse and human genomic fragments containing nPE1 and nPE2 is functionally conserved; (5) POMC expression in the brain and pituitary is controlled by different and independent sets of enhancers; and (6) conserved and aligned putative transcription factor binding sites are present in these enhancers including canonical sequences for STAT3 and the POU-domain proteins Brn 4.0/OCT-1 in nPE1 and the homeodomain factors Nkx6.1/Brn2.0 in nPE2. Our study advances the understanding of the molecular nature of hypothalamic POMC neurons and will be useful to determine whether polymorphisms in POMC regulatory regions play a role in the predisposition to obesity.

C5 GABA function, alcohol action, and addiction

C5.A
INTER- AND INTRACELLULAR METABOLIC COMPARTMENTATION IN GABA AND GLUTAMATE HOMEOSTASIS

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Glutamate (glu) and GABA are metabolically closely related to the TCA cycle and have other functional roles than being neurotransmitters, this being most pronounced for glu. Glutamine (gln) appears to be precursor for glu well as for GABA via glu. Using cultured cerebellar granule cells and neocortical neurons as model systems for glutamatergic and GABAergic neurons, respectively, details of the synthetic machineries have been investigated using NMR and LCMS. GABAergic neurons were repetitively depolarized using different paradigms to induce release of GABA from the cytoplasmic and the vesicular pool, respectively. The percentage of GABA generated from gln via the TCA cycle decreased after depolarization inducing GABA release from the cytoplasmic pool whereas a significant change in this parameter was not observed after release from the vesicular pool. This indicates that during release from the cytoplasmic pool, the fraction of GABA synthesized directly from gln without involvement of the TCA cycle is more pronounced than that occurring when release involves the vesicular pool. Synthesis of the neurotransmitter pool of glu from gln in glutamatergic neurons involves to a considerable extent the TCA cycle as was the case with regard to synthesis of GABA. Glutamatergic neurons were repetitively depolarized in the presence of 0.5 mM gln and a glu transport inhibitor (TBOA) to prevent reuptake of released glu. It was shown that the intracellular pool of glu was decreased compared to the situation without TBOA. This may indicate that neuronal reuptake of released glu is involved in the maintenance of the neurotransmitter pool and that exogenous gln is inadequate to sustain this pool.

C5.B
MOLECULAR MECHANISMS OF TOLERANCE TO AND WITHDRAWAL OF $GABA_A$ RECEPTOR MODULATORS

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Neuronal plasticity is achieved by regulation of the expression of genes for neurotransmitter receptors such as the type A receptor (GABAAR) for γ-aminobutyric acid. The subunit composition of native GABAA receptors plays a crucial role in defining their function in the physiological and pharmacological modulation of neuronal excitability and associated behaviour. The pattern of GABAAR gene expression is affected by environmental stimuli, physiological processes, and drugs that modulate GABAAR-mediated neurotransmission. However, whether such induced changes in gene expression for a given GABAAR subunit are identical among different neuronal populations that express that subunit in a specific brain area is not known. We now show that prolonged exposure to ethanol had no effect on expression of the δ subunit of GABAARs at the mRNA or protein level in cerebellar granule neurons, it increased the abundance of δ subunit mRNA and protein in hippocampal neurons. Subsequent ethanol withdrawal transiently down-regulated δ subunit expression in cerebellar granule neurons and gradually normalized that in hippocampal neurons. On the contrary the expression of other GABAARs subunits (α4 and γ2) follow the same patter of changes in the two neuronal cell types in culture. These effects of ethanol exposure and withdrawal were accompanied by corresponding functional changes in GABAARs. These findings reveal complex and distinct mechanisms of regulation of the expression of GABAARs in different neuronal types.

C5.C
MOLECULAR ACTIONS OF ALCOHOL ON EXTRASYNAPTIC $GABA_A$ RECEPTORS

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GABA(A) receptors, the major inhibitory neurotransmitter receptors in the brain, have long been implicated as targets for anesthetics and ethanol in mammals. We have recently shown that GABA(A) receptors containing the "extrasynaptic" δ subunit are uniquely sensitive to ethanol with a concentration dependence that mirrors human experience during ethanol consumption. Alcohol non-tolerant (ANT) rats are highly susceptible to the impairment of postural reflexes by alcohol and benzodiazepines and have been shown to carry a mutation in the α6 subunit of the GABA(A) receptor (α6R100Q) (Korpi *et al.*, Nature 1993). When co-expressed with β3 and δ subunits, the α6-R100Q mutation leads to an up to 10-fold increase in the already high ethanol sensitivity of wild-type α6β 3δ receptors in recombinant systems. We found that the α6-R100Q allele is frequent in laboratory rats (a naturally occurring "knock-in" point mutation at the α6 "benzodiazepine site") and show that tonic currents in cerebellar granule cell neurons in slices from non-mutant (α6-100RR) rats are alcohol sensitive. This sensitivity is dramatically increased in mutant homozygous α6-100QQ rats. The α6-100QQ mutant animals show motor impairment already at very low alcohol doses, indicating that the α6-R100Q mutation is sufficient to produce alcohol hypersensitivity. In addition, the benzodiazepine alcohol antagonist Ro15–4513 blocks the ethanol enhancement of α 4/6 β3 δ recombinant receptors, providing further evidence for a link between ethanol and benzodiazepine actions. Our findings suggest that extrasynaptic δ subunit-containing extrasynaptic GABA(A) receptors are important mediators of acute alcohol effects.

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C5.D

ALCOHOL AND REWARD: BEHAVIOURAL AND NEUROCHEMICAL APPROACHES TO STUDY CRAVING AND ADDICTION

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In Fawn-Hooded (FH) rats that exhibit a depressive phenotype in parallel to alcohol-seeking behaviour, anti-craving drugs such as naltrexone and acamprosate display short-term efficacy in reducing alcohol consumption/preference, after which tolerance develops. In contrast, sertraline and desipramine displayed efficacy in reducing alcohol consumption without tolerance developing, which was associated with a down-regulation of μ-opioid receptors in the amygdala and VTA. Interestingly, co-administration of naltrexone with desipramine attenuated the efficacy of desipramine and resulted in tolerance similar to rats treated with naltrexone alone. Furthermore, voluntary consumption of alcohol results in a significant up-regulation of enkephalin gene expression within the amygdala, while withdrawal following chronic alcohol consumption results in adaptive modulation of μ-opioid receptors in the amygdala with a parallel alteration of agonist-stimulated binding of [^{35}S]GTPγS. Collectively, these data suggest that opioid transmission within the amygdaloid complex is sensitive to alcohol, such that modulation of this system can influence alcohol consumption and preference. As such, the amygdala may represent an important locus for the targeting of novel therapeutics to reduce craving and assist in breaking the cycle of alcohol (and other substance) abuse. Our most recent data would suggest that neural plasticity of reward-related neurons is a feature of drug dependence. In these studies, acute bilateral microinjections of brain derived neurotrophic factor BDNF into the nucleus accumbens or amygdala (1 μg/side; 100 nl/side) persistently reduced ethanol consumption in FH rats for a period of 4–5 days compared to vehicle-treated rats. Studies are currently underway to elucidate the mechanisms of action of BDNF at the cellular/molecular level.

W2 RNA interference in the brain and the neurodegenerative process

W2.A
APPLICATIONS OF NUCLEIC ACID TECHNOLOGY IN THE CNS

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A range of nucleic acid technologies are available for gene silencing, ranging from modified antisense oligonucleotides (AO) to the more recent RNA interference (RNAi). We are exploiting these approaches to investigate the functions of genes underpinning neurological disorders (such as the SMN gene in spinal muscular atrophy), to develop approaches for ex vivo gene silencing in neural stem cells (using embryonic neural precursors from ventral mesencephalon), and to develop novel methods for the allele-specific silencing of mutant genes underlying dominant neurological disorders (such as acetylcholine receptor mutations in slow channel myasthenic syndrome and tau mutations underlying forms of dementia, specifically FTDP-17). We are also involved in developing AO methods for exon skipping of mutations. All of these approaches have the potential for wide application to the study of neurodegenerative disease and will be discussed.

W2.B
C. ELEGANS AS A MODEL FOR NEURODEGENERATIVE DISEASES: GENOME-WIDE RNAi SCREEN FOR GENES INVOLVED IN PROTEIN AGGREGATION

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Aggregation of misfolded proteins occurs in various age-related neurodegenerative diseases, including Parkinson's, Alzheimer's, and Huntington's disease. To understand how cells protect themselves against misfolded proteins, we searched for genes that enhance or prevent protein aggregation. C. elegans strains expressing polyglutamine stretches fused to YFP with visible, age-dependent protein aggregation are used as a genetic model. Using a genome-wide RNAi screen, we identified 186 genes that, when knocked down, cause premature protein aggregation. These genes include genes involved in protein synthesis, folding, degradation, vesicle transport and RNA synthesis and processing [Nollen E.A.A., Garcia S.M., van Haaften G., Kim S., Chavez A., Morimoto R.I., Plasterk R.H. (2004) Genome-wide RNA interference screen identifies previously undescribed regulators of polyglutamine aggregation. Proc. Natl. Acad. Sci. U.S.A. 101(17): 6403–8]. To address whether these genes collectively comprise a general protein homeostatic buffer or whether they act specifically to prevent polyglutamine aggregation, we are currently developing a C.elegans model expressing YFP-fusions of alpha-synuclein, which is found to form aggregates in Parkinson's disease. We will report on our progress.

W2.C
RNA INTERFERENCE AS A TOOL TO TREAT OR MIMIC NEURODEGENERATIVE DISEASES

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Endogenous pre-existing mechanisms of RNA degradation, known as RNA interference (RNAi), represent an attractive way to lower expression of targeted gene products. RNAi is an efficient method to manipulate gene function in mammalian cells, either by transfection of short interfering RNAs (siRNAs) or by transcription of short hairpin RNAs (shRNAs) from viral vectors. We have previously shown that lentiviral vectors represent a powerful tool for mediating silencing of a target gene in a specific and efficient manner. We are presently evaluating this technology to silence genes associated with neurodegenerative diseases for either therapeutic approaches or to generate experimental models. Mutations in Cu/Zn superoxide dismutase (SOD1), one of the causes of familial amyotrophic lateral sclerosis (ALS), lead to progressive death of motoneurons through a gain-of-function mechanism. We have shown that in SOD1 mutant transgenic ALS mice, intraspinal injection of a lentiviral vector that produces RNAi-mediated silencing of SOD1 significantly retards both the onset and the progression rate of the disease. Mutations in the parkin gene are associated with autosomal recessive-juvenile parkinsonism and lead to a defective E3 ligase activity of the parkin protein with the accumulation of parkin substrates. Knock-out parkin flies and mice do not exhibit nigral neuron death. The direct injection of lentiviral vectors encoding parkin-specific shRNA in the adult rat substantia nigra may avoid such compensatory mechanisms. We are currently investigating this approach to create a new genetic model of Parkinson's disease and to better understand the pathophysiology of the disease.

W2.D
REVEALING NEURAL SIGNALING AND METABOLISM BY siRNA

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The recent discovery of a whole family of small-RNAs has triggered an exciting interest in the knowledge of this still-unexplored field. Whilst most current studies on the function of this new class of RNA have been confined to a variety of cell lines and small organisms, it is now emerging the notion that the RNA interference (RNAi) strategy using small, double-stranded RNA molecules (siRNA) is a successful way to produce specific loss-of-function in the central nervous system. The combination of RNAi with green fluorescence protein in appropriate expression plasmid vectors is further opening a whole world of possibilities for the study of molecular targets involved in the neurodegenerative process. This talk will focus on recent achievements by our group showing the specific blocking of enzymes of metabolic pathways, such as glycolysis and glutathione biosynthesis, in neural cells in primary culture. We believe that the application of the siRNA strategy may help understanding basic neurochemistry as well as molecular mechanisms underlying neurodegenerative diseases, such as Parkinson's disease.

W3 *In vivo* NMR spectroscopy in neurological disorders and basic neuroscience

W3.A
^{13}C NMR STUDIES IN HEPATIC ENCEPHALOPATHY

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Cerebral metabolism and neuron-glial interactions are critical determinants for the development of brain edema in Hepatic Encephalopathy (HE) and Hyperammonemia (HA) due to acute liver failure (ALF). ^{13}C NMR spectroscopy is a unique tool to study the changes in metabolic fluxes in HE. Some of the new insights into ammonia detoxification by glutamine synthesis and brain energy metabolism are presented. For example, *in vivo* ^{13}C NMR studies in hyperammonemic animals showed an association between brain glutamine synthesis and neurotransmitter cycling (Sibson *et al.*, 1997), and a significant contribution of pyruvate carboxylase to glutamine formation (Tsukada *et al.*, 1998). A further study in rats with acute HA showed that synthesis of lactate exceeded that of glutamine (Behar *et al.*, 1993). *Ex vivo* NMR studies on glucose metabolism in awake animals challenged the view that glutamine accumulation is a major cause of brain edema in ALF. In particular, the accumulation of brain glutamine showed no correlation with ammonia or brain edema while the study emphasizes a concerted contribution of lactate accumulation due to progressively impaired mitochondrial energy metabolism (Zwingmann *et al.*, 2003). Hypothermia did not prevent against brain glutamine accumulation, but normalized brain water content and lactate synthesis (Chatauret *et al.*, 2003). Future ex vivo NMR spectroscopic studies will be valuable in the elucidation of the cerebral metabolic consequences of ALF. Furthermore, metabolic flux analysis permits the study of the Krebs cycle flux, cell-specific metabolism and glutamatergic action *in vivo* (Gruetter *et al.*, 2003), reinforcing the potential of *in vivo* ^{13}C NMR studies in HE.

W3.B
METABOLISM OF EXCITATORY AMINO ACIDS IN CULTURED ASTROCYTES AND NEURONS STUDIED BY MRS

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Glutamate and glucose uptake and metabolism are important functions for astrocytes and neurons. Using 13C labeled precursors it is possible to study cellular events. [U-13C]glutamate is an excellent tool for studying glutamate metabolism and this has been done in cortical neurons (GABAergig), cerebellar neurons (glutamatergic), cortical and cerebellar astrocytes. Magnetic resonance spectroscopy analysis of cell extracts and media revealed that both neurons and astrocytes metabolized glutamate extensively with 13C label appearing in aspartate in all cultures. Additionally, GABA is synthesized in the GABAergic cortical neurons. Labeling of lactate and glutamine was prominent in medium from astrocytes, but not detectable in cortical neurons. Cerebellar granule neurons showed some labeling of lactate. Glutamate derived from the first turn of the tricarboxylic acid cycle is present in all cell types analyzed. However, glutamate derived from the second turn of the cycle was only detected in granule neurons. Pyruvate recycling could be demonstrated in astrocytes but not in neurons.

W3.C
MR SPECTROSCOPIC STUDIES OF GABA AND GLUTAMATE IN PSYCHIATRY

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Magnetic resonance spectroscopy, commonly called MRS in the field of psychiatric research, makes a unique contribution through its ability to make direct measurements of neurotransmitters *in vivo* in healthy subjects and patients before and during treatments or substance withdrawal. One focus is glial–neuronal interaction. Disparate areas reveal possible relationships among excitatory function, glial abnormalities, brain energy consumption, and brain GABA levels. Several studies relate GABA levels and the voltage threshold for cortical excitability are altered by sensory state. During sensory deprivation, GABA levels, cortical excitability, and glucose metabolism decrease. A study of anxiety disorder yielded a report of elevations of Glx orbitofrontal cortex, while another showed decreased GABA in patients with panic disorder, suggesting a reciprocal relationship between GABA and glutamate levels. A reciprocal relationship was also observed in major depressive disorder, where the concentration of GABA was reduced by 30–50% and glutamate increased by 10%. Given reports of reduced glucose utilization in depression, it may be that low GABA is related to decreased glutamate release. Also possible is abnormal glutamate uptake, consistent with reported reductions in glia in depressed subjects. GABA levels in alcohol-dependent subjects after a month of sobriety are reduced by 25% compared to healthy control subjects. In alcohol-dependent subjects, reduced glial density and glial nuclear size are associated with reductions in glucose utilization, again suggesting altered glutamate release and uptake. In summary, GABA is lower in sensory deprivation, glial abnormalities, and reduced glucose utilization. The previously reported relationship between glucose utilization and glutamate-glutamine neurotransmitter cycling suggest that GABA levels may serve as an indicator of excitatory function and glial alterations.

W3.D
GLUCOSE TRANSPORT AND GLYCOGEN METABOLISM STUDIED BY NMR

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NMR spectroscopy can make unique contributions to the study of *in vivo* carbohydrate metabolism, such as the ability to directly quantify brain glucose content [Glc] using either ^{1}H or ^{13}C NMR detection of the Glc signal. The quantification of brain [Glc] as a function of plasma glucose concentration yields insight into the kinetics of glucose transport. Brain Glc has a high physical distribution space *in vivo*, [Glc] is a linear function of plasma Glc, consistent with reversible Michaelis-Menten kinetics. Chronic hypoglycemia was shown to increase brain [Glc], implicating upregulated BBB Glc transport kinetics. In acute hypoglycemia increases in CBF were observed when Glc approached the K_m of hexokinase, which was also the point at which glycogenolysis started. The measurement of brain glycogen (Glyc) using biochemical methods is difficult due to its rapid post-mortem degradation. The non-invasive measurement of Glyc metabolism, uniquely possible

by ^{13}C NMR, showed an active, yet slow turnover in rats and humans alike, when Glc concentration was not rate-limiting for metabolism. The rate of glycogenolysis accounted for the majority of the Glc supply deficit during hypoglycemia and Glyc sustained >1 h of hypoglycemia. Biochemical measurements following focused microwave fixation confirmed the observations made by NMR, specifically the quantitation of [Glyc] in rats, which was 3–4 μmol/g ww, similar to that in humans. We conclude that brain Glc and Glyc levels and metabolism can be reliably quantified by NMR and that Glyc is an important store of glucose equivalents in the brain.

Gene expression, regulation and proteomics

P.1

CHARACTERIZATION OF TWO NOVEL VERSICAN SPLICE VARIANTS IN RAT

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Versican is a large chondroitin sulfate proteoglycan which interacts with hyaluronan to form large extracellular complexes in a variety of tissues. In vitro data suggest that it functions amongst others in the restriction of axonal regeneration by the inhibition of neurite outgrowth (Jones *et al.*, 2003). So far four splice variants, named V0, V1, V2 and V3, were identified which differ in the composition of the two large glycosaminoglycan-attachment domains (GAG) alpha and beta. Here we describe the identification and characterization of two novel splice variants on the RNA level. By searching for ESTs encompassing the region on rat chromosome 2 we found two sequences: The human EST BI818462 mapped to the intron downstream of GAG beta. The homologous rat sequence was cloned from dorsal root ganglion (DRG) RNA, whereas analysis in rat brain revealed two ESTs which differ in length. By alternative splicing the putative new exon predicted by EST sequence in both DRG and brain is attached to the GAG beta domain and because it comprises of several stop codons the translated protein will lack the complete C-terminus. The second EST, BF545755, is located in the intron between the GAG domains. By RT-PCR with primers specific for the EST and the GAG alpha domain we determined the 5′ end of the new exon. In addition we conclude that the new exon is part of a mRNA including GAG alpha. Our data show that on RNA level there are at least two more versican splice variants. Because the newly identified transcribed sequences are noncoding exons the assumed translational products will lack 307 aa and 2043 aa, respectively, at the C-terminus.

P.2

FORSKOLIN STIMULATION OF EMBRYONIC RETINAL CELL LINE R28: CHANGES IN CHOLINERGIC RESPONSE

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During development cholinesterase expression shows a co-regulation between acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). BChE is related to proliferation while AChE is expressed in differentiated cell types. Antisense studies towards both cholinesterases revealed an interaction between BChE expression and protein kinase C. It was also shown that AChE is regulated by a cAMP dependent pathway in myoblast differentiation. In the present study, the interaction of cholinesterases with protein kinase A pathway was investigated in the rat precursor retinal cell line R28. The R28 cell line is an embryonic cell line bearing the characteristics of both glial and neuronal cell types. The cells were treated with forskolin over a 24 hr period and the expression of ChEs in response to forskolin treatment was assayed by RT-PCR. The cholinergic response to forskolin treatment was also recorded by patch-clamp experiments. Forskolin treatment revealed an acetylcholine response in R28 cells displaying that these cells have the capacity to become cholinergic after forskolin treatment. The formation of synapses and dendrites were also noted after forskolin treatment.

P.3

CHARACTERIZATION OF GENOMIC STRUCTURE AND TRANSCRIPTIONAL REGULATION OF GRM1

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Metabotropic glutamate receptor 1 (mGlu1) is widely but discretely expressed throughout the brain, specifically in neuronal cells. Its expression is tightly regulated and undergoes dramatic changes during development, in response to environmental modifications and pathological contexts. The mGlu1 gene (GRM1) gives rise to several splice variants, which have different cell-specific expression patterns. The complex mechanisms resulting in the discrete expression of mGlu1 are largely dependent on its genomic structure. Therefore, we have determined the exon/intron arrangement of human, mouse and rat GRM1 and characterized the 5′-end of GRM1 by means of 5′-Random Amplification of cDNA Ends, performed on human and murine anchored cDNA libraries. The results indicate the presence of two distinct clusters of transcription initiation sites upstream from exons I and II in both species, strongly suggesting the existence of two promoter regions. Mouse GRM1 encodes for two additional alternative first exons, Ib and Ic. Identification of the core promoter region and characterization of transcription factor responsive elements is presently ongoing. Expression of the alternative mRNA forms starting within each 5′-alternative exon has been assessed in several brain areas by RT-PCR. We have also identified a novel mGlu1 splice variant expressed in mouse and rat cerebellum, characterized by a short intracellular domain. In this study we have elucidated the genomic structure of an important glutamate receptor, discretely expressed in the brain, and provided the basis for the characterization of its cell- and context-specific expression.

P.4

Abstract has been withdrawn.

P.5

EVALUATION OF GENE EXPRESSION BY STATIC MAGNETISM IN CULTURED RAT HIPPOCAMPAL NEURONS

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We have previously demonstrated that sustained magnetism would modulate mechanisms associated with cellular maturation and development in cultured rat hippocampal neurons. To determine the possible expression of genes responsive to static magnetism as a guidepost for possible long-lasting functional alterations in the brain, we have carried out differential display (DD) analysis in cultured rat hippocampal neurons exposed to static magnetic fields. Hippocampal neurons were cultured for 11 days *in vitro* (DIV) and then exposed to static magnetic fields for 15 min at 100 mT, followed by incubation for

3 h and subsequent extraction of total RNA for DD analysis. A particular gene was upregulated in response to brief magnetism and identified as N-terminal asparagine amidase (NTAN1), encoded for N-terminal amidohydrolase, which is involved in the N-end rule pathway in the ubiquitin-proteasome system. Northern blot analysis revealed a significant increase in expression of mRNA for NTAN1 3 to 6 h after brief magnetism, while brief magnetism significantly decreased the expression of microtubule-associated protein-2 (MAP2) in a manner prevented by a proteasome inhibitor in cultured rat hippocampal neurons. Overexpression of NTAN1 gene led to a similarly significant decrease in MAP2 expression in cells not exposed to magnetism as revealed by Western blotting. These results suggest that brief magnetism may result in long-lasting alterations of cellular integrity and/or functionality through a molecular mechanism relevant to expression of NTAN1 gene and subsequent degradation of MAP2 by ubiquitin-proteasome in cultured rat hippocampal neurons.

P.6

COMBINATION OF THE TWO ROR RESPONSE ELEMENTS IN THE Bmal1 PROMOTER CONFERS ROBUSTNESS TO THE MOUSE Bmal1 OSCILLATION

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Bmal1 is a transcription factor that functions as a positive regulator for the negative components of clock genes such as *Per1, Per2*, and *Cry1*. In the suprachiasmatic nucleus (SCN), the center for circadian rhythm in mammals, *Bmal1* mRNA oscillates with the peak occurring during the subjective night. Conversely, *Per1* and *Per2* are expressed during the subjective daytime. To investigate the mechanism of *Bmal1* expression, we characterized the mouse *Bmal1* promoter using a luciferase assay and real-time monitoring of *Bmal1* promoter activity. Within the region 1 kb upstream from the transcriptional start site for the *Bmal1* promoter, there are three ROR response elements (ROR-RE), which are named ROR1, ROR2, and ROR3 in descending order toward the start site. The enhancement of *Bmal1* promoter activity by RORα was decreased to 18 and 27% of the full enhancement with the intact promoter with deletion of ROR2 or ROR3, respectively, and the enhancement was abolished completely with the deletion of both ROR2 and ROR3. The real-time monitoring system showed that the amplitude of the luciferase activity of the *Bmal1* reporter was reduced to about 20% of the control activity by a single deletion of ROR2 or ROR3, preserving the circadian oscillation. Deletion of the ROR1 site had minimal effects on both the basic activity and induction of the *Bmal1* promoter by RORα. These results indicate that combination of the two proximal ROR-REs confers robustness to the *Bmal1* oscillation.

P.7

EXPLORING Nogo-A FUNCTION: A TRANSCRIPTOMIC/PROTEOMIC COMBINED APPROACH

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Nogo-A is a myelin protein which belongs to the Reticulon family. It is known to play an important role in inhibiting axonal regeneration and plasticity in the central nervous system (CNS); the molecular mechanism is still unknown. No other functions have been identified yet. To investigate the role of this protein, Nogo-A knockout mice have been generated. They are viable, fertile and with no obvious developmental or neurological abnormalities. Biochemical analysis of these mice showed a strong upregulation of the shorter splice isoform Nogo-B and regulation of some isoforms of Reticulon-3. In a silver gel approach, a strong upregulation of two not yet identified bands (30 and 70 kDa) has been found. A combined approach based on Transcriptomics and Proteomics has been used to find mRNAs/proteins which could be influenced by Nogo-A in the CNS. Affymetrix GeneChips have allowed the identification of up- and downregulated mRNAs and 2DimensionalGels have permitted to recognize regulated proteins. We have recognized general patterns of regulation at different function levels through a System Biology approach. Interesting regulated molecules will be further investigated to establish if they are involved in Nogo-A function or if they could permit to clarify its molecular mechanism of action.

P.8

DEVELOPMENTAL AND ESTROGEN-MEDIATED REGULATION OF NEUROSTEROID HYDROXYLASE CYP7B1 IN HUMANS

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CYP7B1 catalyzes 7α-hydroxylation of several steroids, including the neurosteroids dehydroepiandrosterone (DHEA) and pregnenolone (PREG). DHEA and PREG accumulate in the brain and are believed to affect memory function and other cognitive processes, through yet unclear mechanisms. CYP7B1-mediated 7α-hydroxylation of these steroids has been suggested a possible biosynthetic pathway to form active hormones. In the current study, human CYP7B1 mRNA levels were examined in different tissues and regulation of CYP7B1 by estrogens and androgens was studied using human embryonic kidney 293 (HEK293) cells as a model for extrahepatic regulation of CYP7B1. CYP7B1 mRNA was quantitated by real-time PCR in several human tissues of adult and fetal origin. The results showed markedly higher CYP7B1 mRNA levels in almost all fetal tissues, including brain, compared with the corresponding adult ones, except in the liver where levels were higher in adults. This indicates a tissue-specific, developmental regulation of CYP7B1 in humans. Transfection with estrogen receptor α and treatment with 17β-estradiol significantly increased CYP7B1 catalytic activity and mRNA levels in HEK293 cells, and stimulated a human CYP7B1 luciferase reporter gene. These data indicate that estrogen may be important for regulation of human CYP7B1, in brain and elsewhere. Effects on CYP7B1-mediated neurosteroid metabolism is a previously unknown means by which estrogen may affect brain function.

P.9

INVESTIGATION OF THE RAT BRAIN POSTSYNAPTIC DENSITY PROTEOME

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Chemical synapses are major communication sites between neurons. The postsynaptic density (PSD) beneath the postsynaptic membrane is a cytoskeletal specialization where neurotransmitter receptors are asso-

ciated to signaling- and scaffolding-proteins thus organizing signal transduction pathways near the postsynaptic membrane. The protein composition of PSDs is dynamically regulated by neuronal activity. This is thought to be the molecular basis of neuroplasticity. Therefore, there is growing interest to identify the molecular constitutents of the PSD in order to gain more insight into structural reorganization processes during changes of synaptic efficacy. In the present study, ICAT-based correlation profiling was used to assess the degree of enrichment of PSD proteins as compared to the proteins of the synaptic membrane protein fraction. We confirm that the core PSD proteins were enriched in PSD preparation. Other groups of proteins with various functions such as cytoskeleton-associated proteins, protein kinases and phosphatases, components and regulators of signaling pathways, and proteins involved in energy production may be associated to multiple organelles and multiprotein complexes, and consequently as groups they were enriched in the PSD fraction to a lesser extent. Finally, mitochondrial proteins and transporters were generally strongly depleted indicating that they were likely contaminants of the PSD preparation.

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P.10

DIVERSITY OF NEURONS IN THE RAT LATERAL AMYGDALA REVEALED BY ELECTROPHYSIOLOGICAL, SINGLE-CELL RT-PCR AND CLUSTER ANALYSIS

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Based on unsupervised cluster analysis, a classification of neurons ($n = 74$) was performed in neurons of the rat lateral amygdala (LA) in vitro. Multiple electrophysiological and molecular parameters studied by patch-clamp or single-cell mRT-PCR, respectively, were compared. A mRT-PCR protocol was designed to simultaneously detect the expression of vesicular glutamate transporter I (VGLuT1), GAD67, calbindin, parvalbumin, calretinin, NPY, VIP, SOM, and CCK. Four groups of neurons with distinctive features were determined. The majority of neurons ($n = 43$) represented projection neurons (class I), characterized by low firing rates, frequency adaptation in response to prolonged depolarizing stimuli, as well as high occurrence of VGLUT1. The second type of neuron (class II, $n = 6$) displayed high frequent activity of fast spikes, early adaptation, and expressed VIP predominantly. The third type of neurons (class III, $n = 10$) showed high-frequency firing with little adaptation. In contrast to class II neurons, these cells displayed significant SOM expression (70%), but not calbindin nor VIP expression. Class IV neurons ($n = 7$) possessed a molecular profile similar to that of projection neurons (class I), but distinctive electrophysiological properties. The morphological analysis of neurons stained with biocytin after patch-clamp recording ($n = 70$) revealed that class I neurons were large, spiny cells and that the other neuronal classes displayed smaller somata and spine-sparse dendrites. We propose that four types of neurons with a distinctive molecular, electrophysiological and morphological profile seem to exist in the rat LA, including one class of projection neurons and at least two classes of GABAergic neurons.

P.11

COMPARISON OF STATISTICAL CLUSTER METHODS IN ELECTROPHORETIC PROTEIN PATTERN ANALYSIS

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Standard electrophoresis methods were used in the qualitative and quantitative protein analysis of cerebrospinal fluid. Disc electrophoresis was carried out for detection of oligoclonal IgG bands in cerebrospinal fluid on polyacrylamide gel. Pairs of CSF and serum were taken from 30 patients mainly with multiple sclerosis and other central nervous system dysfunctions polyradiculoneuritis, known as Guillain-Barre syndrome, encephalitis, paraproteinemia and analysed. ImageMaster 1D Elite and GelPro specialized software packages were used for fast accurate image and gel analysis. The results obtained from different hierarchic cluster analysis methods were compared. The application of different cluster methods does not produce same results. In some cases, despite the substantial similarities which exist between electropherograms, different cluster methods produced different dendrograms and the method should be used cautiously. Cluster analysis methods offer only additional diagnostic information of the inflammatory conditions of the central nervous system and coupled with conventional electrophoresis lead to better medical relevance of the method.

P.12

HUMAN LETHAL(3)MALIGNANT BRAIN TUMOR [h-l(3)mbt] GENE REGULATES CELL PROLIFERATION AND HOX EXPRESSION

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The Drosophila lethal(3)malignant brain tumor [l(3)mbt] is a tumor suppressor gene whose disruption causes malignant overgrowth of optic neuroblasts. In addition, the domain structure of l(3)mbt protein shows significant similarity to those of several Polycomb group gene products that regulate homeotic gene expression. To investigate the function of mammalian l(3)mbt homolog, we generated adenovirus vectors expressing human l(3)mbt [H-L(3)MBT] and structurally related Polycomb group gene, SFMBT (Scm-related gene containing four mbt domains). Transient expression analyses demonstrated that H-L(3)MBT protein was co-localized in the nucleus and co-immunoprecipitated with SFMBT. Expression of H-L(3)MBT or SFMBT resulted in the reduced cell proliferation rate in a similar manner. Expression of H-L(3)MBT or SFMBT up-regulated the mRNA levels of HOXA1 and HOXB7, and expression of simultaneous H-L(3)MBT and SFMBT increased HOX mRNAs compared with single gene expression. These data indicate that H-L(3)MBT is not only structurally but also functionally related to SFMBT, and that H-L(3)MBT is involved in the regulation of cell proliferation as well as the homeotic gene expression. We are now generating the null-mutant of mouse L(3)mbt homolog by gene targeting technology to analyze its functions *in vivo*.

P.13

SUBTRACTIVE COPY DNA LIBRARIES AS A STRATEGY FOR FINDING EPENDYMA-SPECIFIC GENE PRODUCTS

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The ependyma is a single-layered columnar to prismatic epithelium covering the ventricular surfaces and the spinal canal of the mammalian central nervous system. Its function is presently unclear and ependymal research is severely hampered by the lack of a truly cell type-specific marker. The principle of suppression PCR was employed to subtract ependyma-free bovine brain cDNA from bovine ependymal cDNA. In rat, the expression of mRNAs corresponding to several resulting clones was shown to be confined to ependymal primary cultures, testis and lung, as determined by real-time RT-PCR. In case of the clone designated EPE10, this expression profile was confirmed by Western blot analysis with an antiserum raised against a deduced peptide coupled to keyhole limpet hemocyanin. Expression in testis begins after 18 d, concomitant with the onset of spermatogenesis. Upon separation of bull sperm heads from tails, EPE10 immunoreactivity is found in the tail fraction. In ependymal cell cultures, the EPE10 expression level correlates with the number of ciliated cells. Immunostaining experiments localize the antigen to the cytosol of cilia-bearing cells both in culture and *in vivo*. EPE10 codes for a 68 kDa translation product belonging to the WD protein family. Such proteins, the most well-known of which is beta-transducin, adopt the beta-propeller fold and frequently participate in protein-protein interactions. EPE10 seems to be a marker protein for mature kinocilia-bearing cells, is upregulated during ciliogenesis and may have regulatory rather than structural functions. Subtractive ependymal cDNA libraries may facilitate the discovery of further yet unknown proteins involved in ciliogenesis.

P.14

Abstract has been withdrawn.

Intracellular signaling pathways

P.15

PROTEIN KINASE ACTIVITIES MODULATE SCRAMBLING OF MEMBRANE ASYMMETRY; A PREREQUISITE FOR APOPTOTIC DEATH IN OLIGODENDROGLIAL CELLS

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The possible interplay between extracellular signal-regulated protein kinase (ERK) and protein kinase C (PKC) activation and ethanolamine phosphoglycerides (PG) membrane bilayer translocation following oxidative stress (OS; 0.5 mM H_2O_2/0.05 mM Fe^{2+}), was examined in oligodendroglia OLN93 cells with altered plasma membrane PG composition. Cells supplemented with 0.05 mM docosahexaenoic acid (DHA, 22:6n3) to increase the number of potential double bond targets for OS in ethanolamine-PG (EPG) and plasmalogens were compared to cells with diminished content of EPG, attained by the addition of 0.5 mM N,N-dimethylethanolamine (dEa). After 30 min OS, various oxidized PG species were detected and EPG bilayer translocation accompanied by sustained ERK activation and culminated by apoptotic cell death (ACD) was found in DHA-supplemented cells, in contrast to a brief ERK activation and no ACD in DHA/dEa-supplemented cells. In DHA-supplemented cells the MEK inhibitor U0126 prevented ERK activation, EPG translocation and ACD. PKC activation by TPA slightly enhanced ERK activation upon OS as well as cell death. In contrast, the PKC inhibitor GF triggered ERK nuclear translocation and protected from ACD, indicating an interrelation of PKC activity and ERK intracellular compartmentalization, both upstream events determining EPG translocation and cell death or survival. These findings suggest an intimate link between ERK signaling and changes in EPG membrane asymmetry indicating that ERK activation is an indispensable component for the signaling cascades leading to EPG translocation but only activation of the latter is leading to OS-induced ACD.

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P.16

REGULATION OF COLLAPSIN RESPONSE MEDIATOR PROTEIN PHOSPHORYLATION IN ALZHEIMER'S DISEASE

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Collapsin response mediator protein 2 (CRMP2) is an abundant microtubule-binding protein that is almost exclusively expressed in the central nervous system. We have previously shown that CRMP2 can be phosphorylated by glycogen synthase kinase 3 (GSK3) and that phosphorylation of CRMP2 by GSK3 regulates axon elongation in primary hippocampal neurons. Importantly, CRMP2 is phosphorylated by GSK3 at sites that were previously reported to be hyperphosphorylated in neurofibrillary tangles that are found in the brains of Alzheimer's disease patients, thus providing a direct link between deregulation of GSK3 activity, hyper-phosphorylation of CRMP2 and the development of Alzheimer's disease. Here, we have investigated changes in the levels of CRMP2 phosphorylation (as well as other CRMP isoforms) following alteration of the activities of the priming kinase, Cdk5, or the phosphatase, PP1. Also, we have identified a number of physiological stimuli that can affect the levels of CRMP2 phosphorylation in SH-SY5Y neuroblastoma cells and primary hippocampal neurons, including Abeta peptide and hydrogen peroxide treatment, which are common neurotoxic insults associated with neurodegeneration. Our data is consistent with hyper-phosphorylation of CRMP proteins by GSK3 occurring during the development of Alzheimer's disease.

P.17

INHIBITION OF JNK2 AND JNK3 PROTECT NEURONS AGAINST MITOCHONDRIAL CYTOCHROME C RELEASE AND 6-OH-DOPAMINE

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We have studied the JNK isoforms responsible for 6-OH-dopamine (6-OHDA) mediated degeneration. In PC12 cells, 25 µM 6-OHDA enhanced the JNK activity in the cytoplasm, nucleus and at the mitochondria. Inhibition of JNKs by 2 µM SP600125 and transfection with dominant-negative (dn) JNK2, but not dnJNK1, rescued more than 60% of the otherwise dying PC12 cells. In contrast to the constitutively present JNK1, the JNK2 isoform increased in the nucleus and at the mitochondria (PC12 cells do not contain JNK3). JNK-inhibition by SP600125 or transfection of dnJNK2 reduced the pool of active JNKs in the nucleus, inhibited the translocation of JNK2, but not of JNK1, to the mitochondria and the release of cytochrome c. Moreover, dnJNK2 abrogated the activation of MKK4 at the mitochondria, but did not affect its presence. Transfection with dnJNK1, however, had no effects on the translocation of JNKs to the mitochondria, the release of cytochrome c or the phosphorylation of MKK4. Finally, dnJNK2 prevented the 6-OHDA induced increase in bim. Transfection of JNK3 did not affect either cell death or cytochrome c release. The reason for this failure might be the inefficient activation of the transfected JNK3 which translocated without being phosphorylated to both, nucleus and mitochondria. In the adult mouse brain, JNK3 ko and c-JunAA mice protected dopaminergic neurons in the substantia nigra compacta (SNC) against death following intrastriatal injection of 6-OHDA or transection of medial forebrain bundle. Thus, specific JNK isoforms mediate 6-OHDA provoked neuronal death.

P.18

OLIGODENDROCYTE VESICLES TRANSPORT: INTERACTION OF rRab22b AND OCRL-1

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Myelin biogenesis in oligodendrocytes requires coordination of numerous events governing lipid and protein synthesis, intracellular trafficking of membranes and cytoskeletal reorganization. Membrane trafficking is necessary both for delivery of structural myelin components and for movement of molecules involved in myelin formation. Knowledge of the molecular mechanisms that regulate and coordinate these membrane trafficking pathways is needed for understanding how the disruptions of these mechanisms affect myelin biogenesis and stability. Our study showed that:1) *rRab22b*, a novel protein cloned from a cDNA oligodendrocyte library, regulates the transport of newly synthesized lysosomal hydrolases from the trans Golgi network (TGN) to

endosomes. 2) Traffic of membranes between TGN to endosomes occurs via clathrin coated vesicles traveling along microtubules. 3) rRab22b synchronizes the trafficking from the TGN to endosomes by regulating vesicle formation in the TGN. How does rRab22b regulate vesicle formation in the TGN?. Our studies using a) yeast two hybrid systems, and b) GST-rRab22b pull down experiments show that rRab22b interacts with OCRL-1. This phosphatase play a key role in the regulation of PI(4)P and PI(4,5)P2, signaling molecules involved in formation of carrier vesicles and in their fusion. The importance of these observations is emphasized by the fact that mutation of OCRL-1 causes OCRL, an X-linked disorder associated with alterations in CNS white matter such as loss of white matter, formation of multiple sharp-edged cysts in deep white matter, and demyelination. These results show that the mechanism in which rRab22b is involved in the formation of carrier vesicles from TGN depends on OCRL-1. Disruption of the rRab22b trafficking pathway would therefore lead to demyelination.

P.19

RELEASE OF GROUP IIA SECRETORY PHOSPHOLIPASE A_2 FROM RAT BRAIN MITOCHONDRIA: DOES IT CORRELATE WITH THAT OF CYTOCHROME c?

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Different secretory PLA_2 ($sPLA_2$) have been identified in brain tissue but the role of each enzyme is far for being clearly definite (Farooqui *et al.* J. Neurochem. 69:889, 1997). It has been proposed that their activation might contribute to cell damage and that are likely linked to necrotic and/or apoptotic cell death. The presence of group IIA $sPLA_2$ (GIIA) in the inner mitochondrial membrane and the release of the enzyme under reduced membrane potential (Macchioni, L. *et al.,* J. Biol. Chem. 279:37860, 2004) induced to speculate on the mechanism of release and the possible correlation with cytochrome c (cyt c) release. We have previously demonstrated that potassium phosphate (Pi) at concentration producing mitochondrial permeability transition pore (mPTP) opening, did not affect GIIA release. The same effect was observed when mitochondria were exposed to Ca^{2+}, a mPTP inducer, at concentration of 0–100 μM. We also evaluated the effect of cyclosporine A (CsA), a potent inhibitor of mPTP pores that partially prevent mitochondrial swelling from brain tissue (Kobayashi, T. *et al.,* Brain Res. 960:62, 2003); CsA did not affect the GIIA release. These findings strongly suggest that the release of GIIA may occur via an mPTP-independent mechanism and the effects of Pi, Ca^{2+} and CsA were the same observed for cyt c release. GIIA release was inhibited in cardiolipin (CL)-enriched mitochondria as observed for cyt c release (Piccotti, L. *et al.* J. Biol. Chem. 277:12075, 2002). These data suggests a possible correlation between mitochondrial GIIA and cyt c release and induces to speculate on the role of the enzyme in cellular life or death.

P.20

CHARACTERIZATION OF NUCLEAR SECRETORY PHOSPHOLIPASE A_2 ($sPLA_2$)

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The presence of phospholipase A_2 (PLA_2) activities in mammalian brain has been reported in several studies (Farooqui *et al.*, J. Neurochem. 69:889, 1997). The identity and subcellular distribution of various enzymes, belonging to different groups (G), are still largely unknown. Various groups of PLA_2s mRNAs are ubiquitously expressed in brain, with relatively similar levels detected in all regions (Molloy *et al.*, Neurosc. Lett. 258:139, 1998). Using confocal immunofluorescence and immuno electron microscopy, we showed that GIIA $sPLA_2$ and GV $sPLA_2$ are both present in neural cell lines (U251 astrocytoma and PC12 pheocromocytoma) but with different localization. GIIA is largely localized in perinuclear mitochondria while GV appears to be concentrated in the nucleus (Macchioni *et al.*, JBC, 279:37860, 2004). Aim of this study is to establish whether or not nuclei prepared from rat brain cortex contained $sPLA_2$ activity and eventually provide the identification of the enzyme (s). To this aim, glial and neuronal nuclei were purified from rat brain cortex (Thompson J. Neurochem. 21:19, 1973) and PLA_2 activity was assayed. Under our experimental conditions, both fractions possessed PLA_2 activity and their biochemical properties indicated the presence of a secretory enzyme. The enzyme was identified as GV by Western Blot analysis and by inhibition studies with Indoxam (Shionogi) (Suzuki, N. *et al.* J. Biol. Chem. 275:5785, 2000), thus confirming previous findings with cultured neural cells. Interestingly, GIIA was not present in the nuclear fractions. We conclude that PLA_2 activity present in the nuclei of neural cells is largely, if not exclusively, due to the presence of GV but its function in this cell compartment it is not known.

P.21

PHOSPHATIDYLINOSITOL 3-KINASE ACTS THROUGH THE SMALL GTPASES Cdc42, Rac AND Rho DURING AGRIN-INDUCED ACETYLCHOLINE RECEPTOR CLUSTERING

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The nerve-derived protein Agrin is required for the formation of the neuromuscular junction. Agrin activates a signal transduction pathway through the muscle-specific kinase MuSK, which promotes the reorganization of the postsynaptic membrane. Activation of MuSK leads to the phosphorylation and clustering of acetylcholine receptors (AChRs) and other postsynaptic proteins. Agrin also triggers the activation of the small GTPases Rac, Rho and Cdc42. This activation is important for the formation of AChR clusters on muscle cells. So far, however, the exact mechanisms downstream of MuSK, which are involved in Agrin-induced signaling, are poorly understood. We were able to identify the phosphatidylinositol 3-kinase (PI3K) as a component of the Agrin-MuSK pathway. PI3K is involved in many signaling events. Thus, it represents a good candidate for a signaling molecule downstream of MuSK. Muscle cells are able to cluster AChRs in response to Agrin. In contrast, muscle cells treated with specific PI3K inhibitors form AChR microclusters, which are significantly smaller than AChR clusters formed in untreated muscle cells. The formation of microclusters has so far been attributed to a reduction of Rac, Rho and Cdc42 activity. Consistent with these data activation of Rac, Rho and Cdc42 is reduced in response to Agrin in the presence of PI3K inhibitors. To investigate the spatial distribution of PI3K in native muscle tissue we performed an immunohistochemical study and could show that the PI3K is highly enriched at the postsynaptic membrane. Due to the fact that MuSK activation is not altered when PI3K activity is blocked, we propose a role for PI3K downstream of MuSK and upstream of Rac, Rho and Cdc42.

P.22

COMPARATIVE ANATOMICAL DISTRIBUTION OF SOLUBLE GUANYLYL CYCLASE SUBUNIT mRNAS IN MAMMALIAN BRAIN

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Soluble guanylyl cyclase (sGC) is the main target of NO in the CNS and cGMP the mediator of its important neuroregulatory actions. sGC is present in most mammalian cells as a heterodimer composed by α

and β subunits. Gene cloning has revealed the existence of two isoforms for each subunit, α1, α2, β1, β2. We used ^{33}P-labeled oligonucleotides complementary to the mRNA coding for these subunits as hybridization probes to study the anatomical distribution of the cells containing these mRNAs in rat and monkey brains. We found that β1 is the most abundant subunit in both species and is highly expressed in caudate-putamen, nucleus accumbens, olfactory tubercle, cortex, dentate gyrus and CA fields of hippocampus, many thalamic and hypothalamic nuclei, amygdala, some brainstem motor nuclei, and Purkinje and granule cells of cerebellum. In contrast, β2 mRNA could not be detected in rat or monkey brains. The α subunits present a differential distribution with α1 mRNA being more ubiquitous than α2 mRNA. In rat brain α1 mRNA is expressed in striatum, olfactory tubercle, hippocampus and external cortical layers. In monkey brain α1 mRNA is also found in caudate and putamen, hippocampus and internal cortical layers. α2 mRNA is expressed in cerebellum, all cortical layers, caudate and putamen, hippocampus and in some brainstem nuclei in both species. The distribution of sGC subunits will be compared with that of the related enzymes neuronal NO synthase and cGMP-specific phosphodiesterases and their possible interaction for the regulation of cGMP levels will be discussed.

P.23

ESTRADIOL INDUCES PKA ACTIVATION THROUGH THE PUTATIVE MEMBRANE RECEPTOR IN THE HIPPOCAMPAL NEURON

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This is the first report on real time imaging of estrogen-stimulated PKA activation in living neural cells as observed by fluorometry. H19-7, the immortalized rat hippocampal neural cell line was stimulated by 17beta-estradiol for 45 sec. and the intracellular PKA activity was assayed by DR2 fluorometry. DR2 is a cell-permeable fluorescent PKA substrate. DR2 consists of a fluorescent probe and an amino acid sequence comprising part of regulatory domain 2 of PKA, that contains a specific serine autophosphorylation site. The fluorescence started to decrease 45 sec. after estradiol application in parallel with PKA activation and reached a minimum at 195 sec. Exposure to either Rp-cAMPS, a PKA inhibitor or ICI182,780 prior to estradiol application inhibited the decrease in fluorescence intensity. Instead of beta-estradiol, we added either BSA-conjugated beta-estradiol or alpha-estradiol to H19-7 cell and then DR2 fluorometry was done. The BSA-conjugated beta-estradiol caused as much PKA elevation as beta-estradiol, while alpha-estradiol induced no change in PKA activity. Intracellular Ca ion concentration was also imaged by Fura2 fluorometry on the same cell in which estrogen-stimulated PKA elevation was confirmed. As the result, it was observed that PKA activation followed Ca ion elevation. It is concluded that estradiol induces PKA activation through the putative membrane receptor in the hippocampal neuron.

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REACTIVE OXYGEN SPECIES REGULATE ALPHA-SYNUCLEIN PHOSPHORYLATION STATE IN HUMAN DIFFERENTIATED NEUROBLASTOMA CELLS

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Reactive oxygen species (ROS) are known to occur as natural by-products under physiological condition and have been implicated in the neuronal alterations observed in a variety of age-related neurophatological conditions. Biomarkers of production of reactive oxygen/nitrogen species have been seen to be associated with phospho-alpha-synuclein-immunopositive lesions detected in different neurodegenerative diseases known as synucleopathies. The phospho-serine 129 alpha-synuclein promotes fibril formation in vitro, suggesting the importance of phosphorylation of the filamentous protein in the pathogenesis of these diseases. In the present study, we have analyzed the regulation of alpha-synuclein phosphorylation state following increased levels of ROS induced by pro-oxidant agents in viable human differentiated neuroblastoma cells. We report that alpha-synuclein is constitutively expressed and phosphorylated in serine 87 and 129 in SH-SY5Y differentiated cells as detected by Western blot analysis using specific phospho-antibodies. In addition we found that the phosphorylation state of alpha-synuclein is regulated by high levels of intracellular ROS. These results demonstrated that supraphysiological levels of ROS induced post-translation modification of the alpha-synuclein protein supporting the notion of the role of oxidative stress in neurodegenerative disease characterized by alpha-synuclein positive lesions.

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P.25

NEUROGRANIN: A CRITICAL MOLECULE AT THE NEXUS OF 3 MAJOR SIGNALING PATHWAYS AND MOOD MODULATION

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Neurogranin (RC3) is a brain-specific postsynaptic protein involved in synaptic plasticity, stress, and found in forebrain regions implicated in mood. Identified as a CaM binding PKC substrate under regional and temporal control by thyroid hormones, neurogranin also regulates CaMKII and PKA. Behavioral and cellular studies to investigate neurogranin in affective-like behavior was carried-out in neurogranin (+/+), (+/–), and (–/–) animals. Neurogranin knock-out (–/–) mice showed a decrease in immobility in the forced-swim test similar to wild-type mice undergoing a sustained (10 day) antidepressant treatment. A decrease and increase in autonomous and total CaMKII activity, respectively, occurred in the frontal cortex and hippocampus of neurogranin (–/–) mice. Phosphorylated levels of αCaMKII (Thr 286) and GluR1 at a PKA recognition site (845) in the frontal cortex was decreased in the presence of attenuated PKA activity in these mice. Imipramine in wild-type mice increased activity and levels of all above-mentioned CaMKII and PKA parameters in the forebrain. Phosphorylated levels of neurogranin in the forebrain increased following imipramine treatment in wild-type mice. Imipramine treatment was incapable of further impacting altered CaMKII activity and αCaMKII (Thr 286) levels in neurogranin (–/–) mice suggesting a neurogranin–CaMKII link. Phosphorylated levels of MaRCKS at a non-specific CaMKII site mirrors changes in total CaMKII activity in the frontal cortex of neurogranin (–/–) and wild-type mice undergoing imipramine treatment. Neurogranin is crucial in CaMKII and PKA signalling and to the effect of imipramine on these pathways.

Ischemia

P.26

BRAIN ISCHEMIA CAUSES BINDING OF CYTOCHROME C TO $Insp_3R$ AND RyR IN ENDOPLASMIC RETICULUM

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Calcium signaling mediates many cellular functions, including programmed cell death. Recent study *in vitro* provided evidence that in early stages of apoptosis, cytochrome c translocates from mitochondria to endoplasmic reticulum (ER), where it binds to inositol (1,4,5) trisphosphate receptor ($InsP_3R$) resulting in cytosolic calcium increase *(Nat Cell Biol 2003;5:1051)*. Previously we have demonstrated a biphasic increase in cytosolic cyt c in the gerbil hippocampi subjected to transient ischemia *(Mol Brain Res 2004;121:50)*. Here we have verified the hypothesis that also in ischemic and reperfused gerbil hippocampus cyt c is translocated from mitochondria to ER, and binds to $InsP_3R$, and ryanodine receptor (RyR), the two systems mediating calcium mobilization from ER. Gerbils were subjected to 5 min bilateral ligation of common carotid arteries and 30 min reperfusion. The hippocampi were processed to obtain subcellular fractions containing membrane and mitochondria (P2) cytosol (S2) and endoplasmic reticulum (P3). In control hippocampi, cyt c was present mostly in P2 and to a lesser extent in S2 and P3, as revealed by western blot. In the post-ischemic hippocampi, the level of cyt c was markedly increased in S2 and P3, as compared to the control amount in these cell compartments. Immunoprecipitation with $InsP_3R$ and RyR antibodies, followed by WB analysis, revealed formation of cyt c-$InsP_3R$ and cyt c-RyR complexes in the post-ischemic P3 fraction. These results were futher confirmed by the immunoprecipitations with cyt c antibody. Similarly as *in vitro*, *in vivo* in the post-ischemic brain, cyt c translocates to ER, and binds to $InsP_3R$ as well as to RyR, what suggest the role of cyt c in ischemic signal transduction.

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DIFFERENTIAL REGULATION OF PROSTAGLANDIN E2 RECEPTOR SUBTYPES, EP4/EP2 IN A RAT MODEL OF ISCHEMIC INJURY AND TOLERANCE

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Prostagladin E2 (PGE2) is known to have various physiological functions in the nervous system. Recent studies have shown that activation of PGE2 EP2 receptor mediates a significant neuroprotective effect in cerebral ischemia. We investigated the temporal and regional expression patterns of 2 subtypes of PGE2 receptors, namely EP2 and EP4 in a rat model of transient forebrain ischemia and ischemic tolerance induction. Expression of EP2 and EP4 was induced 12 h after ischemic injury, and was most prominent in the CA1 and the hilar region at 3 d, with expression sustained for at least two weeks. Double-labeling experiments revealed that the cells expressing EP2 and EP4 were GFAP-expressing reactive astrocytes. In rats subjected to 3 min of ischemia, expression of EP2 and EP4 was induced 4 h, and was most prominent in the pyramidal cells in the CA1 region at 3 d, with expression sustained for at least 7. These results provide the distinct pattern of EP2 and EP4 expression in the rat hippocampus after ischemic injury and tolerance induction. A long-lasting upregulation of EP2 and EP4 in reactive astrocytes of the post-ischemic hippocampus reflects that they may be involved in the astrocytic response to an ischemic insult. In addition, a sustained and selective expression of EP2 and EP4 in neuronal subpopulations destined to survive, ie, in CA1 pyramidal neurons after ischemic tolerance induction suggests that PGE2 may play a role in cell survival by activation of EP2 and EP4 receptors.

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EXPRESSION PROFILES OF HYPOXIA-INDUCIBLE FACTOR-1 AND PROLYL HYDROXYLASES IN NEONATAL RAT BRAIN

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Hypoxic preconditioning (HP) can protect against subsequent hypoxic/ischaemic (H/I) insults. The protection is thought to be mediated by the transcription factor, hypoxia-inducible factor-1 (HIF-1), a key factor involved in adaptive responses to ischaemic or hypoxic conditions. HIF-1 is a heterodimer of two protein subunits – HIF-1α and HIF-1β. Levels of HIF-1 expression are highly dependent on the breakdown of HIF-1α subunit that changes with cellular oxygen availability and requires hydroxylation by enzymes called HIF-1 prolyl-4-hydroxylases (PHDs; isoforms PHD1, 2, 3). The current project investigates the mRNA expression patterns of HIF-1 subunits and PHDs in newborn rat brain. Postnatal day 6 Sprague-Dawley rat pups were exposed to HP (3 h, 8% O_2), and 24 h later were subjected to right common carotid artery occlusion followed by 3 h hypoxic exposure. HP showed significant protection of the brain against subsequent H/I injury (histopathological score: hypoxia 0.53 vs normoxia 1.78; $P < 0.05$). A further group of pups ($n = 5–6$) were exposed to HP, sacrificed immediately or 1–24 h after treatment and brains removed. Standard *in situ* hybridisation histochemistry procedures were performed on coronal sections using [^{33}P]-labelled oligonucleotide probes for HIF-1α/β and PHD1/2/3. Results in control brains demonstrated differential expression of HIF-1α/β and PHD2 in various brain regions including anterior olfactory nuclei, olfactory bulb, subventricular zone, hippocampus and superficial cortical layers. HIF-1α mRNA labelling was noted in striatum. Further investigations are examining the distribution patterns of mRNAs for HIF-1 subunits and PHD isoforms in normoxic and HP brains. These studies will provide new insights into the regulatory roles of PHD/HIF-1 expression in neonatal rat brain.

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HYPOXIC PRECONDITIONING IN NEONATAL RAT BRAIN INVOLVES REGULATION OF EAAT2 AND ER ALPHA

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Exposure of the brain to a sublethal insult can protect against a subsequent brain injury. Hypoxic preconditioning induces tolerance to hypoxic-ischemic injury in neonatal rat brain and is associated with

changes in gene and protein expression. To study the involvement of excitatory amino acid transporters (EAAT1 and EAAT2) and estrogen receptors (ERα and ERβ) in neonatal hypoxia-induced ischemic tolerance, we examined changes in expression of these proteins in the cortex, hippocampus and striatum of newborn rats at different time points after exposure to sublethal hypoxia (8% O_2, 3 hours). Preconditioning with hypoxia 24 hours before hypoxia-ischemia afforded marked brain protection compared with littermate control animals as determined by morphological assessment. Immunoblot analysis showed that EAAT2 and ERα were significantly increased by 55% and 49%, respectively, in cortex at 24 hours after hypoxic-preconditioning. Surprisingly, at the same time point, a significant decrease of EAAT2 by 48% in striatum was observed. In contrast, hypoxic preconditioning had no effect on the levels of EAAT1 and ERβ in any of the brain regions studied at any of the time points analyzed. The similar pattern of changes in EAAT2 and ERα levels provide the first *in vivo* indication that ERα might be a further part of some type of functional complex involving EAAT2 and Na^+/K^+ ATPase in cortex. The endogenous molecular mechanisms modulated by hypoxia preconditioning may contribute to the development of hypoxia-induced ischemic tolerance, and may provide novel therapeutic targets for the treatment of cerebral ischemia.

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THE MECHANISMS OF CYTOPROTECTIVE EFFECT OF CYCLOSPORIN A ON ASTROCYTES EXPOSED TO SIMULATED ISCHEMIA IN VITRO

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Neuroprotective effect of cyclosporin A (CsA) has been reported in many experimental models of ischemia *in vivo* and in vitro, although the precise mechanisms is unclear. We evaluated the mechanism of cytoprotective effect of CsA on the primary rat astrocyte cultures in a model of simulated ischemia in vitro. To study these mechanisms, the CsA-mediated modulation of the extracellular signal regulated kinases 1 and 2 (Erk1/2) and calcineurin (CaN)-dependent pathways was determined. At the 21st day in vitro, primary cultures of astrocytes were turned to ischemia simulating conditions (92% N_2, 5% CO_2, 3% O_2 at 37°C) for 8 h and exposed to CsA (0.25, 1 and 10 microM). We demonstrated that exposure of astrocytes to low concentration of CsA (0.25 microM) in ischemic conditions in vitro significantly activates the Erk1/2 kinase signaling pathway. Moreover, we have showed that low concentration of CsA protected glial cells against ischemia-induced apoptosis through the increase of Bcl-XL expression, mitochondrial function restoration by increase of transmembrane potential across the inner membrane, caspase-3 activity inhibition and NFkappaB nuclear binding elevation. These data establish also the cross talk between the action of 0.25 microM CsA on CaN and Erk1/2 kinases in anti-apoptotic signal transduction pathways. We also demonstrated that CsA protective effect at concentration of 10 microM is related mainly to strong CaN inhibition. Finally, in our study 1 microM CsA does not show antiapoptotic action on ischemic astrocytes. These results indicate that CsA might act as a protective agent through different mechanisms on ischemic astroglial cells depending on the concentrations used.

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17β-ESTRADIOL INHIBIT DELAYED NEURONAL DEATH FOLLOWING ISCHEMIA/RECIRCULATION IN GERBIL HIPPOCAMPUS

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Glutamate transporters can maintain the glutamate concentration in extracellular space at low level by taking the amino acid into glial or neuronal cells. Dysfunction of the transporters lead to cause neuronal damage by allowing accumulation of cytotoxic glutamate around neurons and synaptic cleft region to trigger neuronal degeneration. Application of transient ischemia to Mongolian gerbil brain caused excessive release of glutamate from neurons, producing selective neuronal death several days after the insult. Although definite molecular mechanisms are unclear yet, intraperitoneal (i.p.) injection of 17β-estradiol (17β-ED) to the treated animal can produce some protective effect against ischemia-induced neuronal death. We measured some glutamate transporters such as GLAST, GLT-1 and EAAC1 in the hippocampus tissue and they all decreased after ischemic insult. Daily i.p. administration of 17β-ED to the gerbil after the ischemic attack caused significant recovery of these three glutamate transporter protein levels. These result that 17β-ED can protect from neuronal damage caused by ischemia through conserving these glutamate transporter system in the brain.

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EXPRESSION OF DIFFERENT GLUTAMATE RECEPTORS IN BRAIN HEMISPHERES OF RATS WITH MIDDLE CEREBRAL ARTERY OCCLUSION

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Adult male Wistar rats weighing 150–250 g were subjected to middle cerebral artery occlusion (MCAO) for different periods, followed by decapitation 24 h after reperfusion and subsequent dissection of coronal sections for vital staining with 2,3,5-triphenyltetrazolium chloride (TTC). Regional cerebral blood flow was occasionally monitored by a laser-Doppler flow meter. Marked vital damages were seen in the ipsilateral cerebral hemispheres of animals subjected to MCAO with a severity dependent on the duration of occlusion from 1 to 8 h, but not in the contralateral cerebral hemispheres, on TTC assays. Animals were thus subjected to MCAO for 2 h, followed by decapitation 1 and 7 days after reperfusion and subsequent dissection of ipsilateral and contralateral cerebral hemispheres from forebrains for Western blotting using specific antibodies against MAP-2, GFAP and CD11b. A significant decrease was seen in the expression of MAP-2, but not GFAP, in ipsilateral hemispheres dissected 1 and 7 days after reperfusion, while MCAO for 2 h led to a transient increase in the expression of CD11b in ipsilateral hemispheres dissected 1 day after reperfusion. Moreover, MCAO for 2 h resulted in a significant decrease in the expression of NR1, NR2A and NR2B subunits in ipsilateral hemispheres dissected 7 days, but not 1 day, after reperfusion on Western blotting. However, no significant changes were seen in the expression of GluR1, GluR2 and GluR6 subunits in ipsilateral hemispheres dissected 1 and 7 days after reperfusion. These results suggest that MCAO would lead to the disturbed and abnormal functionality and integrity related to maltifunctions of NMDA receptors rather than non-NMDA receptors in rat brain.

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EXPRESSION AND ACTIVATION OF ERK AND JNK IN THE HIPPOCAMPUS OF RATS EXPOSED TO GLOBAL CEREBRAL ISCHEMIA

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Aims: Effects of the global cerebral ischemia on the ERK and JNK expression and activation signals in the hippocampus of rats exposed to global cerebral ischemia were studied.

Procedure: Animals were exposed to global cerebral ischemia (20 min duration) and after different reperfusion periods (5 and 10 min; 1; 6 and 12 h after ischemia), ERK and JNK expression and activation signals were determined. Transient global cerebral ischemia was induced by the four-vessel occlusion method described by Pulsinelli and Buchan (1988). Total protein (35 µg)was loaded for each sample onto a 12% polyacrylamide gel. Membranes were probed with anti-ERK/JNK and anti-phospho ERK/JNK antibody (diluted 1 : 5000 in blocking buffer) overnight at 4°C. A a-rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1 : 4000 in 5% low fat milk in TBS+T) was utilized to allow detection of the appropriate bands using enhanced chemiluminescence reagent and film. Three animals in each experimental group were used to analyze the levels of proteins by Western blot.

Results: The expression levels of ERK and JNK in the hippocampus remained constant during all reperfusion periods examined. The levels of phosphorylated ERK and JNK increased with time during the first 6 h of reperfusion. After 6 h, the amount of phosphorylated JNK had returned to control level. The levels of ERK appeared to return to peak levels after 12 h of reperfusion in the second wave of kinase activation.

Conclusions: Our results indicated that expression and activation of ERK and JNK were implicated in the early and the later periods of reperfusion injury in hippocampi of global cerebral ischemia exposed rats.

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IMPACT OF NEUROPROTECTANTS ON OXIDATIVE MODIFICATION OF NEURAL PROTEINS IN RAT FOREBRAIN ISCHEMIA

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Time course of oxidative modification of neural proteins was compared in rat global cerebral ischemia/reperfusion treated with Ginkgo biloba extract Egb761 (Schwabe) and simvastatin (40 or 20 mg/kg) for 7 days. Animals were subjected to 4VO/15 min. After ischemia and at 2 to 72 h of reperfusion, the lipoperoxidation-dependent and direct oxidative modification protein markers were measured in the forebrain homogenate. Ischemia causes significant changes of tryptophan and bityrosine fluorescence when compared to controls. All tested parameters altered significantly at later reperfusion stage. Content of carbonyl group in re-flow period steadily increased and culminated at 48 h, the highest increase in bityrosines was detected after 24 h of reperfusion and was statistically significant to both sham operated and ischemic groups. The changes in fluorescence of tryptophan decreased during reperfusion time. Formation of lysine conjugates significantly increased only at later stages of reperfusion. Both the Egb761 and simvastatin decreased number of hippocampal CA1 neurons with neurodegeneration. In addition, formation of direct oxidative- or lipoperoxidative dependent products of modified proteins was significantly lower compared to non-treated samples. Our results suggest that global ischemia/reperfusion initiates both the lipoperoxidation-dependent and direct oxidative modifications of neural proteins which can be lowered by neuroprotectant treatment as a part prospective post-ischemic antioxidant therapy.

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TAURINE ATTENUATES THE SYNAPTIC COMPONENT OF ISCHEMIA-INDUCED GLUTAMATE RELEASE

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The sulfur amino acid taurine may be protective in ischemia due to its neuroinhibitory effects. The aim of this study was to evaluate the ability of taurine to attenuate the glutamate release evoked by ischemia and determine which component of this release is affected. The study was carried out on superfused murine corticostriatal slices and the release of D-[^{3}H]aspartate (a non-metabolized analog of glutamate) was measured. Preincubation of corticostriatal slices with 10 mM taurine decreased D-[^{3}H]aspartate release evoked by chemical ischemia (0.5 mM sodium cyanide in glucose-free medium). The effect of taurine was not antagonized by taurine uptake inhibitor guanidinoethanesulfonate (5 mM) and antagonists of strychnine-sensitive glycine receptors and $GABA_A$ receptors (strychnine and bicuculline, 0.1 mM). To determine which component of ischemia-induced glutamate release is affected by taurine, three pathways of this release were modeled pharmacologically. D-Aspartate (0.5 mM) and hypo-osmotic medium ([NaCl] reduced by 50 mM) evoked D-[^{3}H]aspartate release via homoexchange and hypo-osmotic release pathways, respectively. These pathways were not attenuated by taurine. However, taurine suppressed the synaptic release of D-[^{3}H]aspartate evoked by voltage-gated sodium channel opener veratridine (0.1 mM). This effect was not prevented by guanidinoethanesulfonate, strychnine or bicuculline. Our study shows that taurine suppresses glutamate release under ischemic conditions, affecting the synaptic component of the release. Taurine acts extracellularly but its molecular target still remains unknown.

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EXPRESSION OF ENDONUCLEASE G AFTER GLOBAL CEREBRAL ISCHEMIA

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After a global cerebral ischemic insult in the rat, neurons within the hippocampal CA1 region die by delayed cell death. Several, both caspase-dependent and independent cell death pathways have been suggested, including involvement of different endonucleases. In this study we used immunohistochemistry on rat brain paraffin sections to detect the expression of Endonuclease G (EndoG). Endo G is normally located to the mitochondria, but is translocated to the nucleus if involved in cell death. Twenty rats were equally divided into five groups. Three groups, inc. a total of 12 rats, were subjected to two-vessel occlusion (2-VO) with hypotension for 15 min and allowed to survive 2, 3 or 4 days. The two remaining groups were respectively sham operated and served as naïve controls. Neurons destined to die were identified by intense eosinofilic staining in the cytoplasm by hematoxylin-eosin. In naïve and sham operated controls Endo G was detected in the cytoplasm of pyramidal neurons and interneurons. Two days after ischemia nuclear expression of Endo G was detected in a

few dying neurons in the ischemic sensitive region of the thalamic reticular nucleus. The Endo G expression here was more pronounced at days 3 and 4. In the hippocampal CA1 region no nuclear Endo G was detected at day 2, but at day 3 dying pyramidal neurons in the CA1 displayed nuclear expression of Endo G. One day later the expression of Endo G in CA1 neurons had almost disappeared. Our results indicate that Endo G is involved in fragmentation of DNA in cells dying by delayed cell death after global cerebral ischemia both in the hippocampal CA1 region and in thalamic reticular nucleus.

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PARAOXONASE-1 55/192 POLYMORPHISMS AND OXIDATIVE STRESS WITHIN 1ST AND 5TH DAYS FOLLOWING STROKE EVENT

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Ischemia appears to increase free radical production markedly, as evidenced by lipid peroxidation, protein oxidation and depletion of the antioxidants. Paraoxonase (PON1) can hydrolyze lipid peroxide. In the present study, we investigated PON1 55/192 polymorphisms and PON1 and glutathione reductase activities and malondialdehyde levels following the onset of stroke within 1st and 5th days. Seventy-seven patients with ischemic stroke and seventy-five normal controls were enrolled. PON1 55/192 polymorhisms were carried out with restriction enzyme digestion after PCR amplification. The allele frequencies of L/55 (78.1%) and B/192 (75.9%) were significantly higher in patients than in controls (50% and 52.9% respectively). AA (47.1%) and MM (50%) genotypes were significantly overpresented among controls as compared to patients (24.1% and 21.9%, respectively). In patients, PON1 activity was significantly reduced both at 1st and 5th days when compared to 1st day's values in controls. Both 1st and 5th days serum MDA levels in patient group were significantly higher than in controls ($P < 0.01$). 1st day MDA levels in patients carrying BB genotype were found higher than patients without BB genotype. We could not find significant difference between 1st and 5th day serum MDA levels in patients. 5th day patients' GSH-Rd levels were lower than both 1st day's patient and control groups ($P < 0.05$). Our results support that B 192 and L55 variantss were associated with ischemic stroke and PON1 activity was reduced after stroke event.

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EFFECT OF ISCHEMIA ON TRANSCRIPTION OF FACTORS CONTROLLING MITOCHONDRIAL BIOGENESIS IN RAT HIPPOCAMPUS

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Mitochondrial dysfunction is considered to be a central process leading to neuronal injury and death following brain ischemia. The aim of presented work was to study effect of ischemia on factors controlling mitochondrial biogenesis in rat hippocampus. Rats were subjected to transient cerebral ischemia by four-vessel occlusion of 15 min duration, followed by 1, 3, and 24 h of reperfusion. Total RNA was isolated from the hippocampus, and reverse transcribed into cDNA. Using specific primers, cDNA of nuclear respiratory factor 1 (NRF1), peroxisome proliferator-activated receptor gamma coactivator-1a (PGC1a), mitochondrial transcription factor A (TFAM) and heat shock protein 70 kDa (HSP70) was amplified. In respect of HSP70, we examined transcription of both HSP70.1 and HSP70.3 genes. Transient cerebral ischemia did not affect any of investigated genes. Similarly, reperfusion had no effect on transcription of NRF1, PGC1a, TFAM and HSP70.3. After 1 h of reperfusion, the level of HSP70.1 mRNA was significantly increased to 154% of control. The level of HSP70.1 mRNA declined to 140% and 126% of control after 3 h and 24 h of reperfusion, respectively. Hsp70 exerts an important function in the folding and import of mitochondrial proteins encoded by nuclear DNA. However, an ischemia-induced increase of HSP70.1 mRNA levels is associated with either stress response or post-ischemic recovery process different from mitochondrial biogenesis, since transcription factors controlling mitochondrial biogenesis were not affected after transient ischemia.

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COMPARISON OF THE EFFECTS OF CYCLOSPORIN A, MINOCYCLINE AND DIAZEPAM ON COMPROMISED CA1 NEURONS *IN VITRO* AND *IN VIVO*

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Selective neuronal death in CA1 sector of hippocampus has been studied in vitro on organotypic tissue culture and *in vivo* in gerbil ischemic model. To compare the neuroprotective potential of cyclosporin A (CsA), FK506, diazepam, MK801, IGF-1 and minocycline cell injury was induced by tert-butylhydroperoxide (TBH) or NMDA treatment in vitro or by transient (5′) brain ischemia *in vivo*. Cell death was monitored by 24 h in vitro or after 7 days of recovery *in vivo*. We have found that CsA > diazepam > minocycline showed strongest cell protection in vitro reducing the number of propidium iodide labelled cells (average about 50% in non-protected controls) for more than half, in both studied models of injury (TBH and NMDA). In the model of transient gerbil brain ischemia, the selected drugs were administered in a single bolus injection intra-arterially (CsA) or i.p. (diazepam) after the insult or i.p. pre- and after ischemia (minocykline). The extent of cell damage of the CA1 hippocampal region was quantified on paraffin-embedded, 10 um-thick sections stained with haematoxylin/eosin. Following the ischemic episode less than 15% of neurons remained intact. CsA and diazepam provided a significant neuroprotection; in both cases more than 60% of CA1 hippocampal neurons survived the insult when treated with these drugs. However, the positive effect of diazepam is probably due to the induced hypothermia. Our data indicate, that among drugs tested here, cyclosporin A and diazepam are the most promising neuroprotective agents in preventing delayed cell death after brain ischemia.

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ACETYLCHOLINE RELEASE IN THE HIPPOCAMPUS OF RATS WITH CHRONIC CEREBRAL ISCHEMIA

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Using operant conditioning task, we evaluated the deficit of discrimination learning and acetylcholine (ACh) release in the hippocampus by a microdialysis method during the task in rats with permanent bilateral common carotid artery occlusion (two-vessel occlusion:2VO). The operation (2VO or sham) was performed before the start of training and a guide cannula for microdialysis was implanted after the end of 30 training sessions. Subjects' lever press behaviour was reinforced only in the trials in which the key light was illuminated. The discrimination ratio (number of responses made during the 'illuminated' trials as a per-

centage of total number of responses) in the 2VO group was significantly lower than that in the control group. The occlusion resulted in obvious impairment of the discrimination performance. In both 2VO operated and sham operated rats there were significant elevation of ACh diffusion in the hippocampus just after the start of learning session. However, the elevation pattern of the ACh differed between groups. The elevated ACh level decreased gradually in all 2VO rats whereas ACh release in the control increased markedly and decreased to the basal level rapidly following the discontinuance of a session. These findings suggested that the 2VO produced persistent learning deficits from early stage of treatment and that the impaired discrimination learning performance might be related to the changes in the diffusion state of ACh in the hippocampus.

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NEURON DEGENERATION DEVELOPED PROPORTIONALLY TO THE CONTENT OF ACETYLCHOLINESTERASE IN BRAIN AFTER ISCHEMIA

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Acetylcholinesterase (AChE) is one of the essential enzymes maintaining the normal function of the nerve. Previously some reports showed AChE activity was altered after focal cerebral ischemia but contrary results were also provided. In order to know the relationship between the content of AChE but not the enzymatic activity and brain damage the amount of AChE in the brain at different duration after ischemia was determined by ELISA with monoclonal antibody 2E6 directed specifically to brain AChE but neither reacted with AChE from erythrocyte nor did with butyrylcholinesterase from serum. Sixty SD rats, weighing 350 ± 30 g, were divided randomly into 6 groups, one of which consisting of 10 rats. The ischemia was made using the middle cerebral artery occlusion model. 6 of 10 brains each group were obtained, homogenized and centrifuged. The supernatants diluted were coated and AChE was determined by ELISA. The pathological morphology of four brains each group was observed under microscope. The results showed the content of AChE increased continually for 24 h after ischemia and declined to about normal level since then until 96 h. The neuron degeneration developed proportionally to the contents of AChE in the first 24 h. It suggests that ischemia could result in increasing the content of AChE in brain and AChE may play a role in the brain lesion induced by ischemia.

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TRANSIENT FOREBRAIN ISCHEMIA INHIBITS FAK-DEPENDENT SIGNALING PATHWAY IN GERBIL HIPPOCAMPUS

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Focal adhesion kinase (pp125FAK) is a non receptor tyrosine kinase responsible for the flow of information from extracellular matrix to the cell interior. The function of FAK is believed to be tightly linked to its autophosphorylation and association with Src kinase necessary for reciprocal activation/phosphorylation of both enzymes in response to adhesion-dependent signals. Activated FAK may interact directly also with adaptor molecules and cytoskeletal structural proteins, perhaps providing a pathway by which ECM may regulate cell viability. Thus modulation of FAK activity may affect the linkage between ECM and various signaling cascades to which it is connected with profound effect on cell survival. These prompted us to evaluate the possible involvement of FAK-coupled pathway in the evolution of ischemia-induced delayed neuronal death in gerbil hippocampus. For this purpose we determined FAK activation/phosphorylation and its interaction with Src kinase and adapter protein p130Cas. Short-term (5 min) ischemia reduced markedly FAK phosphorylation level starting from 24 h of recovery. At 72 h the level of phosphorylated form demonstrated only 50% of the control value. Down-regulation of FAK activity was followed by the loss of its capacity to associate with investigated proteins. The amount of Src and p130Cas co-precipitated with FAK decreased to 50 and 60% of control value, respectively, at 24 h of recovery and remained on the lower level up to 72 h. These results are indicative of an involvement of FAK-dependent pathway in the postischemic delayed neuronal degeneration in hippocampus.

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Learning and memory

P.43

MATRIX METALLOPROTEASE-9 IS REQUIRED FOR LONG TERM POTENTIATION IN MICE

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Long term plasticity is dependent on remodeling of the synapse. Recently it was suggested that extracellular matrix is involved in this remodeling. As matrix metalloprotease-9 (MMP-9) is expressed in the brain following toxic-neuronal excitation, here we examine the role of MMP-9 in Long Term Potentiation in the hippocampus. We have used slices from the hippocampi of 2–4 month MMP-9 knock-out mice and their control littermates. We have measured extracellular field excitatory postsynaptic potentials induced in Schaffer collateral to CA1 pathway. Following tetanization, LTP was activated in slices from MMP-9 knock-out and control mice amounting to about 170% of the baseline signal. In knock-out mice, LTP was deactivated within 20 to 40 minutes, while in control mice it was stable for more then 90 minutes. These results show that MMP-9 is required for synaptic plasticity.

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PERSISTENCE OF A FEAR MEMORY IS DEPENDENT ON HIPPOCAMPAL PROTEIN SYNTHESIS DURING A RESTRICTED TIME WINDOW

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To the present time, memory research has been extensively and successfully devoted to the study of the mechanisms and brain circuits involved in long term memory formation. It is widely accepted that consolidation for several learning tasks is dependent upon hippocampal de novo protein synthesis. In this sense, it has been demonstrated that many learning tasks, including Inhibitory Avoidance (IA), require de novo protein synthesis during the first 6 hr after acquisition. However, the role of early hippocampal protein synthesis in memory persistence has never been addressed. Intra-hippocampal inhibition of protein synthesis by anisomycin caused amnesia for IA learning in a 7-day retention test, but not in a 2-day retention test in a time-dependent manner. A learning-dependent increase in the level of five hippocampal proteins that peaked at 24 hr was detected by immunoblot. Anisomycin infusion that caused a memory deficit at 7 days, prevented c-Fos, Homer-1a and Akt increases at 24 hr. Our results reveal that early and time-restricted protein synthesis in rat hippocampus is required after acquisition for persistence, but not formation of IA memory. This hippocampal mechanism for memory persistence may involve c-Fos and Homer-1a expression around 24 hr after acquisition.

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SPATIAL LEARNING INDUCE PHOSPHORYLATION AND MOVEMENT TO TRITON INSOLUBLE COMPLEX OF NMDA RECEPTORS DURING SPATIAL LEARNING

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Evidence demonstrates that NMDA receptor activation is necessary for spatial learning in hippocampus and insular cortex. NMDA receptor signaling depends on the receptor phosphorylation state, alternative splicing, subunit composition and trafficking. Phosphorylation of NMDA receptors has been implicated in memory formation. We found that 5 days training in Morris water maze induce NR2B tyrosine phosphorylation in the hippocampus but not in the insular cortex. However from the first training day, spatial learning in the cortex induce a rapid (measured immediately after training) NR2A and NR2B increase in detergent-insoluble complex isolated by centrifugation in buffer with Triton X-100 (no ionic detergent). Our results show a differential NMDAR regulation between structures, and suggest that NMDA receptors translocate to insoluble microdomains during memory formation.
Acknowledgements: Supported in part by CONACYT 42657Q and DGAPA IN212503.

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THE ROLE OF THE NEURONAL NITRIC OXIDE (NO)-SYSTEM IN SPATIAL WORKING AND OBJECT RECOGNITION MEMORY

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The actual role of NO in learning and forms of memory is yet not well understood. We used nNOS-KO mice in the T-maze alternation task (T-AT) and the non-spatial object recognition test (ORT) to reveal the role of neuronal NO-system in cognitive functions. The impact of NO-cGMP system of the nucleus accumbens (NAc) in OR was studied in rats. In the T-AT the nNOS-KO mice showed an increased exploratory activity when compared to the wild-type controls (WT). This is in accordance with published performance in the open field test. The alternation rate of nNOS-KO was not significantly different from WT when the bias of enhanced exploratory activity was eliminated, indicating that their spatial working memory was not impaired. In the ORT nNOS-KO mice showed a lower preference to explore novel objects when compared to WT. This reveals that the performance of nNOS-KO to discriminate new from familiar objects is reduced, suggesting that nNOS is required for full capacity in this form of episodic memory. In rats, bilateral injections of the nNOS inhibitor 7-nitroindazole (7NI) into the NAc disrupted object memory as indicated by abolished exploratory preference for the novel object, without affecting locomotor activity. The inhibition of object memory following 7NI was restored with accumbal microinjection of phosphodiesterase-5 inhibitor UK-114,542. Administration of this inhibitor in untreated rats exhibited a nootropic effect versus vehicle treated control group. These results suggest that the NO-system is involved in visual object recognition and the pathway of NO-cGMP signalling in the NAc is important for this type of episodic memory.

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CYTOKINES INDUCE CHANGES IN THE FUNCTIONAL ASSOCIATION OF BRAIN CELLS DURING OLFACTORY LEARNING

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Social learning of olfactory cues was used to test the hypothesis that cytokine signaling is used for associating relevant information at the cellular level in the nervous as well as the immune system. Naïve rats were caged for 1 h with a scavenger rat that had recently consumed either familiar (chow) or novel (blueberry bar or cheese) food, then tested for food preference prior to sacrifice 2 h later. Total RNA was extracted from hippocampus, amygdala, olfactory bulb, and insular cortex and screened for expression of genes for cytokines, cell adhesion molecules, and neurotrophins by DNA hybridization arrays and qPCR. Naive rats preferred the food they had smelled on the breath of the scavenger. Numerous chemokines, interleukins, and fibroblast growth factors were expressed in all regions examined. Several of these, including an interleukin 1 receptor and tumor necrosis factors were preferentially expressed by rats exposed to the novel odors. Many genes for extracellular matrix and adhesion factors were also expressed robustly across brain samples. In the amygdala, especially, exposure to novel odors affected gene expression for a number of factors, including certain integrins, laminins, cadhedrins, and metalloproteinases. Generally, the behavioral preference as well as the change in gene expression was greater with exposure to blueberry bars than to cheese. These results confirm that cytokine signaling correlates with acquisition of olfactory memory, and suggest that the nature of the response relates to the context and survival value of the information being stored.
Acknowledgements: *Supported by NIH grants from NICDC (to LI) and the RCMI program of the NCRR.*

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NUCLEAR FUNCTIONS OF PROTEIN PHOSPHATASE-1 DURING MEMORY-ASSOCIATED PROCESSES

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Protein kinases and phosphatases are essential mediators of long-term memory formation and associated synaptic modifications. In particular, the protein phosphatase 1 (PP1) is an abundant serine/threonine phosphatase in the brain and a potent negative regulator of memory. Previous work in our laboratory has shown that the genetic inhibition of PP1 improves object recognition and spatial memory. This memory improvement is accompanied by elevated levels of phosphorylation of CREB transcription factor and CREB-dependent gene expression, a process required for acquisition and storage of long-term memory. These results suggested that PP1 regulates long-term memory, in part, by modulating nuclear processes. To delineate the mechanisms of PP1 regulation of nuclear events, PP1 was inhibited selectively in the nucleus of forebrain neurons by overexpressing the nuclear specific inhibitor of PP1 (NIPP1) in an inducible transgenic mouse system. The targeted forebrain regions include the hippocampus, amygdala, and cortex, which are critical for proper memory formation. The NIPP1 transgene is expressed transiently in adulthood and its effect on memory-associated functions are examined. In particular, possible modifications of histones and chromatin-associated molecules, such as CREB transcription factor, histone deacetylases and acetyltransferases are being addressed to detect possible correlations with the expected changes in transcriptional activation and transcription-dependent memory formation and synaptic plasticity. These transcription-regulatory molecules are thought to be involved in memory processes and can interact with and be regulated by PP1. Thus, they are excellent candidates for mediating PP1-regulated transcription processes.

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Abstract has been withdrawn.

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AMNESIA INDUCED BY ANTIBODIES TO THE NEURAL CELL ADHESION MOLECULE (NCAM)

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Antibodies directed to the neural cell adhesion molecule (NCAM) induce amnesia of animal learning paradigms if delivered to the ventricles at the 6–8 h post-training time. This phenomenon has now been observed in three separate laboratories for discriminative avoidance tasks in chicks and both passive avoidance and appetitive learning in rats. To establish if spatial learning could be similarly affected, we determined the consequence of administering anti-NCAM to postnatal day 80 Wistar rats at 6 h following a single 5-trial session in the Morris water maze. Following training, aliquots (5 ml) of anti-NCAM (generous gift of Elisabeth Bock) were delivered by cannula to the ventricles of unrestrained animals. Surprisingly, latencies to reach the hidden platform were unaffected in subsequent water maze trials at 24 h and 48 h following administration of anti-NCAM. As controls, the antibody was found to effectively induce amnesia of an avoidance task and to recognise all NCAM isoforms by an immunoblotting procedure. These results confirm subtle differences to underpin spatial and non-spatial forms of learning.
Acknowledgements: Supported by Enterprise Ireland and the EU 5th Framework. CMR and KJM are Science Foundation Ireland Investigators.

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TRANSCRIPTIONAL REGULATION OF MMP-9 IN THE RAT HIPPOCAMPUS *IN VIVO* AFTER NEURONAL ACTIVATION

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MMP-9 (matrix metalloproteinase 9) is a protein which expression is regulated mainly transcriptionally. It is involved in the formation of the long-term synaptic plasticity, and as a consequence in the learning and long-term memory formation. Despite of the essential physiological functions, there is no data concerning MMP-9 mRNA regulation in the normal brain. We found that 2 hours after neuronal excitation MMP-9 mRNA expression is strongly induced in the rat hippocampus. This molecular event is correlated with the release of the repressive transcription factor Yin Yang 1 (YY1) from mmp-9 proximal promoter in the rat hippocampus *in vivo*. Simultaneously, there is a change in the composition of the dimeric transcription factor activator protein 1 (AP-1), which occupies the promoter in non-active as well as in activated hippocampal neurons *in vivo*.

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SYNAPTIC LOCALIZATION OF EPENDYMIN IN FISH AND POSSIBLE INVOLVEMENT OF RELATED MAMMALIAN MERPS IN MOUSE BRAIN AFTER LEARNING

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Fish ependymins (FEPs) are brain specific glycoproteins of the extracellular matrix synthesized by, and secreted from meningeal fibroblasts.

They comprise the HNK-1 cell adhesion epitope and serve a variety of cellular functions in neuronal regeneration, memory formation and acclimatization to cold-stress. In situ hybridizations of goldfish trained to avoid electric shocks after a conditioned light signal revealed a marked increase of *FEPs* mRNA in the meninx 3 hours after learning. By RIAs, immunofluorescence and immunogold labeling of brain sections FEPs were found to be distributed throughout the extra-cellular fluid. Particularly high immunoreactivity was observed at synapses of tectal neurons that receive synaptic input from the retina and from the longitudinal torus, i.e., at synapses implicated in the processing of information pertinent to the training task. Here, FEPs immunoreactivity appeared to increase after avoidance learning. We demonstrated immunological cross-reactivity of FEPs with the adult murine hippocampus and cultured hippocampal neurons from embryonic rats, and mammalian ependymin-related proteins (MERPs) were recently identified by differential display analysis (Nimmrich *et al.*, 2001). In order to test for a possible involvement of murine MERPs in memory formation, mice were trained on the Morris water maze. Two hours later, expression levels of *MERP* mRNA were analyzed by RT-PCR. Results obtained with 4 brains from learning mice indicate that murine *MERP2* mRNA was increased as compared with brains from untreated litter mates. Therefore, it is tempting to expect a functional involvement of MERPs in memory consolidation analogous to the role of fish ependymins.

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A DOPAMINE TRANSPORTER GENETIC VARIATION AFFECTS THE RESPONSE OF THE HUMAN MIDBRAIN IN EPISODIC MEMORY FORMATION

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Recent data from animal studies raise the possibility that dopaminergic neuromodulation promotes memory formation in the hippocampus, possibly by facilitating long-term encoding of novel stimuli. Here we investigated a possible role for dopaminergic neurotransmission in human hippocampus-dependent memory encoding, by measuring how polymorphisms in dopamine inactivation pathways affect encoding-related brain activity (functional magnetic resonance imaging, fMRI) in an episodic memory task. In young, healthy adults, encoding-related fMRI activation in dopaminergic midbrain structures was higher in carriers of the (low expressing) 9-repeat allele of the dopamine transporter (DAT1) when compared to subjects homozygous for the 10-repeat allele, who express DAT1 at higher levels. Catechol-O-methyl transferase (COMT) polymorphism, on the other hand, affected encoding-related activity in right prefrontal and occipital brain regions. For both DAT1 and COMT polymorphisms, the activation differences observed were specifically related to successful episodic memory encoding, but not to level of study processing. There were, however, no differences in memory performance or reaction times associated with DAT1 or COMT genotypes. Our results provide evidence for a role for dopaminergic midbrain areas in human episodic encoding. The two polymorphisms appear to affect anatomically separable subprocesses of episodic memory encoding, compatible with DAT1 being highly expressed in midbrain and COMT being particularly involved in prefrontal dopamine degradation.

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EXCITATORY AND MODULATORY SYSTEMS IN CONSOLIDATION OF AUDITORY CORTEX-DEPENDENT MEMORY

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The auditory cortex is critical for discriminating frequency-modulated tones (FM). Previous studies on Mongolian gerbils revealed that in this paradigm the initial maintenance of memory for 1 day and the regulation of its long-lasting persistence require the activation of different protein synthesis-dependent consolidation processes in the auditory cortex. Here we elucidate the functional significance of excitatory and modulatory neurotransmission for long-term FM discrimination memory. Injections of NMDA-type glutamate receptor blockers into the auditory cortex shortly before or after the initial training session led to retention deficits in a retraining session 24 h later. In contrast, injections of a D1-like dopamine receptor agonist 24 h before or shortly after the first training facilitated subsequent FM discrimination learning. This effect was sensitive to a D1-like receptor antagonist and to protein synthesis inhibitors. The agonist-induced improvement was confined to auditory cortex-dependent aspects of the task, i.e., discriminating the conditioned stimuli and/or associating them with their respective meanings, whereas learning of the escape reaction in response to foot shock and of conditioned reactions in response to FM tones were not affected. Acquisition performance was normal, implying that D1-like receptor activation supports retention and/or retrieval of the task. Together, our findings suggest that in the gerbil auditory cortex the activation of excitatory glutamatergic neurotransmission is required for the maintenance of FM discrimination memory for 24 h whereas the activation of D1-like dopamine receptors facilitates subsequent consolidation-relevant processes.

Neurodegenerative diseases Alzheimer

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NEW MARINE COMPOUNDS WITH CALCIUM CHANNEL BLOCKING PROPERTIES: POSSIBLE CONTRIBUTION TO ALZHEIMER'S DISEASE TREATMENT

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder which aethiology and pathogenesis remains unclear. The cholinergic hypothesis gave place to neuropharmacological strategies using acetylcholinesterase (AChe) inhibitors that would produce a cholinergic enhancement (Winkler, J. *et al.*, 1998, J Mol Med, 76:555–67). AChe levels are augmented near amyloid-beta deposits and this peptide could increase AChe expression by voltage-dependent calcium channels (VDCC) activation (Sberna *et al.*, 1997, J Neurochem, 69, 1177–84). We postulate that the combination of calcium channels blocking activity that prevent or diminish AChe over expression and AChe inhibition could be a good approach in AD treatment. We have performed a screening of 1750 marine compounds on SH-SY5Y cells exposed to KCl 120 mM, testing survival 24 hours later. Compounds that exhibited a neuroprotective effect were assayed in SH-SY5Y cells loaded with Fluo-4 to measure their effects on changes in intracellular calcium after depolarization with KCl. Three compounds isolated from the sponge Aplysina cavernicola have shown calcium channel blocking properties in SH-SY5Y cells. The patch-clamp technique was used to examine directly the effects of these compounds on VDCC in bovine chromaffin cells. The assayed compounds partially blocked VDCC in a concentration-dependent and reversible manner in this model. These compounds also showed inhibition of AChe and BuChe activity in the 10–15 M range.

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PRETREATMENT WITH L-CARNITINE ENHANCED ACETYLCHOLINESTERASE INHIBITION FOLLOWING GALANTHAMINE ADMINISTRATION IN RATS

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Most of Alzheimer disease (AD) treatments have been focused on the inhibition of cholinesterases. The influence of pretreatment with L-carnitine (CAR) on cholinesterase inhibition caused by galanthamine (GAL) administration in rats is the aim of this study. Male Wistar rats, weighing 250–290 g, were used. The first group ($n = 7$) was administered with water p.o. during 3 consecutive days and then saline was injected (i.m.); 30 min later, the animals were killed and blood, liver, hypophysis, and brain parts (frontal cortex, basal ganglia, septum and hippocampus) were collected. The all tissue samples were homogenized 1 : 10 in distilled water. In tissue homogenates and plasma, activities of acetyl-(AChE)and butyryl-(BuChE)cholinesterase were determined. In experimental groups, CAR was administered daily during 3 consecutive days in dose of 250 mg/kg (p.o.) and after administration of the last CAR dose, GAL was injected (i.m., 10 mg/kg). Highest activity of AChE in BG was observed; BuChE activity in plasma was lower than that in liver. Following GAL administration, both cholinesterases were significantly inhibited. Pretreatment with CAR followed by GAL administration showed higher brain AChE inhibition in comparison with group without pretreatment. Thus, pretreatment with CAR enhanced AChE inhbition in brain of the rat following GAL administration.

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MASS SPECTROMETRY STUDY OF LIPID MODIFICATIONS IN ALZHEIMER'S DISEASE

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Abnormalities of major lipid classes have been found in Alzheimer's disease, but it does not appear yet clearly whether they are early manifestations of the disease. Lipid modifications were studied by ESI mass spectrometry and MSn in temporal lobe, frontal lobe area 10 of Brodmann and cerebellum (as control area) in view of establishing correlations with the stages of Alzheimer's disease which were determined by neuropathological criteria according to Brack. Lipid structures characterization was performed in the negative ion mode using an ESI-ion trap mass spectrometer used in MSn mode. A triple quadrupole instrument was employed in precursor and neutral loss scan mode to obtain a quantitative determination of certain lipid species using specific internal standards. Sulfatides were quantified by precursor ion scan of sulfate SO4H- (m/z 97) using negative ion mode with triple quadrupole. Synthetic C14:0 sulfatide standard was added prior to the extraction and used as internal standard. We found a significant reduction of sulfatides. Galactosylceramides and ceramides were increased. Phosphatidylserine was detected at m/z 788 and m/z 834 and characterized by MS/MS and appeared to be also significantly depleted. Phosphatidylserine and sphingolipid isoforms were identical in control versus Alzheimer disease. Modifications of brain lipids were observed between control and patients with Alzheimer disease in relation to brain areas and stages.

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HEPARIN PROMOTES ACTIVATION AND AUTOCATALYTIC CLEAVAGE OF BACE1

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The β-site APP cleaving enzyme 1 (BACE1) is an aspartic protease that generates the N-terminus of the β-amyloid protein from the β-amyloid precursor protein (APP). BACE1 is a key target for Alzheimer drug development, however little is known about the physiological regulation of the enzyme. Heparin can promote β-secretase processing of APP

in cell culture. However, heparin reportedly inhibits purified BACE1 in vitro. To clarify the role of heparin in regulating BACE1, we examined the effect of various concentrations of heparin on the activity of recombinant human BACE1 in vitro using a fluorescence assay. Low concentrations (1 μg/ml) of heparin were found to stimulate BACE1 (8 μg/ml), whereas higher concentrations (10–100 μg/ml) were inhibitory. Heparin affinity chromatography demonstrated that heparin bound strongly to the partially active proenzyme form of BACE1. Heparin also bound to a peptide homologous to the prosequence of BACE1. Most interestingly, low concentrations of heparin promoted autocatalytic cleavage, resulting in a decrease in the concentration of the proenzyme. On the basis of these results, we propose that high affinity binding of heparin to the pro-domain of BACE1 activates the enzyme and thereby promotes autocatalytic cleavage. Our study provides support for the view that heparan sulfate proteoglycans may regulate endogenous APP processing via the β-secretase pathway.

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EFFECTS OF β-AMYLOID25–35 ON THE EXPRESSION OF GLUTAMINE TRANSPORTERS AND VESICULAR GLUTAMATE TRANSPORTERS IN RAT CORTICAL CELL CULTURES

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Alzheimer's disease (AD) is a neurodegenerative disorder and is the commonest form of dementia in the elderly. It is characterized by pathological features consisting of extracellular β-amyloid (Aβ) deposits, associated with degenerating and apoptotic neurons showing filamentous aggregates of hyperphosphorylated tau protein (neurofibrillary tangles). Glutamine and glutamate are essential amino acids for brain metabolism and function. Within neurons, glutamate is transported into synaptic vesicles by specific vesicular glutamate transporters (VGLUTs) before exocytotic release. Systems A and N mediate glutamine uptake to both neurons and astrocytes. System A glutamine transporter 1 (SAT1) is a member of the system A transporters and is a highly efficient neuronal glutamine transporter. We investigated the effect of Aβ on the transporter proteins in rat cortical cell cultures, incubated with Aβ 25–35 at concentrations of 3 μM and 10 μM for 12 and 24 hr. Quantitative analyses of immunoreactivities in individual neurons were performed by confocal fluorescence microscopy. The result suggested that Aβ does not affect VGLUT1 or 2 in neurons but significantly reduces SAT1 (at 10 μM of Aβ, 12 or 24 h). Thus Aβ may impair neuronal function in part by reducing the neuronal capacity for glutamine uptake.

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'HYBRID' ANTIOXIDANT – POTENTIAL NEW GENERATION MEDICINE FOR THERAPY OF ALZHEIMER'S DISEASE

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As it follows from literature under Alzheimer's (AD) conditions the increase of fluidity of lipid phase takes place alongside with the intensification of lipid peroxidation (LPO) in cell membranes. Those data testify to failures of the system of LPO homeostasis in membranes which could result in functional shifts. The purpose was to develop the approach to AD therapy, based on using 'hybrid' antioxidants (AO) of new generation. As parent compound AO from the group of hindered phenols was used. We synthesized and studied 'ichphans' – substances including AO part and choline moiety, ensuring anticholinesterase activity, and also long-chain hydrocarbon fragment promoting penetration through blood-brain barrier and able to influence membrane fluidity. On the basis of experiments in vitro the preparation was chosen from a number of analogues within optimally combined anticholinesterase and antioxidant properties. Intraperitoneal administration of ichphan resulted in long-term inhibition of soluble and membrane cholinesterase activity of mice brain to which inhibition of cytosolic cholinesterases brought the main contribution. By ESR technique, using paramagnetic probes located in surface area of membrane lipid bilayer and in neighbouring to protein domains, it was found that in contrast with 'usual' AO, icphan is capable to suppress LPO and rigidize lipid bilayer at the same time. The combination of those properties of described AO may be useful for 'membrane' therapy of AD by correction of the system of LPO homeostasis control, broken owing to the pathology.

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DI-INDOLES: NOVEL INHIBITORS OF ALZHEIMER β-AMYLOID AGGREGATION

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Di- and polyanionic compounds have been shown to inhibit in vitro aggregation of amyloidogenic proteins, including the Alzheimer peptide, Aβ. These highly charged, polar molecules, however, suffer from poor pharmacokinetics and minimal penetration through the blood-brain barrier. Our objective was to develop compounds having activity similar to that of anti-amyloidogenic anionic species but with improved pharmacokinetics. This led to the identification of a novel series of synthesized compounds containing two indole groups, i.e. 'di-indoles'. Rather than forming cationic-anionic contacts at positively charged residues of Aβ, the di-indole compounds may instead bind to these sites through cation-π interactions. In a kinetic Thioflavin T (ThT) fluorescence assay, the compounds potently inhibited Aβ1–40 aggregation, with the most active compounds showing potency similar to that of morin, an anti-amyloidogenic polyphenol present in wine. Circular dichroism (CD) experiments confirmed the ThT results. When Aβ1–40 was incubated on its own, the secondary structure of the peptide was seen by CD to shift from predominantly random coil to β-sheet over a period of two to three days. The addition of a di-indole compound delayed or eliminated this transition, with some compounds having greater activity than morin at equimolar concentrations. Given their low-to-medium polarity, the compounds are expected to possess higher blood-brain barrier permeability and slower clearance (i.e. longer half-life) than highly charged molecules. The di-indole compounds identified here thus constitute a new class of Aβ aggregation inhibitor whose absorption and pharmacokinetics are anticipated to be superior to those of anti-amyloidogenic polyanionic compounds.

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MINOCYCLINE REDUCES NEURONAL CELL DEATH AND IMPROVES COGNITIVE IMPAIRMENT IN VITRO AND AN ANIMAL MODEL OF ALZHEIMER'S DISEASE

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Minocycline is a second-generation tetracycline that effectively crosses the blood-brain barrier. Minocycline has been reported to have significant neuroprotective effects in models of cerebral ischaemia, traumatic

brain injury, amyotrophic lateral sclerosis, and Huntington's and Parkinson's diseases. In this study, we demonstrate for the first time that minocycline has neuroprotective effects in an in vitro and in a rat model of Alzheimer's disease (AD). We found that minocycline attenuates, the phosphorylation of eucaryotic translation initiation factor-2 alpha, the activation of caspase 12 induced by Aβ 1-42 treatment, the expression of the C-terminal fragments of amyloid precursor protein, or endoplasmic reticulum (ER) stressors such as brefeldin A or tunicamycin in neuronal cells, which suggests that minocycline exerts its neuroprotective effects by targeting ER-mediated apoptotic pathways. In addition, minocycline was found to improve learning and memory impairment by reducing neuronal death in the hippocampal area in an Aβ 1-42-infused AD rat model, which suggests that minocycline treatment could be effective in AD.

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MITOCHONDRIAL ABNORMALITIES IN ALZHEIMER BRAIN: MECHANISTIC IMPLICATIONS

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Reductions in cerebral metabolism sufficient to impair cognition in normal individuals also occur in Alzheimer Disease (AD). The degree of clinical disability in AD correlates closely to the magnitude of the reduction in brain metabolism. Therefore, we tested whether impairments in tricarboxylic acid cycle (TCA) enzymes of mitochondria correlate with disability. Brains were from patients with autopsy-confirmed AD and clinical dementia ratings (CDR) before death. Significant ($P < 0.01$) decreases occurred in the activities of the pyruvate dehydrogenase complex (−41%), isocitrate dehydrogenase (−27%), and the α-ketoglutarate dehydrogenase complex (−57%). Activities of succinate dehydrogenase (Complex II) (+44%) and malate dehydrogenase (+54%) were increased ($P < 0.01$). Activities of the other four TCA cycle enzymes were unchanged. All of the changes in TCA cycle activities correlated with the clinical state ($P < 0.01$), suggesting a coordinated mitochondrial alteration. The highest correlation was with PDHC ($r = 0.77$, $r^2 = 0.59$). Measures to improve TCA cycle metabolism might benefit AD patients.

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THE FIRST INHIBITORS FOR HUMAN GLUTAMINYL CYCLASE (QC)

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The first screened and subsequently designed inhibitors for human Glutaminyl Cyclase (QC) are presented. The recently described ability of human QC to convert N-terminal glutamate of the peptide Glu3-Abeta(3–11) and Glu3-Abeta(3–21) of the Amyloid-beta-peptide (Abeta) into the respective pGlu3-Abeta(3–11) and pGlu3-Abeta(3–21) suggests the hypothetical involvement of QC in the formation of the neurotoxic plaques in the Alzheimer's syndrome (AD). Hence, human QC might be involved in the pathophysiological initiation of AD, exposing the enzyme as a new potential drug target. Starting from imidazole exhibiting a binding constant (Ki) of 100 μM, compounds derived from 1-imidazolalkyl-thiourea, serving as lead with binding constants in the low micromolar range, were found. Based on that scaffold, a library of compounds was synthesized resulting in an efficacy improvement of three orders of magnitude. In addition, investigating the substrate specificity of QC, we found a preference for aromatic side chains in the penultimate position to the N-terminal glutamine in tripeptide substrates. Performing a flexible alignment of the best inhibitors and the best substrate resulted in a good match of certain characteristic features of both molecules, suggesting a similar binding mode for both ligands in the active site of QC and resulting the prediction of a potential shape of the pharmacophor.

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MEASUREMENT OF THIRTEEN BIOLOGICAL MARKERS IN CSF OF PATIENTS WITH ALZHEIMER'S DISEASE: FOCUS ON MCP-1 AND NGF

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Cerebrospinal fluid (CSF) biological markers may be of valuable help in the early diagnosis of Alzheimer's disease (AD). The aim of the present study was to evaluate CSF levels for beta-amyloid (Ab42), total tau (T-tau) and phosphorylated tau181 (P-tau181) and to compare it with CSF levels for 10 different growth factors, cytokines and chemokines. We studied 25 age-matched controls, 23 patients with AD, 5 persons with frontotemporal lobe dementia, 10 patients with alcohol dementia and 11 depressive patients. The CSF levels of Ab42 were significantly decreased, while T-tau, P-tau181 as well as the ratio P-tau181/Ab42 were significantly increased in patients with AD compared to controls. The ratio P-tau181/Ab42 provided best diagnostic accuracy in distinguishing patients with AD from healthy control subjects (sensitivity 91%, specificity 91%) and all other tested groups. CSF levels of monocyte chemoattractant protein-1 (MCP-1) significantly increased with age and remained unchanged in AD. Depending on the degree of neurodegeneration (as expressed by ratio of P-tau181/Ab42), the patients with AD displayed significantly increased CSF-levels of nerve growth factor (NGF) as compared to the healthy control persons. Our data demostrate the suitability of ratio P-tau181/Ab42 by diagnosis of AD, while CSF levels of NGF and MCP-1 are less specific and reliable for AD. It is suggested that CSF-levels of NGF depend on the degree of neurodegeneration and the levels of MCP-1 on the age.

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STRESS PROMOTES LEARNING AND MEMORY IMPAIRMENTS AND MODULATES AMYLOID DEPOSITION IN APPV717I-CT100 MICE

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Despite extensive progress in genetics, cell-biology, and animal models, many puzzles remain about the etiology and the precise molecular and pathological mechanisms of Alzheimer's disease (AD). There is a link between stress and memory or many neuropsychiatric disorders such as depression and schizophrenia. However, little is known about the relationship between stress and the onset and development of AD. In order to investigate the effects of long-term stress on the AD developments, APPV717I-CT100 mice expressing human APP-CT100 containing London mutation (V717I) was exposed to immobilization stress throughout from 3 to 12 months after birth, respectively. Passive avoidance test showed that continuous stress for 8 months is enough to

induce a significant cognitive impairment in APPV717I-CT100 mice. In social transfer of food preferences test, at one hour and 24 hours following the demonstrator-observer interaction, the mice which received stress for 9 months showed lower cued food preference than non-stress group. In parallel with learning and memory impairments, western blot and immunohistochemistry also showed a dramatic elevation in amyloid deposition and C-terminal immunoreactivity in the cortex and hippocampus of stress group. This study provides the first evidence that chronic stress may give faster cognitive impairments and amyloid depositions in AD and thus reducing stress may be one of potential strategies for the prevention and retardation of AD.

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MEFENAMIC ACID SHOWS NEUROPROTECTIVE EFFECTS, IMPROVING COGNITIVE IMPAIRMENT IN *IN VITRO* AND *IN VIVO* ALZHEIMER'S DISEASE MODELS

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Non-steroidal anti inflammatory drugs (NSAID) exert anti-inflammatory, analgesic and anti-pyretic activities and are involved in the suppression of prostaglandin synthesis by inhibiting cyclooxygeneases (COX), an enzyme that catalyzes the formation of prostaglandin precursors from arachidonic acid. There is epidemiological observation that long-term treatment of patients suffering from rheumatoid arthritis with NSAID results in reduced risk and delayed onset of Alzheimer's disease (AD). Here, we investigated the therapeutic potential of mefenamic acid, one of the commonly used over-the-counter NSAID that is a cyclooxygenase (COX)-1 and COX-2 inhibitor, for AD. We found that mefenamic acid protected neuronal cells against amyloid beta peptide 1-42, Swedish double mutation (KM595/596NL) of amyloid precursor protein and C-terminal fragments of amyloid precursor protein, decreased the production of free radical including nitric oxide (NO), and cytochrome c release from mitochondria in neuronal cells. In addition, we first demonstrate that mefenamic acid improves learning and memory impairment in the Aβ 1-42-infused AD rat model, suggesting that mefenamic acid could be an effective therapeutic agent for AD.

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INHIBITION OF Wnt SIGNALING PATHWAY INDUCES CHANGES IN TAU PHOSPHORYLATION IN NEURONAL CELL CULTURES AND IN RAT BRAIN SLICES

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Alzheimer's disease (AD) is the most common neurodegenerative disease and is characterized by neuronal and synaptic lost. The two neuropathological hallmarks are senile plaques, composed by β-amyloid peptide and neurofibrillary tangles, formed by bundles of hyperphosphorylated forms of the microtubule associated protein tau. During development Wnt signaling pathway plays a crucial role in cellular adhesion and in cell fate determination. Wnt proteins are secreted signal molecules that interact with Frizzled receptors to promote gene transcription resulting from β-catenin translocation into the nucleus. When Wnt proteins are absent, glycogen synthase kinase-3β (GSK3β) is activated and phosphorylates β-catenin which in turn is degraded by the proteasome system. GSK3β is a proline-directed protein kinase that phosphorylates tau protein in vitro, and interestingly, colocalize with tangles in the brains of AD. However, at present it is not known the relationship between the control of the GSK3β activity through Wnt pathway and tau phosphorylation, which is the aim of the present work. We used primary cortical cultures and metabolically active brain slices from young and aged rat brain to study the effect of Wnt inhibitors (sFRP-3, Frizzled-8 and Dkk-1, 50 and 100 ng/ml). We found that inhibition of Wnt pathway significantly increase tau phosphorylation in those epitopes recognized by the PHF-1 antibody concomitant with a slightly increase in total tau revealed by the tau-1 antibody. These results suggest a role of the Wnt signaling pathway in tau phosphorylation possibly mediated trough activation of GSK3β.

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ALZHEIMER'S DISEASE: TARGETING OF NICASTRIN TO SUB-CELLULAR COMPARTMENTS AND ITS INFLUENCE ON G-SECRETASE COMPLEX ACTIVITY

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Alzheimer's Disease is a progressive neurodegenerative disorder, which is characterized by the accumulation of senile plaques, predominantly composed of amyloid-Beta (ABeta) peptide and neurofibrilary tangles. ABeta peptide is generated by proteolytic processing of amyloid Beta precursor protein (APP) through sequential cleavages by the beta- and gamma-secretases. The active gamma-secretase complex is composed of at least four noncovalently bound proteins: Presenilin (PS), Nicastrin, APH-1 and PEN-2. Nicastrin, a type I transmembrane protein containing multiple glycosylation sites, appears to be the initial binding partner of the g-secretase complex, since in the absence of PS the immature form of Nicastrin accumulates in the endoplasmic reticulum. The transmembrane domain of Nicastrin has been identified as being required for assembly of g-secretase complex, but is not sufficient to mediate activity. The ectodomain is required for g-secretase complex activity, while the deletion of the cytosolic domain did not influence association or activity. In order to partially target the g-secretase complex to specific cell compartments, target signal sequences were inserted in the cytosolic domain of Nicastrin. Nicastrin was partially targeted to the endoplasmic reticulum, to the trans-Golgi compartment, to the lysosomes and to the plasma membrane. Localization of Nicastrin target constructs was confirmed through confocal immunofluorescence microscopy. Complex association and transport, APP and Notch cleavage assays were used to analyze the g-secretase activity and specificity of these Nicastrin target constructs.

P.70

ABETA25-35 AGGREGATION AND TOXICITY

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The present study is aimed to elucidate the micro-environmental conditions driving amyloid-β peptide (Aβ) toxic conformational changes and assembly, with particular attention to protein glycation. Aβ 25-35 (GSNKGAIIGLM), the shortest active fragment of Aβ, was incubated (aged) at 50°C for 72 h in PBS (metal free, pH7.4) and in the presence of well known Advanced Glycation End products forming agents such as copper ions (Cu^{2+}), ribose, glycolaldehyde, and of an antioxidant/anti-aggregating peptide, carnosine (β-alanyl-1-methyl-L-histidine). The status of aggregation, measured by Congo Red (CR) absorbance spectrum shift, increased after incubation, particularly in the presence of Cu^{2+}, and was surprisingly reduced by ribose. While the increased aggregation induced by glycolaldehyde was prevented by Cu^{2+}. The addition of Carnosine caused an overall anti-aggregating effect. The

toxicity of the different Aβ 25-35 preparations, measured as cell viability (MTT assay) in rat astrocyte enriched primary culture, not always matched with the CR binding activity. Indeed the peptide aged in PBS alone and not aged showed to be equally toxic, while glycolaldehyde induced aggregation did not produce toxic aggregates. However the pattern of aggregation with ribose and Cu^{2+} fully matched the toxicity. The peptide toxicity was prevented by the anti-RAGE antibody, carnosine and threalose. The same level of protection was obtained by the addition of iNOS inhibitor (NMMA). The not aged Aβ 25-35 is already toxic, therefore, it could be speculated that compounds able to interact with Aβ 25-35 might interfere with the folding affecting differently the CR binding activity and the toxic conformation.

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INCREASED LEVELS OF GM1 AND GM2 IN RAFTS FROM ALZHEIMER BRAINS

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Lipid rafts are functional domains in the cells plasma membrane as well as intracellular. Disturbances in molecular composition, cellular distribution and/or formation of lipid domains might have implications in pathophysiological events. In fact, rafts have been indicated to be of relevance for Alzheimers disease. In this study, frontal and temporal brain tissue from Alzheimer disease was investigated and compared to age matched control brains. Analysis of ganglioside composition in detergent resistant membranes (DRMs) revealed that GM1 and GM2 were significantly elevated (2-fold and 4-fold respectively) in rafts from frontal as well as temporal Alzheimer brains, compared to control material. Immunohistochemical studies with confocal microscopy showed that these alterations were restricted to certain cell types of the brain. Lipid rafts might thus strongly be considered in the pathology of Alzheimers disease.

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PHARMACOKINETIC ANALYSIS OF BBB TRANSPORT OF ^{125}I-Aβ40 IN WT/AD TRANSGENIC MICE AND ITS IMPLICATION FOR AMYLOID PLAQUE FORMATION

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Amyloid plaques are formed in the extracellular space of Alzheimer's disease (AD) brain due to the accumulation of amyloid β proteins (Aβ) such as Aβ 40. The relationship between Aβ 40 pharmacokinetics and its accumulation within and clearance from the brain in both wild type (WT) and AD transgenic mice (APP, PS1) was studied to understand the mechanism of amyloid plaque formation and the potential use of Aβ 40 as a probe to target and detect amyloid plaques. In both WT and APP,PS1 mice, the ^{125}I- Aβ 40 tracer exhibited bi-exponential disposition in plasma with very short first and second phase half-lives. The ^{125}I-Aβ 40 was significantly metabolized in the liver >>> kidney > spleen. Co-administration of exogenous Aβ 40 inhibited the plasma clearance and the uptake of ^{125}I-β 40 at the BBB in WT animals but did not affect its elimination from the brain. The ^{125}I-Aβ 40 was shown to be metabolized within and effluxed from the brain parenchyma. The rate of efflux from APP, PS1 brain slices was substantially lower compared to WT brain slices. Since the Aβ 40 receptor at the BBB can be easily saturated, the blood-to-brain transport of Aβ 40 is less likely to be a primary contributor to the amyloid plaque formation in APP, PS1 mice. The decreased elimination of Aβ 40 from the brain is most likely responsible for the amyloid plaque formation in the brain of APP, PS1 mice. Furthermore, inadequate targeting of Aβ 40 to amyloid plaques despite its high BBB permeability is due to the saturability of Aβ 40 transporter at the BBB and its metabolism and efflux from the brain.

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THE HMG-CoA REDUCTASE INHIBITOR PRAVASTATIN INCREASES NEURITE OUTGROWTH IN CULTURED RAT HIPPOCAMPAL NEURONS

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Epidemiological studies demonstrate a relationship between treatment with statins (HMG-CoA reductase inhibitors) and a reduced risk of developing Alzheimer's disease. However, reduction in cholesterol levels does not reliably correlate with disease development, which suggests that statins may exert their beneficial effect via a cholesterol-independent mechanism. To determine whether statins affect the development of neurons in the hippocampus, we treated cultured rat hippocampal neurons with pravastatin, a potent HMG-CoA reductase inhibitor. After 24 h, pravastatin treatment significantly increased the number of neurites produced by each cell, and caused a corresponding increase in the levels of the membrane phospholipid phosphatidylcholine (PC). Pravastatin treatment also significantly increased neurite length and branching. Co-incubation with cholesterol did not inhibit the stimulatory effect of pravastatin on neurite outgrowth; by contrast, co-incubation with mevalonate abolished the effect. The small GTPase RhoA is known to be involved in regulation of neurite formation. Neurons treated with pravastatin for 24 hours contained significantly lower RhoA levels, compared to levels in control cells. Co-incubation with cholesterol did not prevent the reduction in RhoA levels caused by pravastatin, whereas geranylgeranylpyrophosphate, farnesylpyrophosphate and mevalonate prevented this effect. These data suggest that pravastatin treatment increases neurite outgrowth and may do so via inhibition of RhoA.

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NEW SYNTHETIC ALPHA-CONOTOXINS FOR CHARACTERIZATION OF NICOTINIC ACETYLCHOLINE RECEPTORS

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Alzheimer's and Parkinson's diseases are accompanied by impairment of cholinergic transmission and by a decrease in certain neuronal nicotinic acetylcholine receptors (AChR). To distinguish particular AChRs, α-conotoxins (Ctx) are widely used. To obtain more selective and potent Ctx, we synthesized analogs blocking muscle-type or neuronal AChRs. The uncharged or negatively charged residues in α-conotoxins GI, MI, SI, SIA and PnIA were substituted for Lys or Arg. The analogs were tested in binding to *Torpedo* AChR or to acetylcholine-binding proteins (AChBP) from *Lymnaea stagnalis* and *Aplysia californica* snails, current models for the extracellular domains of AChRs. The incorporation of positive charge in position 12 of Ctx acting on muscle-type AChRs considerably increased their affinity for *Torpedo* AChR. Similar D14K substitution in the Ctx PnIA(A10L), targeting neuronal α7 AChR, increased several-fold its affinity for this

receptor and 10-fold for *Lymnaea*, but not for *Aplysia* AChBP. Against *Lymnaea* AChBP, D12K-analog of Ctx SIA was equipotent to Ctx ImI targeting α7 AChR. With lower affinity both AChBP bound Ctx specific for neuronal α3β2- or α3β4 AChR, suggesting that a structure of AChBP complex with a Ctx is appropriate for modeling the antagonist-binding sites in diverse AChRs.

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SEROTONIN DYSFUNCTION AT EARLY DEVELOPMENTAL STAGES OF DOWN SYNDROME

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Down syndrome (DS), trisomy of chromosome 21, is the most common genetic cause of mental retardation that affects approximately 1 in 700 live births and culminates in a variety of mental and physical dysfunctions including an absolute predisposition to Alzheimer's disease (AD). It is known that adult DS patients show signs of an altered serotonergic system. Serotonin dysfunction has been observed in adult DS brains which exhibit AD as there is significant reduction in serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) concentration in areas including frontal cortex, temporal cortex, hippocampus, caudate putamen. It is not clear when the disruption of the serotonergic system occurs, as information on the fetal brain in DS is limited. To help address this lack of information concerning the serotonergic system in fetal DS we have analysed 5-HT, and its metabolite 5-HIAA, in brain tissue taken from the frontal cortex of male DS fetuses ($n = 6$) aged 17 to 24 weeks gestation versus age matched control brains ($n = 4$). Analysis was conducted using High Performance Liquid Chromatography coupled with electrochemical detection. Result of this analysis was significant reduction in 5-HT (41%, $P < 0.05$) concentration in DS samples and this was paralleled with decreased 5-HIAA (43%, $P < 0.05$) concentration compared to control fetal brains. The results suggest that significant 5-HT dysfunction is present already in the developing fetal brain which continues throughout life in DS patients.

Neurotoxicity, neuroprotection

P.76

CYTISINE AND DERIVATIVES PARTIALLY PREVENTS THE STRIATAL DOPAMINE DECREASE IN AN EXPERIMENTAL MODEL OF PARKINSON'S DISEASE

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There is a negative correlation between smoking and the appearance of Parkinson's disease. Several experimental evidences support the protective role of nicotinic acetylcholine receptors (nAchR) against a variety of insults in cells in culture and *in vivo*. Utilizing the intranigral 6-OHDA injection model of experimental parkinsonism we have demonstrated the protective effects of intermittent, subcutaneous nicotine administration against striatal dopamine decrease. To study the protective capacity of other nAChRs agonists on the dopaminergic terminals, the same experimental paradigm was applied in the case of cytisine and novel brominated derivatives. An intermittent administration of 2 mg/kg of cytisine and 1 mg/kg of 5-Br-cytisine, 4 h before and 20, 44 and 68 h after 6-OHDA, significantly prevented the striatal dopamine decline induced by the nigral lesion. The effect was not well correlated with the $\alpha 4\beta 2$ or $\alpha 7$ ligand binding potency and with the potency and efficacy to induce dopamine release in the corpus striatum.

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MALNUTRITION DOES NOT PREDISPOSE THE BRAIN TO DRUG NEUROTOXICITY

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Noting that a large percentage of the World's population is malnourished and given to various debilitating diseases, which in most cases are treated with drugs that have neurotoxic side-effects, it becomes necessary to verify whether malnutrition itself protects the brain against drug neurotoxicity. This study utilized adult male mouse that were subjected to protein-malnourished (3% casein) diet right from birth. The mothers were given malnourished diet from pregnancy until weaning; thereafter the mice continued with the malnourished diet until adulthood. They were divided into 4 groups; each group received 10 mg/kg/mouse of either pilocarpine or 3 nitropropionic acid or D-glutamic acid, or methamphetamine administered intraperitoneally once and left for 7 days on the malnourished diet before sacrifice. Brains were removed and frozen sections of the cerebral cortex and hippocampus were stained by Nissl method. Control group received normal mouse chow (20% protein) and were subjected to all other treatments as the malnourished ones. Examination of the Nissl-stained sections did not reveal any significant difference in the brain of the malnourished compared to the control; suggesting that brain is capable of protecting itself against these neurotoxins even in the presence of malnutrition.

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CARBONYL STRESS AND NMDA RECEPTOR ACTIVATION CONTRIBUTE TO METHYLGLYOXAL NEUROTOXICITY

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The present study extends previous studies on the metabolic effects of AGEs in human brain and investigates the mechanism of methylglyoxal (MG) toxicity in human SH-SY5Y neuroblastoma cells. Short-term exposure of SH-SY5Y cells to MG was associated with an early depolarization of the plasma membrane, glutamate release and formation of reactive oxygen species (ROS). In addition, long-term exposure (24 h) of SH-SY5Y cells to MG caused a decrease in cell viability, intracellular ATP as well as in rhodamine-123 (Rh-123) fluorescence. ATP depletion and the decrease in Rh-123 fluorescence were prevented by carbonyl scavengers, the nitric oxide synthase (NOS) inhibitor L-NAME, and by N-methyl-D-aspartate (NMDA) receptor antagonists at very low concentrations. β-estradiol counteracted MG-induced ATP depletion, with an increase in lactate production in contrast to the other compounds investigated. Furthermore, MG-induced glutamate release and the loss in cell viability were prevented by NMDA receptor antagonists. Therefore, MG renders cells more vulnerable to excitotoxicity. In conclusion, carbonyl scavengers as well as NMDA receptor antagonists may represent effective therapeutic tools to reduce the risk of pathophysiological changes associated with carbonyl stress in neurodegenerative diseases.

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HYPERCAPNIC ACIDOSIS FOLLOWING HYPOXIA IN NEWBORN PIGLET, A POTENTIAL NEUROPROTECTANT?

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Perinatal asphyxia occurs in approximately 4 per 1000 live births each year in Australia and can lead to permanent neurological impairment and in some cases result in infant death. A newborn piglet model of asphyxia has been developed in our laboratory to study the effects of hypoxia-ischemia in the brain [Foster KA, *et al.* (2001) *Brain Research* 919:122–131]. The aims of this project are to 1) determine whether hypercapnic acidosis reduces hypoxic-ischemic neuropathology in newborn piglets and 2) establish a timeline of events in the cell injury

cascade to better understand the 'window' in which this and other therapies may be applied. One-day old piglets were anaesthetised and hypoxia induced by decreasing inspired O_2 to 4% and maintaining the EEG amplitude <5 μV for 30 minutes. Hypercapnia was induced immediately post-insult for 1 h to maintain acidosis; end tidal CO_2 60–70 mmHg. Piglets were euthanased at 1, 8, 24 and 72 h post-insult. H&E staining, MAP2 and activated Caspase-3 IHC are currently being graded for neuronal injury by a blinded observer. Preliminary analysis of MDA levels in brain tissue from hypoxic animals shows an increase of 21% of baseline at 1 h post insult, increasing to 49% of baseline at 8 h post-insult. At 24 h MDA levels returned to baseline and at 72 h, levels had decreased to 68% below baseline. Plasma MDA levels responded in a similar manner with an increase of 130% and 102% at 1 and 8 h post-insult respectively. At 24 h levels had to returned to baseline however unlike brain tissue, plasma MDA remained at baseline level 72 h post-insult.

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NMDA PRECONDITIONING PROTECTS MICE HIPPOCAMPAL AGAINST NECROTIC CELLULAR DEATH INDUCED BY QUINOLINIC ACID

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NMDA preconditioning has been used to prevent cellular death induced by glutamate or NMDA in cultured cells. Quinolinic acid (QA)-induced seizures is used to evaluate NMDA receptor-evoked neurotoxicity in animal models. NMDA preconditioning protects mice against seizures and hippocampal damage induced by QA. The purpose of this study was to elucidate the participation of intracellular proteins associated with neuroprotection or cellular death in the NMDA preconditioning mechanism. The activation of caspase-3, DNA fragmentation and expression of HSP-27 were evaluated. Mice were pretreated with non-convulsant doses of NMDA for different times before i.c.v. QA infusion and observed for the occurrence of seizures. QA induced cellular damage in the hippocampus 4 hours after animals displayed seizures and it was maintained up to 72 hours. The cortical and hippocampal samples from mice treated with QA or NMDA preconditioning plus QA were assayed to DNA fragmentation. DNA laddering formation was not observed. The subfields CA1, CA3 and DG of hippocampal slices from mice were obtained. Mice preconditioned with NMDA and infused with QA without seizures behavior, showed no caspase-3 activation in the subfield CA1 24 hours after treatment. In these animals, an increased expression of HSP-27 was observed. These results suggest that i.c.v. QA infusion on mice lead to necrotic cellular death. NMDA preconditioning protects cellular damage and seizures induced by QA with involvement of HSP-27 in the hippocampus.

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NEUROPROTECTIVE EFFECTS OF 17B-ESTRADIOL ON SPINAL CORD INJURY IN YOUNG AND OLD RATS

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There is growing evidence that supports a role of estradiol in the nervous system during development and repair throughout life. Estradiol is now being studied as a neuroprotective agent in brain injury and disease but has only been preliminary examined in spinal cord injury (SCI). Also, there are no studies about the effect of estradiol on spinal cord injury in the postmenopausal period. The objective of this study is to evaluate the effect of estradiol pretreatment on motor function and neuronal death after SCI in young and aged rats. One week prior SCI, female young and middle-aged rats were ovariectomized and implanted with a silastic capsule containing either estradiol (180 μg/ml) or vehicle (sesame oil). Subsequently, a 3-seconds crushed injury at Th-8 was produced. Behavioral analysis was conducted at 3, 7, 14 and 21 days post-injury via BBB. Spinal cords were collected and processed for histology. Results demonstrated that estradiol protected motor neurons and decreased apoptotic cell death in both young and old rats. Normal estradiol level in young non-ovariectomized rats protected motor neurons and decreased apoptosis but not in non-ovariectomized aged rats. Behavioral testing indicated that hind limb motor function was improved in rats treated with estradiol in both young and old rats more than vehicle treated rats. In non-ovariectomized rats, locomotion was improved in the young, but not the post-menopausal rats. This suggests that the native neuroprotection seen in young rats is lost in post-menopausal rats. Importantly, exogenous application of estradiol in post-menopausal rats was protective.

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THE INHIBITORY EFFECT OF MELATONIN ON ENDOTHELIAL NITRIC OXIDE SYNTHASE IN BOVINE CEREBRAL ARTERIES

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In the present study, we have identified the existence of melatonin receptors and the function of melatonin on endothelial nitric oxide synthase (eNOS) expression in bovine cerebral arteries. The results indicated that mt1A melatonin receptor mRNA was expressed in bovine cerebral arteries. The relative levels of mt1A melatonin receptor mRNA expression in anterior, posterior, middle and vertebral cerebral arteries were examined. The data showed the highest and lowest levels of mRNA expressions in the middle and vertebral cerebral arteries, respectively. The melatonin receptor subtypes in different regions of cerebral arteries were identified and further characterized by using selective 2-[125I] iodomelatonin binding assay. Saturation studies revealed that the binding represented a single site of high affinity binding for the melatonin receptor. In addition, hydrogen peroxide induced induction in eNOS protein level has been demonstrated in the bovine isolated cerebral arteries and the said effect was abolished by melatonin. This is the first evidence showing data of the expression of mt1A melatonin receptor and its function in the bovine cerebral arteries. However, further studies are necessary to understand the significant role of melatonin and its receptors in regulating physiological function.

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SUSTAINED CALPAIN BUT NOT CASPASE ACTIVATION DURING GLUTAMATE-INDUCED LESIONS IN RATS TREATED WITH THE MITOCHONDRIAL TOXIN 3-NP

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During the last years a close relation between the energy state of the cell and glutamate neurotoxicity has been demonstrated. It has been shown that glutamate-toxicity threshold is reduced when energy metabolism is impaired. Exacerbated neuronal death during energy deficit is related to decreased ATP levels, mitochondrial dysfunction, loss of calcium homeostasis, and activation of different proteases. It is also suggested that caspase activation is related to an apoptotic phenotype of neuronal death, whereas calpain activation is related to a necrotic phenotype. In this report we aimed to study caspase and calpain activation during glutamate neuronal death when a previous mitochondrial inhibition exists. Results show that a subtoxic dose of injected 3-NP (i.p.) does not induce caspase nor calpain activation in the striatum of rats. In contrast, a subtoxic dose of glutamate injected intrastriatally induces a transient activation of calpain but not of caspases. On the other hand, exacerbated glutamate-induced lesion in the striatum of rats previously treated with 3-NP, is related to a large and sustained activation of calpain but not of caspases. Results suggest that sustained calpain activation is involved in the exacerbation of glutamate-induced lesions in rats previously treated with 3-NP, because glutamate alone only induces transient calpain activation without injuring the striatum. Results also suggest that glutamate-induced neuronal death during mitochondrial inhibition might show a necrotic phenotype.

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COMBINED ISONIAZIDE/RIFAMPICIN ADMINISTRATION IS NEUROTOXIC ON THE CEREBRAL CORTEX OF ADULT WISTAR RATS

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Combined Isoniazide (INH) and Rifampicin administration still remain as two of the main drugs of choice in the treatment regimes of tuberculosis (TB) world wide, particularly in the tropics. This study examined the effects of chronic administration of combined Isoniazide/Rifampicin on the cerebral cortices of adult rats. Adult wistar rats (average weight – 250 g) maintained under standard laboratory conditions were randomly grouped into treatment and control ($n = 8$). The treatment group A received combined INH + Rifampicin (50 mg/kg each, i.p) while the control group B received normal saline (0.5 ml, i.p.) only; daily at 1600 hrs for 15 days. Animals were sacrificed by cervical dislocation for whole brain weight determination ($n = 4$) and by whole body perfusion following anaesthesia for neurohistology with Nissl staining and immunohistochemistry for flourescent GFAP immunoreactivity and TUNEL method for the detection of apoptosis, ($n = 4$) from each group. The treatment group showed highly reactive intracortical and subpial GFAP positive astrocytes, mostly localized as clusters, compared to the control. There were large and widespread presence of pyknotic neurons, affecting cortical layers IV and V. However, no loss of cortical lamination was observed in the treatment group, compared to the controls. The treatment group showed intense darkly stained discrete cells, positive for the TUNEL signals. These microanatomical and microchemical changes suggest neurotoxicity of these drugs.

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ROLE OF NITRIC OXIDE SYNTHASE ISOFORMS ON KAINIC ACID-INDUCED CHOLINERGIC DAMAGE AND NO LEVELS IN RAT STRIATUM

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We studied the role of free radicals and of neuronal and inducible nitric oxide synthase activity (nNOS and iNOS) on the cholinergic neurotoxicity induced by kainic acid (KA 0.5μg). Selective inhibitors of nNOS and iNOS, 7nitroindazol (7NI, 25 mg/kg) and s-methylisotiourea (MIT, 15 mg/kg), and melatonin (MEL, 2.5 mg/kg), a scavenger of free radicals, were used. The compounds 7NI, MIT, MEL alone or in combination, were administered i.p., 20 min before, immediately and 1, 2 and 3 hr after the intrastriatal injection of KA. After three days, the animals were sacrificed and the striatum dissected. Choline-acetyltransferase (CAT) activity was assessed radio-enzymaticaly and NO metabolites(NOX) (nitrates + nitrites) quantified using the Griess reaction. Histological changes were also evaluated. CAT activity was expressed in nmol/mg protein/hr, and NOX in pmol/mg tissue. 7NI increased, while MIT and MEL reduced KA-induced cholinergic damage. Intrastriatal injection of KA, per se, did not alter NOX basal levels; however KA administered with 7NI or MIT reduced ($20 \pm 9\%$ and $26 \pm 8\%$) and KA+MEL did not affect NOX levels. Interestingly, simultaneous administration of KA with MEL+MIT increased cholinergic neurotoxicity ($50 \pm 3\%$) and prevented the reduction of NOX induced by MIT. We conclude: 1)Inhibition of nNOS increases the cholinergic neurotoxicity induced by KA, suggesting that nNOS isoform protects the cholinergic neuron from KA. 2)The inhibition of iNOS protects against cholinergic damage, through the reduction of free radicals related to NO. 3)MEL interferes with MIT, possibly preventing the protective effect of MIT on NOX and thus on cholinergic activity.

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EARLY CATHEPSIN D ACTIVATION AND A ROS BURST CAUSE THE RAPID CEREBELLAR GRANULE NEURONS DEATH INDUCED BY DOXORUBICIN

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Neurons show a high sensitivity to antineoplasic agents and a large number of experimental evidences support that the cellular toxicity of doxorubicin is mediated by reactive oxygen species (ROS), [Barnabe *et al.* (2002) Free Radic. Biol. Med. 33, 266]. On these grounds, we have evaluated the possibility that doxorubicin can induce oxidative stress-mediated cerebellar granule neurons (CGN) death in culture. Rat CGN were grown in culture as indicated previously [Martin-Romero *et al.* (2002) J. Neurochem. 82, 705]. At 7 days in vitro the medium was replaced with serum-free medium supplemented with different concentrations of doxorubicin. Cell viability was measured at different times after the change of the medium, leading to an IC_{50} of 20 micromolar for doxorubicin-induced neuronal death. The CGN death induced by doxorubicin was not prevented by caspases inhibitors nor was accompanied by the internucleosomal DNA fragmentation characteristic of apoptosis nor by a rise of intracellular calcium. However, in parallel to CGN death doxorubicin induced a large intracellular ROS burst as monitored by dihydroethidium and dihydrodichlorofluorescein diac-

etate. Both, pepstatin A, the specific inhibitor of cathepsin D, and the lysosomal stabilizer guanabenz protected against doxorubicin-induced CGN death, and produced a decrease in the doxorubicin-induced intracellular ROS burst which correlated with the extent of protection against CGN death.

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BOTH NECROSIS AND APOPTOSIS ARE INVOLVED IN NEURONAL CELL DEATH IN CA2 SECTOR OF RAT HIPPOCAMPAL SLICE CULTURES AFTER BICUCULINE STIMULATION

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We recently reported that the CA2 is the most vulnerable sector to bicuculline (BiC) exposure in rat hippocampal slice cultures. To investigate the underlying mechanisms, we first asked whether apoptosis is involved in BiC induced neuronal cell death. If it is the case, we wanted to know what is the relationship between apoptosis and necrosis. To address above questions, the hippocampal slices were prepared from Wistar rats of postnatal 7–8 days and cultivated by the interface method for one week. In order to induce significant neuronal cell death, 20 μM roscovitine was added along with 6 μM BiC for 12–24 h. The neuronal cell death was evaluated by the viability marker propidium iodide (PI) and immuno-staining for active form of caspase-3. When the slices were exposed to BiC for 12 h, selective PI uptake was induced only in the CA2 sector. In the CA2, 64 ± 5.3% of the cells were both PI uptake positive and immuno-reactive to caspase-3 antibody, while 31 ± 2.8% were positive for PI uptake only and 4 ± 1.2% were positive for caspase-3 only. After 24 h, PI uptake could be observed also in other sectors, especially the CA3 sector. PI uptake in the CA2 sector was significantly increased but the percentage of cells positive for both PI uptake and caspase-3 was decreased to 38 ± 6.1%. These results suggest that both necrosis and apoptosis are involved in BiC-induced neuronal cell death in hippocampal slice cultures with more significant role played by necrosis pathway.

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DIFFERENTIAL ROLE OF EXCITOTOXICITY AND OXIDATIVE STRESS IN CELL DEATH INDUCED DURING GLYCOLYSIS INHIBITION IN CULTURED NEURONS

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Oxidative stress plays an important role in different pathological conditions, and its contribution to cell death has been implicated in ischemia and neurodegenerative diseases. It has been demonstrated that the influx of calcium through NMDA receptor stimulates the production of reactive oxygen species (ROS). Treatment with antioxidants prevents the damage induced by glutamate. In the present study we investigated the effect of iodoacetate (IOA), an inhibitor of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, on cell death and ROS production. In addition, we studied the effect of vitamin E and MK-801, an antagonist of NMDA receptor, on cell death and ROS generation induced by IOA. Our results show that incubation of cultured hippocampal neurons with IOA (50, 100 μM) induced 50 and 90% decrease, respectively, in cell survival at 24 h. Neuronal death induced by moderate glycolytic inhibition was prevented by both vitamin E and MK-801, while neuronal death induced during acute glycolytic inhibition was only significantly prevented by vitamin E. IOA exposure increases of ROS production, assessed by fluorogenic markers, which depends on the exposure time and IOA concentration. Vitamin E partially prevents ROS generation. Results suggest that neuronal damage induced during moderate or acute glycolysis inhibition might involve different mechanisms depending on the intensity of the energy deficit. During moderate glycolysis inhibition an excitotoxic component is involved, while during acute inhibition oxidative stress might play a major role.

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AMPHETAMINE (AMPH) EVOKES NEUROTOXICITY IN STRIATUM (ST) AND PREFRONTAL CORTEX (PFC) VIA M1/M4 ACETYLCHOLINE RECEPTORS (AChRS)

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Oxidative stress has often been implicated in dysfunctional states and neurodegeneration in ST and PFC. In the rat model of acute AMPH neurotoxicity (5 mg/kg ip; 3x in time intervals of 2 h) an increase of striatal ACh is associated with elevated nitric oxide (NO) synthesis and raised generation of lipid peroxidation (LPO) in PFC and ST. The interrelationship of these processes is not well understood. In our study, the rise in NO (determined by electron paramagnetic resonance) and LPO production (determined from thiobarbituric acid reactive substances) by AMPH was prevented in PFC and ST by M1/M4 AChR blockade with pirenzepine (30 μg icv) and/or pre-treatment of the ST with microinjected MT7 (2 μg), a selective and irreversible M1 antagonist. Activation of M1/M4 AChRs with McNA-343 (200 μg icv) enhanced generation of NO by about 70% and LPO about twofold in the striatal and cortical tissue. The increases in these parameters of oxidative stress were prevented by pirenzepine (30 μg), L-nitroarginine (100 mg/kg ip) and quinacrine (40 mg/kg ip). These results indicate that striatal M1/M4 AChRs stimulate NO synthase and phospholipase A2 (PLA2), inducing oxidative stress leading to phospholipide breakdown. M1 AChRs via NO synthesis and PLA2 activation play an important role in the neurotoxic effect of AMPH in ST and PFC. Probably the hypercholinergic state induced by AMPH leads to the M1 AChR activation. The described neuronal damage-promoting pathway may be of importance in neurological disorders such as dementia, Parkinson's disease and addiction.

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ADULT EFFECTS OF PRENATAL EXPOSURE OF LINDANE ON CEREBRAL AND HEPATIC CYTOCHROME P450s

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The consequences of prenatal exposure to environmental chemicals could be enormous owing to the limited xenobiotic metabolizing capabilities of the developing fetus and neonate. Studies were initiated to investigate whether low-level exposure to pesticides such as lindane, an organochlorine pesticide, has the potential to affect the ontogeny of xenobiotic metabolizing Cytochrome P450s (CYPs) in the offsprings and predispose the offsprings to the toxic effects of environmental chemicals. Prenatal exposure to, 0.0625-, or 0.125- or 0.25 mg/kg (1/720th, 1/480th and 1/240th of LD50) of lindane was found to produce a dose dependent increase in the expression and activity of

CYP1A1, 1A2, 2B1, 2B2 and 2E1 in brain and liver of the exposed offsprings at postnatal day (pnd) 21, as determined by RT-PCR, western blotting and enzymatic studies. Interestingly, the effects were found to persist upto pnd 60. This increase in the expression of CYPs was found to be associated with altered spontaneous locomotor activity of the exposed offsprings especially at pnd 21, where a significant increase in distance traveled, ambulatory time, stereotypic time and horizontal count and a decrease in resting time was observed. Furthermore, when the offsprings (= 6 wks old) prenatally exposed to lindane (0.25 mg/kg b.wt., p.o. to mother) were subsequently challenged with a single dose (30 mg/kg b.wt.) of lindane, it lead to the worsening of the toxicity symptoms. While the onset of convulsions was decreased, the incidence was increased indicating higher vulnerability of the offsprings in the prenatally lindane exposed group.

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17β-ESTRADIOL REDUCES NEURONAL DEGENERATION FOLLOWING TRAUMATIC BRAIN INJURY IN RATS

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17β-estradiol (E2) has shown neuroprotective activity in several models of neuronal injury, but few studies have examined neuroprotective activity of E2 following traumatic brain injury (TBI). Therefore, this study was carried out to investigate effects of treatment with exogenous E2 on neuronal degeneration caused by lateral fluid percussion TBI. Female Sprague Dawley rats were divided into 3 groups; ovariectomy with E2 (OVX+E2), ovariectomy with oil vehicle (OVX), and non-ovariectomized controls (nonOVX). Ovariectomy and implantation (s.c.) of silastic pumps filled with oil or E2 (1 mg/ml) were performed 1 wk before TBI. Degenerating neurons were detected in cortex and hippocampus (CA2–3) by Fluoro-Jade (FJ) staining 24 hour after TBI, and quantified stereologically. Group OVX+E2 showed fewer numbers and lower densities ($P < 0.05$) of FJ-positive neurons in CA2–3 of hippocampus compared to the OVX, but not the nonOVX group. The OVX+E2 group also had fewer FJ-positive neurons in cortex than the OVX ($P < 0.01$) and nonOVX ($P < 0.05$) groups, as well as a lower density of FJ positive neurons compared to the nonOVX group ($P < 0.05$). There was also significant decrease in the cortical lesion volume of OVX+E2 rats compared to the OVX, but not the nonOVX group. No significant differences in neuronal numbers, density or lesion volume were found between the OVX and nonOVX groups. Treatment with exogenous E2 prior to TBI reduced neuronal degeneration in both cortex and hippocampus 24 hr after brain injury. Neuronal degeneration in nonOVX rats was similar to that in OVX rats, suggesting that endogenous levels of E2 may not be neuroprotective in TBI.

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GLUTATHIONE PEROXIDASE-1 CONTRIBUTES TO THE RAPID CLEARANCE, AND PROTECTS AGAINST THE IRON-MEDIATED TOXICITY, OF HYDROGEN PEROXIDE

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Glutathione peroxidase-1 (GPx1) is a key enzyme in the antioxidant defence of the brain. To test for the role of GPx1 in peroxide disposal and protection of astrocytes from oxidative stress, we used primary astrocyte cultures from neonatal GPx1-deficient [GPx1(–/–)] or wild type mice. The cells were treated with 100 μM hydrogen peroxide (H_2O_2), and the rate of H_2O_2 clearance and accumulation of oxidised glutathione was measured during peroxide exposure. The rate of peroxide clearance was significantly retarded in GPx1(–/–) astrocytes compared to wild type cells. H_2O_2 disposal in GPx1-deficient astrocytes was strongly impaired by the catalase inhibitor 3-amino triazole (3AT), whereas in wild type cells, 3AT was only effective when the glutathione content of the cells was lowered by preincubation with buthionine sulfoxime (BSO). In contrast to wild type astrocytes, GPx1(–/–) cells did not exhibit a rapid and transient accumulation of oxidised glutathione following application of H_2O_2. While there was not a substantial loss of viability in wild type astrocytes for up to 24 h, about 50% of GPx1(–/–) cells died within 8 h of H_2O_2 application. The viability of both types of astrocytes was strongly compromised by pre-treatment with 3AT and/or BSO. This cell death was almost completely prevented by iron chelators, whereas pre-incubation with FAC increased and accelerated H_2O_2 toxicity. These data demonstrate that GPx1 contributes to the rapid clearance of H_2O_2 and promotes the high resistance of astrocytes to iron-mediated oxidative damage.

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ASPIRIN AND PARACETAMOL PROTECTS AGAINST MPP+-INDUCED MITOCHONDRIAL NEUROTOXICITY

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The cellular damage caused by 1-methyl-4-phenyl pyridinium (MPP+) is primarily the result of the mitochondrial respiratory inhibition at the level of complex I and a reduction in cellular antioxidant mechanisms such as the enzymatic defense system ie. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the antioxidant molecule, glutathione (GSH). The effect of non-narcotic analgesics, paracetamol and aspirin on mitochondrial complex I and electron transport chain activity was investigated, *in vivo*, in rats treated intranigrally with MPP+. Furthermore the effects of these non-narcotic analgesics was also investigated in SOD, CAT, GPx activity and GSH levels in similar treated animals. Intranigral infusion of MPP+ in rats caused severe inhibition of mitochondrial complex I activity and electron transport chain activity which was reversed by systemic post-treatment of rats with aspirin, paracetamol and the combination of aspirin and paracetamol. Furthermore the effect of MPP+ on SOD, CAT and GPx activity was significantly altered by aspirin and paracetamol. The MPP+-induced reduction in GSH levels was significantly attenuated by the administration of these non-narcotic analgesics. While these findings suggest usefulness of non-narcotic analgesics in neuroprotective therapy in neurodegenerative diseases, aspirin appears to be a potential candidate in prophylactic as well as in adjuvant therapy in Parkinson's disease.

Keywords: Non-narcotic analgesics; aspirin; Paracetamol; mitochondria, complex I, electron tranport chain and Parkinson's Disease.

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N-ACETYLCYSTEINE, EBSELEN AND NICOTINE PROTECTS NEURONS EXPOSED TO ARACHIDONIC ACID IN SIMULATED ISCHEMIC CONDITIONS

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Ischemic processes in central nervous system disorders (as stroke or trauma) are responsible for delayed and amplified injury, e.g. through release of free fatty acids, in particular arachidonic acid. Arachidonic

acid and its metabolites have neurotoxic effects related to the oxidative mechanisms. We studied N-acetylcysteine, ebselen and nicotine effects on arachidonic acid (AA), 4-hydroxynonenal (HNE) and menadion (free radical precursor) toxicity in cultured neurons in simulated in vitro ischemic conditions. Rat cortical and cerebellar granule neurons, maintained in supplemented Neurobasal medium, were exposed to simulated ischemia (37°C, 3% O_2, 5% CO_2 with simultaneous glucose deprivation). Some cultures were also exposed to AA, HNE or menadion. Exposure of neurons to simulated ischemia caused a dramatic loss of cellular viability, exaggerated by co-exposure with arachidonic acid, 4-hydroxynonenal or menadion. Their neurotoxicity was attenuated by pretreatment with nicotine, N-acetylcysteine and ebselen. Apoptosis rate followed the similar pattern. Simulated ischemia increased also oxidative stress; the effect was potentiated by co-exposure with arachidonic acid and prevented by nicotine, N-acetylcysteine and ebselen. Glutathione concentration in neurons exposed to arachidonic acid was elevated by N-acetylcysteine and nicotine pretreatment. The results suggest that activation of nicotinic receptors, increase in intracellular thiol level and strengthening of intrinsic antioxidative mechanisms can protect against ischemia and/or arachidonic acid-mediated injury to neurons.

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CALRETININ-IMMUNOREACTIVE HIPPOCAMPAL NEURONS ARE NOT AFFECTED IN TMT-TREATED CULTURES

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Trimethyltin (TMT) is a toxicant inducing neurodegenerative processes, selectively localized in the limbic system and accompanied by behavioural alterations, including cognitive deficits [Balaban CD *et al.* 1988. Neuroscience 26:337–361; Ishida N *et al.* 1997. Neuroscience 81:1183–1191]. Hippocampal neuronal subpopulations expressing the calcium-binding protein calretinin (CR) are selectively spared by TMT-induced neurodegeneration *in vivo* [Geloso MC *et al.* 1997. Exp Neurol 146:67–73; Geloso MC *et al.* 1998. Exp Neurol 154:645–653]. In order to investigate whether the resistance of CR-containing neurons is related to connectivity and/or hippocampal microenvironment or to their intrinsic neurochemical features, we investigated the possible resistance of CR-containing neurons in TMT-treated primary hippocampal neuron cultures. Cell cultures were affected by treatment in a dose-dependent manner. Cell counts also showed that CR-immunostained neurons were spared by TMT treatment. CR expression at transcriptional level was also evaluated by semiquantitative RT-PCR analysis using neuron-specific enolase (NSE) as reference gene. Densitometric analysis showed that an active CR transcription occurs during TMT-induced neurodegeneration, with an increased CR/NSE expression ratio in treated cultures at low TMT concentration. The present data demonstrate that the resistance of CR-containing cells after TMT-treatment is due to the peculiar neurochemical features of these cells (possibly including CR-dependent calcium-buffering action to counteract calcium overload accompanying neuronal death), rather than to connectivity or hippocampal microenvironment.

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EFFECTS OF ALUMINIUM ON 6-HYDROXYDOPAMINE-INDUCED OXIDATIVE STRESS IN MITOCHONDRIA: CONTRIBUTIONS OF ASCORBATE AND GLUTATHIONE

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Aluminium (Al^{3+}) has been proposed as an etiological factor in Parkinson's disease and its ability to promote oxidative stress also reported. Assuming that 6-hydroxydopamine (6-OHDA) causes neurodegeneration by oxidative damage induced by its autoxidation, our study investigates the potential of Al^{3+} to modify oxidative stress caused by 6-OHDA autoxidation and the contribution of ascorbate (Asc) and glutathione (GSH), using crude mitochondrial preparations from rat brain. The determination of TBARS was used as an index of lipid peroxidation and the protein thiol content as an index of protein oxidation. Our results show that Al^{3+} has no effect on lipid peroxidation induced by 6-OHDA. However, the presence of Asc increases the lipid peroxidation induced by 6-OHDA, but this effect is reduced by the presence of Al^{3+}. GSH does not affect lipid peroxidation induced by 6-OHDA, an effect which is not modified by the presence of Al^{3+}. A reduction in protein oxidation by 6-OHDA was observed with the addition of Al^{3+}. Although, the presence of Asc caused an increase in protein oxidation by 6-OHDA, the presence of Al^{3+} provoked a reduction of this effect. The addition of GSH induced a reduction in protein oxidation by 6-OHDA which was exacerbated by the presence of Al^{3+}. We conclude that Al^{3+} have a marked antioxidant effect in the absence of an initiator of the oxidative process.

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EVALUATION OF FLUOROGENIC SUBSTRATES FOR THE DETECTION OF MITOCHONDRIAL AND NEURONAL CALPAIN ACTIVITY

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Calpains are calcium-activated intracellular proteases that play roles in many signaling processes including apoptotic and necrotic cell death. Susceptibility to non-specific proteolysis and lack of cell permeability have hindered the use of fluorogenic substrates for the detection of calpain activity in intact cells. We constructed a small library of fluorescence resonance energy transfer (FRET)-based calpain substrates using known calpain cleavage sites, Cal Fluor Orange fluorescence donor, and BHQ-2 fluorescence quencher. Both commercial and custom fluorogenic calpain substrates were evaluated for the kinetic measurement of calpain activity in isolated brain mitochondria and primary hippocampal neurons (11–14 days in vitro). A novel proteolytic activity was detected in the intermembrane space of brain mitochondria that was inhibited by two chemically distinct calpain inhibitors (calpeptin and MDL28170) but not by calcium chelation. Non-specific proteolysis precluded detection of this activity by two of the commercial substrates. Enhanced proteolysis of a cell permeable fluorogenic calpain substrate following activation of calcium-conducting N-methyl D-aspartate (NMDA) receptors was detected in live hippocampal neurons by single cell imaging. Simultaneous measurement of delayed calcium deregulation in individual neurons using the calcium indicator Fluo-4FF revealed a further augmention of proteolysis coinciding with

loss of calcium homeostasis. Calpain inhibitors are being tested to examine the specificity of the enhancement in substrate hydrolysis. Further refinement of synthetic calpain substrates should yield a viable method for the measurement of intraneuronal calpain activity.

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INTRINSIC TISSUE TOLERANCE IN ARCTIC GROUND SQUIRREL HIPPOCAMPAL SLICES

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Hibernating Arctic Ground Squirrel (AGS), *S.parryii*, survive profound decreases in blood flow, heart rate, respiratory rate, and cerebral perfusion during torpor, and reperfusion during intermittent rewarming periods without neurological damage. Investigation of neuroprotection in hibernating animals has shown pronounced tolerance to traumatic brain injury *in vivo* (Zhou *et al.*, 2001). Tolerance to oxygen and glucose deprivation has also been observed *in vitro* in acute hippocampal slices from a hibernating species (Frerichs *et al.*, 1998). In the latter, however, it is unclear if neuroprotection results from intrinsic tissue properties or from differences in response to acute trauma associated with slice preparation. The goal of this work was therefore to investigate neuroprotection from ischemia in AGS using chronic hippocampal slices to determine whether the hibernation phenotype, characterized by tolerance to an *in vitro* model of ischemia-reperfusion (I/R), persists in culture. A second goal was to address NMDA receptor involvement and channel arrest as potential mechanisms of intrinsic tissue tolerance. Slices from juvenile, hibernating AGS (hAGS) and interbout euthermic AGS (ibeAGS) were cultured at 37°C using organotypic inserts. CA1 cell death was quantified with propidium iodide. Early in culture (onset of insult at 3 h) both hAGS and ibeAGS tolerated I/R (4 h/20 h) and 500 μM NMDA plus KCl. Later in culture (onset of insult at 24 h), this tolerance persisted in hAGS but was lost in ibeAGS. Ouabain (Na,K-ATPase inhibitor), administered at 24 h for 30 minutes, enhanced hAGS survival 24 hours later suggesting that hAGS preserve ion homeostasis without ATP expenditure. Tolerance to I/R in hAGS slices is thus due to intrinsic tissue properties involving NMDA receptors and ion channel arrest.

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ESTROGEN ATTENUATED Ca^{2+} INFLUX AND APOPTOSIS IN A SPINAL MOTONEURON CELL LINE EXPOSED TO GLUTAMATE

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VSC4.1 cells, a spinal motoneuron cell line, were used to study a mechanism of action for 17β-estradiol(EST)-mediated cytoprotection. Cells were divided into 4 groups: control, 100 nM EST for 30 h, 1 mM L-glutamic acid (LGA) for 24 h, and EST (6 h pretreatment)+24 h LGA cotreatment. Cell death was examined by Wright staining, TUNEL assay, and MTT assay. Treatment with 17α-estradiol was used to examine if cytoprotection was EST receptor (ER)-dependent, and BSA-conjugated-EST was used to examine the role of putative membrane ER. Calpain and caspase-3 activities were measured by Western blot analyses. Whole cell recording was performed to measure membrane potential and capacitance, and intracellular free Ca^{2+} was measured by Fura-2. Potential sites of Ca^{2+} entry were blocked with or without EST. LGA-induced cell death was attenuated with EST but not with 17α-estradiol or BSA-conjugated EST, indicating that protection is ER-dependent but not through membrane ER. EST attenuated both calpain and caspase-3 activities. Both membrane potential and capacitance were altered in LGA-treated cells but were restored with EST treatment. The L-type Ca^{2+} channel blocker nifedipine showed comparable cytoprotection in cells treated with LGA. To further characterize EST-mediated cytoprotection, cells were treated with a voltage gated Ca^{2+} channel (VGCC) agonist FPL-64176 (FPL). FPL caused comparable cell death and increases in intracellular Ca^{2+}, which were attenuated by estrogen. These results suggest that EST-mediated cytoprotection in VSC4.1 cells is ER-dependent and may be related to VGCC, preventing pathologic Ca^{2+} influx and downstream protease activation.

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NMDA- AND GLUTAMATE-INDUCED NEUROTOXICITY IN HIPPOCAMPAL SLICES ARE DIFFERENTIALLY MODULATED BY GMP

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Glutamate plays physiological roles in the nervous system by interacting to its receptors and can also cause cell death by necrosis or apoptosis when it is excessively released. Guanine derivatives exert relevant extracellular roles as modulation of glutamate neurotransmition. The mode of cell death induced by glutamate and NMDA in hippocampal slices and a putative neuroprotective effect evoked by GMP, an endogenous modulator of glutamate receptors, were evaluated. Hippocampal slices from young rats were incubated for 1 h with glutamate (1 and 10 mM) or its ionotropic receptor agonist, NMDA (100 μM), in the presence or absence of GMP (1 mM) or the NMDA antagonist, MK-801 (50 μM). After this insult, slices were incubated for 6 h in culture medium in order to evaluate cell viability by MTT reduction and for 18 h to analyze internucleossomal fragmentation of DNA. DNA was extracted and a 60 μg aliquot was submited to a 2% agarose gel eletrophoresis. Glutamate (1 or 10 mM) and NMDA decreased cell viability in 33%, 40% and 22% related to control, respectively. Such effect was inhibited by addition of MK-801. GMP did not alter the reduced cell viability induced by glutamate but blocked the decreased cell viability induced by NMDA. DNA fragmentation induced by glutamate was potentiated by GMP. However, GMP reduced DNA fragmentation induced by NMDA. There was no alteration in cell membrane permeability of hippocampal slices in all treatments. These results indicate glutamate and NMDA induced an apoptotic-like mode of cell death which is inhibited by MK-801. However, GMP protected against the damage caused by NMDA only, suggesting glutamate-induced hippocampal slices damage might occur via glutamate carriers.

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NEUROPROTECTION ASSOCIATED WITH ALTERNATIVE SPLICING OF NMDA RECEPTORS IN RAT CORTICAL NEURONS

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Exposure of cultured cortical neurons to elevated extracellular K^+ (25 mM) induces membrane depolarisation and an increase in action-potential firing. Long-term high K^+ treatment was associated with an increased neuronal cell death. In surviving neurons, multiple changes occurred in the proportion of individual NMDAR subunit 1 (NR1) splice variant mRNA expression, whereas the overall expression of NR1, NR2A and NR2B transcripts remained unaffected. The high K^+-induced changes in NR1 splice variant expression were abolished upon administration of tetrodotoxin (TTX 3 μM). In voltage-clamp recordings performed on neurons resistant to high K^+ treatment, inward currents induced by NMDA (1–1000 μM) were reduced. However, the NMDAR function was not affected in cultures to which high K^+ was concomitantly applied with either TTX or a NMDAR antagonist

(CGS19755 or memantine). In addition, in K^+-resistant cells, the activity of calpains and caspase-3 was diminished compared with controls. In conclusion, the present data indicate adaptive changes in NR1 splice variant expression and a decrease in NMDA receptor function upon a sustained increase in neurotransmission. Accordingly, alternative splicing could be an endogenous mechanism to counteract cellular damage due long-term activation of excitatory NMDAR and may be associated with an impairment of necrotic and apoptotic mechanisms.

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CREATINE KINASE LEVELS AS A PUTATIVE PERIPHERAL NEUROCHEMICAL MARKER OF NEUROLEPTICS-RELATED MYOTOXICITY

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Serum creatine kinase (SCK) levels may serve as a sensible indicator of the skeletal muscle damage. The purpose of this study was to estimate prospectively the SCK activity in psychiatric patients treated with typical (TN) and atypical neuroleptic agents (ANA) as a marker of their neuromuscular toxicity. We screened 590 adult patients who begun their treatment with TN and ANA. Patients with idiopathic hyper-CKemia, suffering from any significant physical disorder, receiving parenteral medication or ECT were excluded. Blood samples for CK determinations were collected at baseline and at weeks: +1, +2, +3, +4, +8, +12 and every 3 months thereafter, up to one year. Results: We recruited 244 eligible patients receiving clozapine ($n = 52$), olanzapine ($n = 54$), risperidone ($n = 55$), quetiapine ($n = 25$), haloperidol ($n = 23$) or perphenazine ($n = 35$). During the study, 1600 blood samples were collected and 11 evaluated patients, treated with clozapine ($n = 6$), olanzapine ($n = 3$), perphenazine ($n = 2$) were found having persistent hyper-CKemia – 545.5 ± 230.7 IU/L, in range 250–950 IU/L. Conclusions: Results of this prospective comparative study indicate that incidence of the persistent hyper-CKemia in our sample (4.5%) is compatible with previous reports. However, the magnitude of hyper-CKemia is less than reported previously. Majority of hyper-CKemic patients received ANA. Routine therapeutic monitoring of SCK levels in neuroleptics-treated psychiatric patients is warranted.

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PROTECTIVE EFFECTS OF PROLYL ENDOPEPTIDASE INHIBITORS IN ASTROCYTOMA CELL LINES AND RAT PRIMARY ASTROCYTES

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The neuroprotective effects of prolyl endopeptidase (PEP) inhibitors are well characterized in a number of experimental in vitro and *in vivo* models. However, several clinical conditions are associated with the death of astroyctes, whose functions are important for the maintenance of neurons. In order to demonstrate the effects of PEP inhibitors on the survival of astrocytes in stress paradigms, the astrocytoma cell lines U-343 and U-138MG as well as rat primary astrocytes were subjected to toxic stimuli by means of staurosporine-, LPS- or oncostatine M-treatment. These treatments resulted in the death of 30–70% of the astrocytes within a period of 24 hours as demonstrated by LDH release into the culture medium. Enzymatic activity assays and immuncytochemistry using an antibody against active caspase-3 revealed the contribution of caspase3/7 in astrocytic cell death under these experimental conditions. To reveal whether or not PEP inhibitors are protective in these in vitro paradigms, co-incubation experiments with the stressors mentioned above and three different PEP inhibitors of distinct chemical classes were performed. We observed a dose-dependent protection from the cytotoxicity for all PEP inhibitors used but with a different potency and kinetics. These data indicate that PEP inhibitors protect astrocytes from cell death induced by different insults. Based on the known functions of astrocytes in neuroprotection/repair it is tempting to speculates that the observed neuroprotective effects of PEP inhibitors in experimental lesion studies *in vivo* are contributed by enhanced astrocytic survival.

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CHANGES IN NITRIC OXIDE SYNTHASE AND PKC ACTIVITIES IN NEONATAL IONIZING RADIATION CEREBELLUM. PARTIAL NEUROPROTECTION BY A TREATMENT WITH 17-BETA-ESTRADIOL

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Exposure to ionizing radiation during neonatal period can generate plastic changes, mainly in highly immature organs, such as cerebellum (CE). PKC regulates a variety of intracellular and extracelullar signals in the developing brain suggesting that might be target of radiation damage. Estrogens promote growth, cellular survival and also prevent axonal pruning. Modulation of nitric oxide synthase (NOS) activity by PKC was just investigated in irradiated rat cerebellum. The aim of the present work was to evaluate motor, structural and biochemical changes induced by neonatal ionizing radiation and to find drugs which could improve changes observed in this neonatal model. Our results show motor alterations in irradiated animals (I) respect to control animals (C) in test of footprint [Control (C) = 0.39 ± 0.06 vs. I = 0.86 ± 0.07 cm], changes in the citoarchitecture of irradiated cerebellum and an increase in the activity of PKC (C = 103 ± 15; I = 305 ± 10 pmol/min/g). The activity of constitutive NOS (C = 1234 ± 9.68; I = 433 ± 4.32 pmol/citrul/g) was diminished and the activity of iNOS, in the long term, was increased (C = 29.8 ± 1.9 vs. I = 2824.1 ± 99.1). Pre-treatment with 17-estradiol restore some of those alterations; the motor syndrome was restored (I+17-estradiol = 0.42. ± 0.02 cm) and cytoarchitecture of irradiated and treated cerebellum resembled the control tissue. These findings show that 17-beta-estradiol exerts a partial neuroprotector effect suggesting that it could be used like adjuvant in neuroprotectant therapies.

Neurotransmitter receptors

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ADENOSINE A_{2A} RECEPTOR INTERACTS WITH THE NEURONAL Ca^{2+}-BINDING PROTEIN 2 (NECAB2)

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Heptaspanning-membrane or G protein-coupled receptors (GPCR) have been shown to be linked to intracellular proteins identified as regulators of receptor anchoring and signalling. Using a yeast two-hybrid screen, NECAB2 (Neuronal Ca^{2+}-Binding Protein 2), was identified as a binding partner for the C-terminal domain of adenosine A_{2A} receptor ($A_{2A}R$). NECAB2 belongs to a family of neuronal proteins that bind Ca^{2+} by means of EF-hand domains. This family is formed by three members that share three homology regions, namely a N-terminal sequence that contains an EF-hand domain, a central unique and highly conserved domain (NHR) and a C-terminal domain called ABP (Antibiotic Biosintesis Monooxygenase) with unknown function and only observed in bacteria. In mammalians, NECAB2 is expressed in brain and its exact physiological function remains elusive. This is the first report describing a partner for this protein. Colocalization, coimmunoprecipitation and pull-down experiments showed a close and specific interaction between $A_{2A}R$ and NECAB2 in transfected HEK-293 cells and also in rat striatal tissue. Also, the interaction of NECAB2 with $A_{2A}R$ was modulated by calcium. Depletion of calcium by means of EDTA increases the ability of NECAB2 to interact with $A_{2A}R$. Conversely, pull-down experiments indicated that the interaction was progressively weakened upon increasing calcium concentration. Interestingly, the interaction between $A_{2A}R$ and NECAB2 abrogated the constitutive activity exhibited by $A_{2A}R$. Overall, these results show that $A_{2A}R$ interacts with NECAB2 and that this interaction could be physiologically relevant for receptor function.

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INVOLVEMENT OF EXTRACELLULAR DOMAINS IN THE TRAFFICKING AND SURFACE EXPRESSION OF AMPA RECEPTORS

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Previous studies have identified a number of regions and motifs important for the trafficking of ionotropic glutamate receptors (GluR); these have been primarily in the intracellular carboxy domain. However, other regions have also been implicated. The AMPA receptor subunits all have an alternatively spliced region – the 'flip/flop' domain – within the S2 loop and part of the ligand-binding domain. This region has been shown to be important in the desensitisation kinetics of the receptor channel. In this study we show this region also has a significant effect upon the trafficking and surface expression of recombinant GluR-A and GluR-D receptors; the flip forms being strongly expressed and the flop forms poorly expressed on the plasma membrane. The difference in surface expression between the GluR-D flip homomeric receptor and the GluR-D flop homomeric receptor was not altered by incubation of transfected cells with either receptor antagonists or potentiators. Furthermore, the behaviour is mimicked by a secretable, soluble construct consisting of only the S1S2 ligand-binding domain. The construction and expression of a series of deletion and point mutants of the full-length GluR-D subunits identified a single position as responsible for this difference. This site, 780 in GluR-D and 772 in GluR-A, encodes a valine in flip and a leucine in flop. Moreover, it is outside the identified ligand-binding cleft and models suggest that its side-chain is directed away from the dimer interface. This relatively conservative difference between the flip and flop isoforms of GluR-D and -A is responsible for the striking difference in surface expression.

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EXTRACELLULAR TRAFFICKING OF NR2 PEPTIDE IN CEREBRAL ISCHEMIA

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Increasing evidence has suggested that receptors for excitatory amino acids play an important role during cerebral ischemia. In this study we monitored the trafficking of NR2A/2B peptide, N-terminal domain of the N-methyl-D-aspartate receptor (NMDAR), from brain to blood after transient cerebral ischemia. The secondary endpoint of the study was the time course of NR2A/2B mRNA expression in brain and NR2A/2B antibodies development in blood of stroke animals. Adult male Sprague-Dawley rats were subjected to a transient focal ischemia induced by right middle cerebral artery occlusion. We found that the ischemia upregulated NR2A/2B mRNA with peak expression at 24 hour after reperfusion in cerebral cortex and at 0 hours in hippocampus. The level of NR2A/2B mRNA expression gradually declined from 2 to 72 hours in hippocampus. In contrast to the expression of mRNA, the level of NR2A/2B protein, examined by Western blot, was reduced in the lesioned cortex and adjacent hippocampus at 0 and 24 hours. The extracellular trafficking of NR2 peptide due selective neuronal damage accompanied with decrease of the NMDAR distribution in the cortex at 0 h after reperfusion. In blood, the level of NR2A/2B peptide was significantly increased from 2 to 72 hours after stroke. In conclusion, our data suggest that cerebral ischemia induces early production of NR2A/2B peptide in bloodstream and may be used as a biomarker for acute brain ischemia.

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MAPPING AND HOMOLOGY-BASED MOLECULAR MODELING OF A BINDING MOTIF FOR PROTEIN PHOSPHATASE 1 IN METABOTROPIC GLUTAMATE RECEPTORS

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An important mechanism in the control of synaptic signal transduction is the modulation of neurotransmitter receptors by kinases and phosphatases. We identified a physical interaction between the gamma-isoforms of protein phosphatase 1C (PP1gamma) and the metabotropic glutamate receptors mGluR1a, mGluR5a, mGluR5b and mGluR7b. The interactions were verified in yeast cells and GST pull-down assays, using recombinant and native protein preparations. Applying confocal laser-scanning microscopy, PP1gamma was detected in dendrites of

retinal bipolar cells expressing the interacting glutamate receptors. Furthermore, we determined the structure of metabotropic glutamate receptors in complex with PP1gamma by homology-based molecular modeling. Combining computational with experimental techniques, we mapped interacting domains and identified two conserved amino acid motifs (KSVSW and KSVTW) in the intracellular C-termini of mGluR1a, mGluR5a, mGluR5b and mGluR7b. Analyzing the individual contribution of each position within this motif, we propose the consensus sequence [HKR][ACHKMNQRSTV][V][CHKNQRST][FW] for proteins binding to PP1gamma. To test the power of this pattern, we predicted and experimentally confirmed previously unknown PP1 interactors by database searches and GST pull-down analysis, including the ion channels ClC7 and TRPC5. Our results define a new phosphatase binding motif that is located in the intracellular C-termini of metabotropic glutamate receptors as well as in several other proteins.
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SELECTIVE ENRICHEMENT OF METABOTROPIC GLUTAMATE RECEPTORS IN THE INTERCALATED CELL MASSES OF THE AMYGDALA

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Metabotropic glutamate receptors (mGluRs) in the amygdala have been implicated in fear-related learning and memory. However, the distribution of these receptors in amygdala remains poorly defined. We have investigated the localization of mGluRs in this area at both light and electron microscopic level. Intense labeling for mGluR1a was observed postsynaptically in a subpopulation of GABAergic neurons in the intercalated cell masses (Im). Immunolabeling for mGluR1a was also observed in the basolateral nucleus (BL), in cortical nuclei and medial amygdaloid nucleus. Double immunofluorescence studies revealed partial colocalization between mGluR1a and calretinin in neurons of the BL, but not in the Im. No colocalization was detected with other calcium binding proteins, or with neuropeptides such as somatostatin, cholecystokinin and vasoactive intestinal polypeptide. Neurons immunopositive for mGluR1a in the Im were targeted by nerve terminals enriched in presynaptic group III mGluRs (mGluR4, 7a and 8a). We showed that neurons of the Im receive glutamatergic afferents enriched in the vesicular glutamate transporter 2 (VGluT2), but not VGluT1, as well as a dense GABAergic input. Recent evidences indicate that Im neurons gate impulse traffic from the prefrontal cortex and BL to the central nucleus and may be involved in the extinction of conditioned fear. Therefore, the marked enrichment of mGluRs in the Im at both pre- and postsynaptic sites might serve important functions in the synaptic integration implicated in the neural mechanisms of extinction.

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EXPRESSION OF GLUTAMATERGIC AND MUSCARINIC RECEPTORS IN BRAIN SLICE CULTURES OF MUNC13 KNOCK-OUT MICE

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Munc13 proteins are presynaptic phorbol ester receptors, which are essential for synaptic vesicle priming and glutamate release. Approximately 90% of synapses are solely dependent on Munc13-1 but in a small number of synapses Munc13-1 and Munc13-2 isoforms are present. Thus, glutamate release is reduced by 90% and 100% in neurons of Munc13-1 knock-out mice and Munc13-1/2 double knock-out mice, respectively. The regulation of Munc13 proteins is partly mediated by binding of DAG to the Munc13 C1 domain. Several biological processes – including the proteolytical processing of APP – depend at least in part on Munc13 function. Phenotypic characteristics observed in Munc13 knock-outs are either directly due to the presynaptic functions of Munc13 proteins (i.e. vesicle fusion and glutamate release) or represent secondary consequences of impaired glutamate release (i.e. lack of stimulation of glutamate receptors). To distinguish between these scenarios, we established organotypic brain slice cultures from newborn Munc13 knock-out mice. These cultures were maintained for different periods of time and characterized with regard to the temporal expression of DAG-linked Type I metabotropic glutamate receptors (mGluR-I) and M1 muscarinic receptors (M1 mAChR) by RT-PCR, radioligand binding, and immunohistochemistry. Our results indicate that both, mGluR-I and M1 mAChR are expressed by neurons in brain slices cultured for 10 to 18 days. Thus, these cultures are useful experimental tools to reveal mechanisms of Munc13 function in pharmacological stimulation experiments.
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EXTRACELLULAR KCl CONCENTRATION MODULATES IONOTROPIC GLUTAMATE RECEPTORS RESPONSES IN RAT CEREBELLAR GRANULE CELLS IN CULTURE

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Cultured rat cerebellar granule cells have been widely used as a model for different neurobiological studies. A survival requirement for these cells is the maintenance of chronic depolarising conditions usually obtained by raising potassium concentration to 25 mM. In the presence of physiological potassium concentration (5 mM) granule cells in culture progressively undergo slow apoptosis. However, several studies have revealed that the response to different stimuli of granule cells cultured in 25 mM KCl for six days and then shifted to low potassium conditions mimics better the response of these cells *in vivo*. The aim of this work was to investigate whether changes in the concentration of potassium in the culture medium influence calcium influx elicited by several glutamatergic agonists (NMDA, AMPA and Kainate). Cells maintained in control culture conditions for 7 days or shifted to low potassium concentrations for 24 hours before the experiment were loaded with Fura-2AM and then stimulated. We found that AMPA-induced intracellular calcium increase was higher in cells maintained in low potassium for 24 hours than in control cells and that the contribution of voltage dependent calcium channel (VDCC) to such response was different in the two experimental conditions. The NMDA response was similar in both control and cell shifted to low potassium concentration but also the contribution of calcium entry through VDCC was different. The regulation by the NO/cGMP pathway of the responses elicited by the different agonists was also different in the two experimental conditions.

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GENOMIC STRUCTURE AND TRANSCRIPTIONAL REGULATION OF GRM8

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Expression of metabotropic glutamate receptors (GRM) is mainly restricted to neural cells and undergoes changes in development as well as in neuropathological disorders or as a consequence of pharmaco-

logical treatments. The differential expression of these receptors is largely dependent on their distinct genomic structure. Understanding the molecular basis of their transcriptional regulation may open up novel strategies for pharmacological intervention on these glutamate receptors. We have elucidated the complete exon–intron structure of human and mouse GRM8. Transcripts of this gene are generated by different alternatively spliced first exons. Transcription initiation of human GRM8, identified by 5′-Rapid Amplification of cDNA Ends (5′-RACE), takes place on two distinct exons: Ia and Ib. In mouse, additional transcription initiation sites were identified, located on a third first exon, Ic. Preliminary data for transcription initiation sites on mouse Grm8 exon Ib, were obtained by ribonuclease protection assay, thus confirming the 5′-RACE results. The regional expression of the 5′ alternatively spliced Grm8 mRNA isoforms was investigated by RT-PCR analysis on total RNA extracted from several mouse brain areas, and was obtained in all areas analysed. In silico analysis of the 5′ flanking regions of the first exons identified no obvious core promoter elements, but a CpG island upstream of and spanning exon Ia, could act as a minimal promoter. To further assess the structure of the promoter we have performed mapping of DNAse I hypersensitive sites. These results suggest that alternatively spliced first exons under the control of distinct promoters contribute to regulatory mechanisms for tissue- and context-specific expression.

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REGULATION OF NETWORK ACTIVITY IN HIPPOCAMPUS BY METABOTROPIC GLUTAMATE RECEPTORS: MECHANISM OF ACTION OF A NOVEL ANTICONVULSANT

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To elucidate the mechanism of action of the potential anticonvulsant, 2-methyl-4-oxo-3*H*-quinazoline-3-acetyl piperidine (Q5) binding and functional studies have been performed. However Q5 inhibited binding of [^{3}H]Glu in rat brain membrane homogenates ($K_I = 0.3\,\mu M$), it did not affect neither the binding of specific radioligands to orthosteric or allosteric sites of ionotropic AMPA, kainate and NMDA receptors nor the transport of Glu. Q5 (10–100 μM) antagonised Glu-induced [^{35}S]GTP γ S binding. Q5 (500 μM) decreased the frequency, but not the amplitude of spontaneous EPSCs of CA3 pyramidal cells. Although being ineffective on $GABA_A$ receptor binding and GABA transport, Q5 lowered both the frequency and the amplitude of spontaneous IPSCs in the absence of TTX. Antagonists of group I, II and III metabotropic Glu receptor (mGluR) subtypes (MCPG, AIDA and CPPG) did not mimick the effects of Q5 on IPSCs. In pyramidal cells, Q5 failed to block IPSC increases, induced by the non-selective mGluR agonist, (1*S*,3*R*)-ACPD (30 μM), whereof intracellular Ca^{2+} ion transients observed in non-pyramidal cells were suppressed by Q5 competitively. Q5 (200–500 μM), but neither CNQX (10 μM) nor GYKI-52466 (100 μM) blocked low-[Mg^{2+}]-induced seizure-like events in hippocampal slices from juvenile (P9–13) rats. These findings suggest that Q5 acts by decreasing the excitability of CA3 neuronal networks, most probaly *via* a mGluR, not identified yet.

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STATUS EPILEPTICUS ALTERS GABAA RECEPTOR SUBUNIT MESSENGER-RNA AND PROTEIN EXPRESSION IN THE DEVELOPING RAT HIPPOCAMPUS

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The gamma-aminobutyric acid type A receptor ($GABA_A$) is the main inhibitory neurotransmitter receptor in the brain. The receptor protein is composed of multiple subunits of different families (α 1–6; β 1–3; γ 1–3; δ; ε; θ; π). Reduced GABAergic inhibition, in particular in the hippocampus, is important for seizure generation in epilepsy. However, it is not known whether the subunit expression is modulated by seizures in the developing brain. We used in situ hybridization to study the acute (6 h), and sub-acute (3 and 7 days) changes in the $GABA_A$ subunit mRNA expression after kainic acid (KA)-induced status epilepticus (SE) in immature, 9-day old rats compared to saline-injected control rats. Oligonucleotide probes for the $GABA_A$ receptor subunits α 1–5, β 1–3 and γ 1–2 were used, and immunocytochemistry was applied to detect the α 1, α 2 and β 3 subunit proteins. In the control rats, the α 1 and α 4 subunit mRNA expression significantly increased between the postnatal days 9 to 16, whereas those of α 2, β 3, and γ 2 subunits decreased in hippocampal subregions. These developmental changes were also confirmed at the subunit protein level for α 1, α 2 and β 3. The normal developmental changes in the expression of α 1, α 2 and β 3 subunit mRNAs were altered after the KA treatment. The subunits α 1, α 4, γ 1 and γ 2 were decreased and β 1 and β 3 were increased in the hippocampal subregions of epileptic rats compared to normal control rats. Our results show that SE disturbs the normal developmental expression pattern of $GABA_A$ receptor subunit mRNAs in the hippocampus during the sensitive postnatal phase of brain development. These perturbations could result in altered functional and pharmacological pro perties of $GABA_A$ receptors.

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MODULATION OF NMDA RECEPTOR FUNCTION BY CAMP IN CEREBELLAR NEURONS IN CULTURE

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The signal transduction pathways involved in the modulation of NMDA receptor by other types of receptors are not clear. One of the second messengers that could be involved in this modulation is cAMP. The aim of this work was to analyse the contribution of cAMP to NMDA receptor modulation in cerebellar neurons in culture. Forskolin increases cAMP and results in increased intracellular calcium and cGMP which are prevented by blocking NMDA receptors. Similar effects were induced by two cAMP analogues but not by 1,9-dideoxyforskolin, an analog of forskolin that does not activate adenylate cyclase. This indicates that cAMP leads to NMDA receptor activation. It has been reported that phosphorylation of Ser897 of the NR1 subunit by cAMP-dependent protein kinase (PKA) activates NMDA receptors. Forskolin increases Ser897 phosphorylation. Neither Ser897 phosphorylation nor cGMP increase induced by forskolin were prevented by four different inhibitors of PKA, suggesting that activation of NMDA receptors is dependent on cAMP but not on PKA.

Inhibition of Akt prevents forskolin-induced phosphorylation of Ser897, suggesting a role for Akt in the mediation of the modulation of NMDA receptors by cAMP. Pituitary adenylate cyclase-activating polypeptide (PACAP) activates its receptors, increasing cAMP, and also leads to phosphorylation of Ser897 of NR1 and activation of NMDA receptors. These results indicate that cAMP modulates NMDA receptor function in cerebellar neurons and may play a role in the modulation of NMDA receptors by other receptors.

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UPREGULATION AND TRAFFICKING OF DELTA OPIOID RECEPTORS FOLLOWING PROLONGED MORPHINE TREATMENT IN THE MOUSE BRAIN

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In recent years, we demonstrated that prolonged (48 h) treatment with selective mu opioid receptor (MOR) agonists induced a translocation of delta opioid receptors (DORs) from intracellular compartments to neuronal plasma membranes in the spinal cord dorsal horn. It remained to be determined whether this phenomenon also occurred in the brain. To resolve this issue, we analyzed by immunogold histochemistry the subcellular distribution of DORs in mice treated or not with morphine (48 h). We focused our analysis to regions of the brain which co-express MORs and DORs, namely the nucleus accumbens, the dorsal neostriatum, and the frontal cortex. We found that morphine was capable of modulating DOR trafficking in the nucleus accumbens and neostriatum, but not in the frontal cortex, indicating that the effects of the drug were region-specific. This effect is likely to be MOR-mediated, as earlier studies have demonstrated that in the spinal cord, morphine-induced DOR trafficking was abolished in MOR-knockout mice. The recruitment of DORs induced by prolonged stimulation of MORs represents a potential endogenous mechanism to maintain opioidergic neurotransmission in conditions of prolonged agonist exposure.

P.117

DESENSITIZATION OF NMDA RECEPTOR CHANNELS DURING MATURATION IN CULTURED RAT CORTICAL NEURONS

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Activation of NMDA receptors is supposed to induce rapid opening of an ion channel permeable to Ca^{2+} ions, often followed by signaling cascade to the nucleus for long-term consolidation in the brain. Rat cortical neurons were cultured in DMEM/F12 in the absence of FBS for 3 to 15 days *in vitro* (DIV), followed by homogenization and subsequent SDS-PAGE for immunoblotting using an antibody against NeuN, NSE or synapsin-I. Expression of these proteins all increased in proportion to the duration of culturing up to 15 DIV. These cortical neurons were incubated with fluo-3 AM for determination of intracellular free Ca^{2+} ions on fluorescence image analysis. As long as cells were continuously exposed to NMDA, the increased fluorescence was sustained at constant levels irrespective of the cellular maturity. When cultured neurons were briefly exposed to NMDA, followed by washing and subsequent second brief exposure to NMDA at the same concentration with an interval of 25 min, however, the second exposure to NMDA led to a less potent increase in the number of fluorescent cells than that found after the first exposure in neurons cultured for 9 and 15 DIV but not in those for 3 DIV. Moreover, a less potent increase was seen in the fluorescence following the second exposure to NMDA with an interval of 45 min than that found after the first exposure even in neurons cultured for 3 DIV. These results suggest that *in vitro* cellular maturation may be crucial for the mechanism underlying desensitization of NMDA receptor channels, which is triggered by dissociation, but not by association, of the agonist NMDA in a manner dependent on the time after dissociation in cultured rat cortical neurons.

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QUINOLINIC ACID, KYNURENIC ACID AND D-SERINE STIMULATE BRAIN METABOLISM

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The *N*-methyl D-aspartate receptor (NMDAR) is partly responsible for mediating inflammatory brain insults. Quinolinic acid (QA), a kynurenine pathway metabolite, is a potent neuroexcitotoxin at NMDAR. Kynurenic acid (KA), also a kynurenine pathway metabolite, is a weak, non selective NMDAR antagonist. Alterations in QA and KA have been implicated in various inflammatory neurological disorders such as AIDS dementia, and cerebral malaria. D-Serine modulates NMDAR response at the glycine binding site. In this work we have examined the metabolic sequelae of addition of a number of different concentrations of QA, KA or D-ser to Guinea pig cortical tissue slices incubated with [3-^{13}C]pyruvate using ^{13}C NMR spectroscopy and metabolomic analysis. Addition of QA (0.5 μM & 100 μM), KA (10 μM & 100 μM) and D-serine (0.5 μM and 5 μM) resulted in increased net flux into glutamate C2, C4 and aspartate C2, C3 consistent with these compounds causing increased cell body Krebs cycle flux, but no increase in glutamate/glutamine cycling [Rae C., Hare N., Bubb W. A., McEwan S. R., Broer A., McQuillan J. A., Balcar V. J., Conigrave A. D. and Broer S. (2003) *Journal of Neurochemistry* **85**, 503–514] The response was significantly different to that seen using a classical NMDA agonist (TZG) or antagonist (CGS19755) [Moussa C.E-H. PhD Thesis, Dept of Anatomy and Histology, The University of Sydney]. Surprisingly, the effect of QA and KA was very similar, although they are respectively classified as agonist and antagonist at NMDAR. The intense stimulation of metabolism by D-Ser is also of interest, as the glycine site has been suggested to be largely fully occupied *in vivo* [Johnson J. W. and Ascher P. (1987) *Nature* **325**, 529–531].

P.119

ACTIVATION OF THE NMDA RECEPTOR-MEDIATED NO-cGMP SIGNAL TRANSDUCTION PATHWAY FOLLOWING ACUTE AMMONIA EXPOSURE. A MICRODIALYSIS STUDY

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Hyperammonaemia is a key factor in the pathogenesis of hepatic encephalopathy in which brain edema and increased extracellular brain glutamate occur. Acute hyperammonemia results in activation of NMDA receptors, increased intracellular calcium and activation of nitric oxide synthase (NOS) (Monfort *et al.*, 2002). Activation of the NMDA receptor-mediated NO-cGMP signal transduction pathway is assessed *in vivo* by measurement of cGMP using cerebral microdialysis in freely moving animals. In order to investigate ammonia's effects on NMDA activation/cGMP production, we studied the local effect of ammonia via the microdialysis membrane. Ammonia perfusion led to increased extracellular glutamate and cGMP production. Ammonia perfusion in conjunction with MK-801 (an NMDA receptor antagonist) led to the attenuation (not normalization) of increased extracellular glu-

tamate and cGMP. These results suggest that a) cGMP production occurs in most part as a result of NMDA activation b) ammonia-induced release of cGMP and glutamate also occur independently of NMDA receptor activation. Such mechanisms include direct ammonia-induced stimulation of NOS.

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NMDA RECEPTOR GLUR ε 2/NR2B SUBUNIT IS CRUCIAL FOR HIPPOCAMPAL SYNAPTIC FUNCTIONS

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Gene targeting studies have shown that NMDA receptor GluR ε 2/NR2B subunit is required for synaptic plasticity and neuronal pattern formation. Because the disruption of this subunit causes neonatal death, it has been impossible to study its molecular functions in adulthood. In order to elucidate the physiological functions of GluR ε 2 in adulthood, a conditional knockout mouse was generated using the Cre/loxP recombination system. We generated a mouse strain that is deficient in GluR ε 2 selectively in the hippocampal CA3 area, and carried out physiological studies without experiencing neonatal death. Immunohistochemical analyses revealed that GluR ε 2 proteins were ablated in the CA3 region of the mutant mouse and GluR ζ 1 /NR1 proteins were also reduced. In contrast, the contents of GluR ε 1/NR2A and PSD95 proteins were not affected by the GluR ε 2 disruption. Although GluR ε 1 and GluR ζ 1 remained in the CA3 area, NMDA receptor-mediated currents were scarcely detected at the three major inputs into CA3 pyramidal neurons. Moreover, NMDA receptor-dependent LTPs were severely impaired at these synapses. These results suggest that GluR ε 2 is crucial to form functional NMDA receptor in the hippocampal CA3 synapses, and its role cannot be compensated by GluR ε 1 subunit.

P.121

IDENTIFICATION OF AMINO ACID RESIDUES IMPORTANT FOR ASSEMBLY OF GABAA RECEPTOR ALPHA1 AND GAMMA2 SUBUNITS

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GABAA receptors are ligand-gated chloride channels consisting of five homologous subunits that specifically recognize one another and assemble around an aqueous pore. The majority of these receptors are composed of two alpha, two beta and one gamma subunit. Recently, the crystal structure of the acetylcholine binding protein (AChBP), a structural homologue of the extracellular parts of the so-called Cys-loop receptors, has been published. Using this structure as a template, homology models of the extracellular domains of different GABAA receptor subunits have been generated. In the present study, we used the comparative models developed by our group for predicting three amino acid residues on the alpha1 and three on the gamma2 subunit, that might form direct contacts with each other. These residues were substituted by cysteines and a possible disulphide bond formation between subunits was investigated on co-transfection into HEK cells. Although disulphide bond formation between subunits could not be observed, results indicated that out of the six residues investigated, three are important for assembly of GABAA receptors. Results indicated that the amino acid residues identified might contribute to novel assembly sites on alpha1 and gamma2 subunits.

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NEUROTENSIN EFFECT ON CENTRAL MUSCARINIC RECEPTORS IS INDEPENDENT OF HIGH AFFINITY NEUROTENSIN RECEPTOR (NTS1)

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Neurotensin (NT) is a tridecapeptide distributed in central and peripheral nervous system, which can behave as a neurotransmitter or neuromodulator of dopaminergic and cholinergic transmission. Previous studies demonstrated that NT is able to inhibit the activity of synaptosomal membrane Na^+, K^+-ATPase and to decrease the binding of tritiated quinuclidinyl benzilate ([^{3}H]-QNB) to rat cerebral cortex, hippocampal and cerebellar membranes. NT action on the enzyme is blocked with SR 48692, a specific antagonist for high affinity NT receptor (NTS1). The purpose of this study was to evaluate the potential participation of NTS1 receptor in the inhibition of [^{3}H]-QNB binding by NT. Rat cerebral cortex, cerebellar and hippocampal membranes were incubated with SR 48692 10^{-6} M dissolved in dimethylsulfoxide (DMSO) 10% v/v in the presence or absence of NT 10^{-6} M. As controls, membranes were incubated with DMSO and/or NT 10^{-6} M plus DMSO. It was observed that NT+DMSO decreased 49%, 32% and 53% [^{3}H]-QNB binding to cerebral cortex, cerebellar and hippocampal membranes, respectively; this inhibition was not observed by the single presence of DMSO. Membranes preincubated with 10^{-6} M SR 48692 did not alter NT effect on binding. SR 48692 10^{-6} M decreased 50% the binding only to cerebral cortex membranes, suggesting a possible direct effect on muscarinic receptors in this area. These results indicate that in ligand binding inhibition to muscarinic receptor by NT, high affinity NT receptor is not involved.

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INHIBITORY EFFECT OF FLUOXETINE ON NMDA RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

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We have previously shown that the SSRI fluoxetine is able to inhibit neuronal nicotinic acetylcholine receptors (nAChRs) in concentrations observed during clinical treatment (Hennings *et al.*, 1999, J. Neurochem. 73:1043). The mechanism of action of this drug is similar to that of mecamylamine, a channel blocker-type anatgonist of nAChRs. Since mecamylamine has been shown to block also NMDA receptors, our aim was to investigate whether fluoxetine may affect the function of these ionotropic glutamate receptors. We studied, therefore, the effect of fluoxetine on the NMDA-induced [^{3}H]noradrenaline release from rat hippocampal slices and on the NMDA-evoked currents in cortical neuronal cultures. The NMDA-induced [^{3}H]noradrenaline release was dose-dependently inhibited by fluoxetine. In whole cell patch clamp experiments on cultured cortical neurons the NMDA-evoked currents were concentration-dependently inhibited fluoxetine with an IC_{50} value of 13.2 μM. The mechanism of action proved to be complex

since the inhibitory effect of fluoxetine on the NMDA-currents was not voltage-dependent but magnesium application almost completely prevented the antagonism. Our data show, that fluoxetine is able to inhibit NMDA receptors in the central nervous system in a clinically relevant concentration range. The plasma concentration of this drug reaches 1–2 μM during antidepressant medication and brain concentration can be even higher due to accumulation, therefore the inhibition of NMDA receptors might contribute to the development of therapeutic and/or side effects of fluoxetine.

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DAILY EXPOSURE TO STATIC MAGNETISM IS PROTECTIVE AGAINST SUSTAINED BLOCKADE OF NMDA RECEPTOR CHANNELS IN RAT HIPPOCAMPAL NEURONS

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Repetitive transcranial magnetic stimulation is beneficial for the treatment of selected patients with depression, bipolar affective disorder and schizophrenia, whereas blockade of N-methyl-D-aspartic acid (NMDA) receptor channels is shown to lead to behavioral abnormalities related to neuropsychiatric disorders such as psychosis. In the present study, therefore, we have attempted to evaluate possible protective properties of repetitive daily exposure to static magnetic fields against the neurotoxicity of sustained blockade of NMDA receptor channels in cultured rat hippocampal neurons. In cells cultured with the antagonist for NMDA receptors MK-801 for 8 days in vitro, a significant decrease was seen in the expression of microtubule-associated protein-2 (MAP-2) on Western blotting, in addition to decreased cell viability. Sustained exposure to MK-801 not only decreased the expression of NR1 subunit, but also increased NR2A expression without affecting NR2B expression. However, the repetitive magnetism prevented decreases in the expression of MAP-2 and additionally increased the expression of NR2A subunit without altering NR1 expression in neurons cultured in the presence of MK-801. Repetitive magnetism was also effective in preventing the decrease by MK-801 in the ability of NMDA to increase intracellular free Ca^{2+} ions, without affecting the decrease in the maximal response, when determined by fluorescence imaging. These results suggest that repetitive magnetism may at least in part counteract against the neurotoxicity of MK-801 through modulation of the expression of particular NMDA receptor subunits in cultured rat hippocampal neurons.

P.125

LOW-MOLECULAR-WEIGHT SERUM COMPONENT(S) ARE NEEDED FOR THE AMPA RECEPTOR-MEDIATED DOWN-REGULATION OF GABA-A RECEPTOR SUBUNITS

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We have previously shown that kainate (KA), an agonist of AMPA and KA subtypes of ionotropic glutamate receptors, drastically down-regulates GABA-A receptor alpha1, alpha6 and beta2 subunit mRNA expression in cultured mouse cerebellar granule cells. The aim of the present study was to investigate whether this down-regulation is mediated by AMPA or KA receptors. The AMPA receptor-selective agonist CPW-399 down-regulated dose- and time-dependently alpha1 and alpha6 mRNA expression. This down-regulation was reversible as removal of CPW-399 from culture medium restored expression of both subunits. The KA receptor (GluR5)-selective agonist ATPA had no effect on alpha1 or alpha6 expression. The CPW-399-induced down-regulation was not observed in cells cultured in serum-free culture medium or in the presence of medium containing 10% dialysed serum. The results indicate that AMPA subtype of glutamate receptors mediate GABA-A receptor mRNA down-regulation and that the down-regulation is dependent on low-molecular-weight component(s) present in serum. Therefore, by altering serum composition of the culture medium, distinct GABA-A receptor subtypes can be studied in cultured mouse cerebellar granule cells.

P.126

IDENTIFICATION OF A NOVEL MUSK BINDING PROTEIN AND ITS ROLE DURING NEUROMUSCULAR SYNAPSE FORMATION

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The muscle-specific kinase MuSK plays an essential role during neuromuscular synapse formation. MuSK knock-out mice fail to form neuromuscular synapses and die shortly after birth due to respiratory failure. MuSK is a receptor tyrosine kinase that is activated by the nerve-derived protein Agrin. This leads to the phosphorylation of several tyrosine residues in the MuSK intracellular domain and is essential for all known aspects of postsynaptic and presynaptic differentiation. The events that follow MuSK activation and lead to AChR phosphorylation and clustering remain largely unknown. Therefore, the identification of new MuSK binding partners represents an important step towards the molecular characterization of MuSK/Agrin signaling. Using a mouse muscle cDNA library we performed a yeast-two-hybrid screen with the cytoplasmic region of MuSK. Thereby we have identified a protein that specifically interacts with MuSK and represents a novel gene. With the exception of a Vps9 domain in the C-terminus, common domains or motifs are missing in the predicted protein sequence. The Vps9 domain was first identified in the yeast vacuolar sorting protein Vps9p and is able to function as a guanine nucleotide exchange factor. When a GFP-tagged form of the protein is expressed in fibroblasts, a punctuated intracellular staining is observed that indicates localization in vesicles or endosomes. Ongoing experiments concentrate on the functional analysis of the newly identified MuSK binding protein with particular emphasis on its potential role as a protein involved in MuSK trafficking.

Nitric oxide

P.127

SELECTIVE ROLE OF THE NITRERGIC SYSTEM IN DOPAMINE MEDIATED NEUROTOXICITY OF SUBSTITUTED AMPHETAMINES

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Recently we have shown that blockade or ablation of neuronal nitric oxide synthase (nNOS) attenuates the reinforcing and locomotor stimulating effects of methamphetamine (METH)-induced dopaminergic neurotoxicity. In the first part of these studies, the neurotoxic effects of amphetamine analogs that produce various degrees of DA/5-HT neurotoxicity on wild type (WT) and nNOS knockout (KO) mice were investigated. METH produced selective depletion of DA markers in the striatum, frontal cortex, hippocampus and amygdala of WT mice, but not KO mice. MDMA (ecstasy) and p-chloroamphetamine caused dual DA/5-HT neurotoxicity in the four brain regions examined in both WT and KO mice. Fenfluramine (FEN) caused selective depletion of 5-HT markers in the four brain regions examined in both WT and KO mice. In the second part of these experiments, the locomotor stimulating effect of METH and MDMA on WT and KO mice was investigated. Repeated administration of METH (1 mg/kg) for 5 days resulted in long-lasting (45 days) locomotor sensitization in WT but not KO mice. However, repeated administration of MDMA (10 mg/kg) for 5 days resulted in locomotor sensitization to MDMA in both WT and KO mice during the first 5 days of drug administration. These results suggest that the induction of sensitization to MDMA, unlike METH, may recruit 5-HT transmission, and the deletion of the nNOS gene does not modulate 5-HT-mediated effects of MDMA. The persistence of the sensitized response, however, may be DA/NO-dependent as it was blunted in both METH and MDMA pretreated KO mice. Taken together, the results suggest a selective role of the nitrergic system in DA-mediated effects of substituted amphetamines.

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NITRIC OXIDE INHIBITS NEUROMUSCULAR TRANSMISSION BY FAVOURING TONIC ACTIVATION OF ADENOSINE A1 RECEPTORS ON MOTOR NERVE TERMINALS

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At the motor endplate, depression of acetylcholine (ACh) release by NO may result directly from its action on nerve terminals or, alternatively, by increasing the production of adenosine activating inhibitory A1 receptors. Therefore, we investigated the role of adenosine on NO-induced inhibition of electrically (5 Hz)-evoked [3H]-ACh release from rat phrenic nerve-hemidiaphragm preparations loaded with [3H]-choline (2.5 μCi/mL). L-arginine (L-Arg, 0.047–4.7 mM), the substrate of NO-synthase, decreased evoked [3H]-ACh release in a concentration-dependent manner. Release-depression by L-Arg (47 μM) was prevented upon inhibiting NO-synthase and guanylcyclase respectively with Nω-nitro-L-arginine (L-NOARG, 100 μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), whereas the extracellular NO scavenger, haemoglobin (10 μM), was without effect. Pretreatment with adenosine deaminase (0.5 U/ml, the enzyme that inactivates adenosine into inosine) and with 1,3-dipropyl-8-cyclopentylxanthine (2.5 nM, an A1 receptor antagonist), but not with the selective A2A antagonist, ZM241385 (10 nM), also blocked L-Arg (47 μM) inhibition. Increasing endogenous adenosine levels by inhibiting the nucleoside uptake with S-(p-nitrobenzyl)-6-thioinosine (30 μM), significantly potentiated L-Arg (47 μM) inhibition. In contrast, activation of A1-inhibitory and A2A-facilitatory adenosine receptors with R-N6-phenylisopropyl adenosine (100 nM) and CGS21680C (2 nM), respectively, failed to affect the inhibitory action of L-Arg (47 μM). Data indicate that NO-induced depression of [3H]-ACh release depends on tonic activation of A1 receptors by adenosine generated endogenously.

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NITRIC OXIDE AND METABOTROPIC GLUTAMATE RECEPTORS IN THE MECHANISMS OF AUDIOGENIC EPILEPSY IN DBA/2 MICE

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Nitric oxide (NO) has been proposed as a key pathophysiological factor in neurological disorders, which involve the neurotoxic effect of glutamate such as stroke and epilepsy. We have previously demonstrated a significant increase of NO generation in cerebral cortical tissue after seizures of various origin including NMDA-induced convulsions. The situation in relation to metabotropic glutamate receptors (mGluR) and free radical formation is less clear. The aim of this work is to study a possible involvement of NO as well as mGluR of 1st subtype (mGluR1) in the pathophysiology of audiogenic seizures in DBA/2. NO generation was directly measured by electron paramagnetic resonance spectroscopy. NO formation was more than 2-fold increased in the brain of DBA/2 mice during tonic-clonic seizures produced by sound stimulation (109 dB for 60 s) in comparison with the control group (no audiogenic stimulation). Specific indexes of lipid peroxidation (i.e., thiobarbituric acid reactive substances, TBARS) in mouse brain were also elevated. Pretreatment with mGluR1 antagonist AIDA, 1 mmol, i.c.v., 30 and 90 before sound stimulation suppressed sound-induced seizures as well as enhanced NO and TBARS levels. The mGluR1/mGluR5 agonist, DHPG, 1 mmol, i.c.v., admitted 120 min prior sound stimulation was proconvulsant in DBA/2 mice. These findings support the notion that increased formation of NO contributes to the pathogenesis of development and propagation of audiogenic seizures. The results of our study might form the basis for designing novel strategies for neuroprotection against epilepsy and mGluR1 antagonists might be considered as potential anticonvulsant drugs.

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MODULATION OF MEMBRANE PROPERTIES AS A MECHANISM OF NITRIC OXIDE SIGNALING IN NEURONS

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Most of the neurons receive nitric oxide (NO) from the outside. In this case the first target for NO is plasma membrane and its structures.

Inside the cell NO can also influence organelles membranes. Modulation of the membrane properties affect the activity of channels, receptors and ATP-ases and thus alters function of the whole cell. We aimed to study the influence of NO on the properties of plasma membrane [ion channel activity and amount of bound Ca^{2+} (Ca_{mb})], mitochondria and microviscosity of cytosomes membrane. These studies were done by means of patch-clamp recording, fluorescent microscopy and Raman spectroscopy. By using interference microscopy we explored NO influence on the neurons' refractive index as an integral cellular characteristic. Our preparations were identified neurons of the pond snail *Lymnaea stagnalis*. As NO donor we used sodium nitroprusside. We have shown that NO activates voltage-dependent K channels and causes desorption of Ca^{2+} from the organelles' and plasma membranes. NO induced depolarisation of mitochondria, which may lead to the exit of mitochondrial Ca^{2+}, and caused the decrease in the microviscosity of cytosomes membrane and in the amount of stores-bound Ca^{2+}. It was shown that NO affects refractive index of neurons due to the induced processes in the plasma membrane and cytoplasm. Changes of the $Ca^{2+}{}_{mb}$ amount and membrane microviscosity affect membrane surface charge and the activity of membrane proteins. Desorption of Ca_{mb} and exit of Ca^{2+} from its stores (e.g. mitochondria) contribute to the redistribution of cellular Ca^{2+} and alter Ca-dependent processes. So, NO via changed membranes properties may influence other cellular characteristics and processes.

P.131
HYPOXIA AND MITOCHONDRIAL DYSFUNCTION IN DEVELOPING CNS

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In some hypoxia models an increase of intracellular levels of nitric oxide(NO) has been described which in turn, stimulates the mitochondrial production of reactive oxygen species like superoxide anion (O2-) and peroxynitrite (ONOO-). The aim of this paper is to determine whether hypoxia/reoxygenation events during embryonic development induce the expression of neuronal nitric oxide synthase (nNOS), and putative effects in nitrosylation/nitration of proteins and mitochondrial function. For this purpose, 12-embryonic-day old chicken embryos were used which underwent severe hypoxia [8% (O_2), 60 minutes], followed by different reoxygenation periods:1 h, 2 h, 3 h and 4 h. nNOS expression potentation and mitochondrial complex content was detected by Western blot in cytosolic and mitochondrial fractions. Activity of respiratory complexes I(CI) and IV (CIV) was recorded by monitoring oxidation of specific substrates. During hypoxia a 50% transient increase of nNOS was observed ($P < 0.05$) in cytosolic fraction and during the first hour reoxygenation period together with an increase of the content of nitrated proteins. Surprisingly, nNOS was translocated to mitochondria (3 fold increase respect to control, $P < 0.01$) during the first two reoxygenation hours followed by intense nitration. Decreased Complex I activity was detected after the first reoxygenation hour without changes in protein expression. In conclusion, the increase of nNOS levels and the formation of peroxynitrite should explain protein nitration and reduction of CI activity in developmental hypoxia.

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THE EFFECT OF LONG-TERM NITRIC OXIDE SYNTHASE INHIBITION IN NEUROPATHIC AND NAİVE RATS

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Nitric oxide (NO) is a free radical gas that has been suggested to play a role in nociceptive processing in the spinal cord. The present study was designed to evaluate the influence of nitric oxide synthases (NOS) inhibition at physiological conditions and after chronic constriction injury (CCI) of the sciatic nerve. Nonspecific NOS inhibitor, N-nitro-L-arginine (L-NNA) was applied intraperitoneally to rats during 11 weeks. The development of mechanical allodynia was studied with regard to changes of immunocytochemically assessed neuronal NOS occurrence in the spinal cord. Long-term inhibition of NOS in CCI rats had antinociceptive effect and significantly decreased the level of nNOS positive neurons in the dorsal horn laminae III–VI. However, administration of L-NNA to naive rats evoked mechanical allodynia and reduced numbers of nNOS positive neurons in the superficial dorsal horn laminae I–II. It is suggested, that NO may be a messenger molecule of different types of neurons and it may exert a dual role in mediating neuronal activity depending on the type of neurons being activated. Our results indicate, that during physiological conditions low level of NO produced by neurons in laminae I–II could be involved in the descending inhibitory modulation of the spinal nociceptive inputs and is essential for physiological nociception. The elevated levels of NO after peripheral nerve injury could influence the excitatory neurons in the deep dorsal horn laminae and result in the development of allodynic symptoms. The effect of NOS inhibitor may therefore vary in different experimental conditions.

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LATERALIZATION OF HIPPOCAMPAL NITRIC OXIDE MEDIATOR SYSTEM

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Studies suggest cerebral lateralization underlying hemisphere specialization and its alterations due to neurodevelopmental and neurodegenerative disorders. On neurotransmitter levels, noradrenergic system is under greater control of the right hemisphere, dopaminergic and cholinergic systems under that of the left hemisphere, on the contrary. Nitric oxide mediator system has not been evaluated yet. Here we estimated the degrees of lateralization in the activity and expression of synthases, in the concentrations of L-glutamic acid, L-citrulline and L-arginine and in the levels of nitrites/nitrates in the right and left autoptic hippocampi of patients with Alzheimer disease, multi-infarct dementia and schizophrenia. Our results indicate: i) the mild right/left asymmetry in the activity of neuronal and endothelial synthases of controls, the right/left increase in schizophrenia and the marked left/right asymmetry in Alzheimer disease but not in multi-infarct dementia, ii) the mild left/right asymmetry in the activity of inducible synthase of controls but no differences among groups, iii) no marked correlation between the activity and expression of synthases, and finally iv) no significant differences among groups for L-glutamic acid, L-citrulline, L-arginine and nitrites/nitrates. Our results support the studies reporting lateral alterations in the hippocampus of Alzheimer disease and schizophrenic patients and the involvement of nitric oxide mediator system in the pathogenesis of brain disorders.

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NITRIC OXIDE IN CULTURED RETINAL CELLS: EFFECTS ON CELL PROLIFERATION, SURVIVAL AND PROTECTION FROM GLUTAMATE EXCITOTOXICITY

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Nitric Oxide (NO) plays important roles in synaptic transmission, plasticity and neuronal survival, suggesting that these multiple effects are

mediated through different signal transduction pathways. Here, using cultures of chick retina cells, we show that NO regulates neuronal survival and blocks glutamate excitotoxicity through the classical cGMP/PKG pathway but also inhibits glial cell proliferation by a cGMP-independent pathway. Cultures of neurons or glial cells and mixed cultures obtained from 8/11-day-old chick embryos (E8-11) were incubated with the NO donor SNAP since the first day in culture (C1). Cell survival was measured 1 day after refeeding E8C3 cultures with fresh medium (that promotes an extensive cell death) or 8 hours after addition of glutamate (1 mM). Cell proliferation was assessed using Thymidine incorporation in mixed or glial cultures. Preincubation for at least 24 hours with SNAP attenuates cell death induced by refeeding the cultures, an effect blocked by ODQ or LY83583 (guanylate cyclase inhibitors), KT5823 (PKG inhibitor), and mimicked by zaprinast (cGMP phosphodiesterase inhibitor). Interestingly, the effect of NO was also blocked by inhibition of PI3 kinase, MEK, CAMK II and PKA. Similarly, cultures were protected against glutamate excitotoxicity, an effect mimicked by 8Br-cGMP and zaprinast. Preincubation with SNAP also reduced basal or ATP-stimulated proliferation in mixed or glial cultures, an effect not mimicked by cGMP or zaprinast, and not related to Erk regulation. The results indicate that NO regulates different cellular functions through different signal transduction pathways.

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NITRERGIC SYSTEM OF THE RAT BRAIN: EFFECTS OF ACUTE AND CHRONIC MORPHINE ADMINISTRATION AND MORPHINE WITHDRAWAL

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The nitrergic system in rat brain regions was studied after a single and chronic morphine administration, as well as during morphine withdrawal. Morphine hydrochloride was injected intraperitoneally at a single dose of 10 or 30 mg/kg or chronically at increasing doses (10–100 mg/kg; twice a day, 6 days). Nitric oxide synthase activity (NOS) and nitrate/nitrite concentration (NO_x^-) were measured in the neocortex, striatum, hippocampus, midbrain, cerebellum and brain stem 15, 30, 60, 120 and 240 min after a single morphine administration, 2 hours after 6 or 12 morphine injections, or 36, 72 and 144 h after 12 morphine injections. A time course for both NOS activity and NO_x^- concentration is described in the above situations. Striatum, midbrain, cerebellum and hippocampus appeared to be more reactive than other brain regions studied. The relations between changes in NO metabolism indices (NOS and NO_x^-) were different depending on the morphine administration regimen and the region studied. The results suggest that changes in NO metabolism in the specific brain regions are involved in the development of opiate dependence.

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CENTRAL ADMINISTRATION OF AMYLOID-BETA PEPTIDE(25–35) SELECTIVELY AFFECTS DIFFERENT ISOFORMS OF NITRIC OXIDE SYNTHASE IN RAT BRAIN

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Nitric oxide synthase (NOS) activity and expression of neuronal and inducible NOS was studied in brain regions of rats intracerebroventricularly injected with the aggregated fragment (25–35) of β-amyloid peptide [β A(25–35); 15 nmol]. A radioisotopic method was used to measure NOS activity, while expression of NOS isoforms was studied using immunohistochemical approach. Administration of π A (25–35) resulted in an increase of NOS activity in the neocortex and the hippocampus 6 days after the surgery. This increase could be attributed to a rise in the constitutive NOS activity since the activity of inducible NOS did not change. The number of neurons expressing neuronal NOS increased in the neocortex, but not in the hippocampus after β A (25–35) administration. The number of cells expressing inducible NOS did not differ from those in control (vehicle-treated) rats in either region. Thus, the rise in neocortical NOS activity induced by β A (25–35) may be the result of an increase in the number of neurons expressing neuronal, but not inducible NOS, while augmented NOS activity in the hippocampus cannot be attributed to an increase in expression of either isoform.

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THE LEVEL OF NITRIC OXIDE AND THE PEAK EXPIRATION FLOW RATE OF THE CASES WITH TOURETTE SYNDROME AND THEIR FAMILY MEMBERS

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Objectives: Tourette syndrome is a movement disorder involved in the immunological dysfunction. Since nitric oxide was found in the etiology and treatment of movement disorders for years, we proposed that the nitric oxide in lung is involved in the pathogenesis of Tourette syndrome.
Methods: 12 drug-free, Tourette syndrome cases without the history of acute repiratory infection or asthma were recruited. The expiration nitric oxide level was measured and compared to the 12 matched control. The peak expiration flow rate was also monitored. We also collected the nitric oxide level of their fathers, mothers and siblings of the children with Tourette syndrome.
Results: There were no significant difference of the level of nitric oxide between the cases with Tourette syndrome and the control group. There was a significant correlation of the level of nitric oxide between the cases and their mothers but not fathers or siblings. However, the cases had significantly higher peak expiratory flow rate than the control group.
Conclusions: Future studies are warranted to investigate the role of nitric oxide in different phenotypes of Tourette syndrome. The higher peak expiratory flow might be the pathophysiology with regard to the neuroanatomy of Tourette syndrome.