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TUESDAY SESSION
Plenary lecture 2

PL2

NEUROTRANSMITTER TRANSPORTERS

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Neurotransmitter transporters present on the plasma membrane contribute to the clearance and recycling of neurotransmitters and can have a profound impact on the extent of receptor activation during neuronal signaling. Our major research efforts have focused on the structure, regulation and cellular physiology of two families of sodium-dependent neurotransmitter transporters: the biogenic amine and the excitatory amino acid carriers. The dopamine, norepinephrine and serotonin transporters (DAT, NET and SERT) are well-established targets for addictive drugs, including cocaine and amphetamines, and for therapeutic antidepressants. Analyses of substrate transport, ion conductances and carrier regulation have provided additional insights into distinct functional states of biogenic amine transporters and suggest different mechanisms by which drugs can affect carrier function. Recent electrophysiological studies show that DAT substrates increase the firing activity of dopamine neurons by activating an anion conductance associated with the carrier, revealing an unanticipated function for these transporters in neurotransmitter signaling. Five different human subtypes of excitatory amino acid transporters (EAAT1-5) and their homologs in several species have been identified. Although these carriers limit CNS concentrations of glutamate, they also possess a ligand-gated chloride channel activity that can regulate neuronal excitability. This lecture will consider some of the novel aspects of neurotransmitter transporter function, and will present the results of molecular genetic, electrophysiological and cell biological approaches aimed at defining the relationships between neurotransmitter transporter structure, substrate transport, and ion permeation.

ISN young scientist lectureship award

YL2

GENE-ENVIRONMENT INTERACTIONS MEDIATING EXPERIENCE-DEPENDENT PLASTICITY IN THE HEALTHY AND DISEASED CEREBRAL CORTEX

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While much progress has been made in recent years in understanding how genetic factors and other molecular mediators contribute to various brain disorders, less is known about how environmental factors and associated gene–environment interactions modulate pathogenesis. Huntington's disease (HD) involves progressive motor, cognitive and psychiatric symptoms, and is caused by a trinucleotide repeat mutation that is common to many other brain diseases, which may therefore involve similar molecular mechanisms. We have shown, using transgenic mouse models of HD, that environmental factors can dramatically modify the disease process. Environmental enrichment of HD mice delays the onset and progression of motor and cognitive symptoms, as well as loss of cortical volume. We have also been investigating putative molecular and cellular mediators in HD and establishing how gene–environment interactions can modulate these pathogenic pathways. We have examined gene expression, neuronal morphology, neurogenesis, cortical plasticity and other measures of experience-dependent changes in specific brain regions of HD mice. For example, brain-derived neurotrophic factor (BDNF) protein levels are downregulated in the striatum and hippocampus of HD mice and environmental stimulation rescues this deficit. In the anterior neocortex, HD does not affect BDNF levels indicating a problem in anterograde protein trafficking from the cortex to striatum, which is rescued by environmental enrichment. Our findings indicate that the modulatory effects of environmental manipulations are mediated by amelioration of specific molecular and cellular deficits and provide experimental paradigms for the identification of novel therapeutic targets for HD and related brain disorders.

S5 Mitochondria: guardians at the gate of neuronal injury

S5.A
MECHANISMS OF MITOCHONDRIAL ROS GENERATION. FACTS AND CONTROVERSIES

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Studies on isolated mitochondria using different respiratory substrates and inhibitors indicated that ROS can be generated by complex I and complex III in the respiratory chain. However, there is a great deal of diversity in reports as to the mechanism and rate of ROS formation, as well as the contribution of the mitochondrial complexes to ROS generation. ROS formation in isolated mitochondria are strongly dependent on the substrates supporting respiration. In mitochondria of high membrane potential in the presence of succinate electrons flow from coenzyme Q to NAD+ and this reverse electron transport via complex I results in a high rate of ROS generation, which is inhibited by rotenone. With NAD+-linked substrates (glutamate plus malate) the rate of ROS generation is smaller and could be increased by rotenone. Our results indicate that the method by which mitochondria are prepared could also have a profound influence on the characteristics of ROS formation. In mitochondria prepared by the method using digitonin and bovine serum albumin (BSA) ROS formation by a reverse electron flow is more significant than that in mitochondria prepared on a percoll gradient with no BSA present. The mechanism of ROS generation by in situ mitochondria is difficult to establish. We have found no evidence for ROS production by a reverse electron flow in isolated nerve terminals. In addition to the respiratory chain, the key Krebs cycle enzyme α-ketoglutarate dehydrogenase (α-KGDH) is also able to generate ROS during its normal catalytic function. α-KGDH-mediated ROS formation is increased by calcium and NADH and inhibited by NAD+. This finding points to a further mechanism of ROS generation that could be of physiological importance.

S5.B
AN NADPH OXIDASE IN ASTROCYTES: EXPRESSION, FUNCTION AND PATHOPHYSIOLOGICAL ROLE IN THE NEUROTOXICITY OF BETA AMYLOID

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β-amyloid (β A) peptides accumulate in the CNS in Alzheimer's Disease and are toxic to neurons in culture. In mixed neuronal/glial cultures from rat or mouse cortex and hippocampus, β A peptides 25–35 and 1–42 caused an increase in $[Ca^{2+}]_c$ and a loss of mitochondrial membrane potential in astrocytes. The latter was reversed by antioxidants and by mitochondrial substrates, suggesting an action of reactive oxygen species (ROS) on intermediary metabolic pathways. β A increased ROS generation in astrocytes, causing glutathione (GSH) depletion in astrocytes and neurons. ROS generation and GSH depletion were calcium dependent and were blocked by inhibitors of the NADPH oxidase (DPI, apocynin; Abramov *et al.*, 2003, 2004). Western blots confirmed the expression of a range of NADPH subunits (gp91phox, p40phox, p47phox, p67phox and p22phox) in astrocyte cultures. Localization to astrocytes was confirmed with immunofluorescence. ROS generation by the NADPH oxidase was stimulated either by the phorbol ester PMA, or by a rise in $[Ca^{2+}]_c$ either through IP3 mediated calcium signals or by the ionophores A23187 and ionomycin. Activation by PMA was blocked by the PKCδ inhibitor, hispidin, activation by calcium was not. All responses were dramatically reduced in cells cultured from gp91 knockout transgenic mice. These data demonstrate that astrocytes express an NADPH oxidase and suggest that the activation of the enzyme plays a central role in the pathophysiology of β A neurotoxicity and perhaps also in Alzheimer's Disease.

S5.C
THE MITOCHONDRIAL ORGANELLE DYSFUNCTION PROGRAM IN NEURONAL APOPTOSIS

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Defects in protein degradation and protein quality control have been implicated in ischemic nerve cell death and in Parkinson's and Alzheimer's disease. We will provide evidence that mitochondria play an important role in neuronal apoptosis as a consequence of defects in protein folding and protein degradation. The Bcl-2 family of proteins contains pro and anti apoptotic proteins that regulate the mitochondrial pathway of apoptosis. There is increasing interest in a subfamily of the Bcl-2 family, the BH3-only proteins and their pro-apoptotic effects within neurons. We will provide evidence that the induction of ER-stress in neurons activates the bb3/PUMA gene, a member of the BH3-only gene family. This results in the activation of the BH3-only protein PUMA, the activation and clustering of the pro apoptotic Bcl-2 family member Bax and the loss of cytochrome-c from the mitochondria. We will also provide evidence for the involvement of the BH3 only proteins BIM and BID during ER stress and during nerve cell death as a result of prolonged proteasome inhibition.

S5.D
MITOCHONDRIAL TRAFFICKING AND NEURONAL DEATH

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Intact mitochondrial function is essential for the maintenance of viability of neurons, while mitochondrial dysfunction may be pathogenic in a number of neurodegenerative disease states. Mitochondria are distributed throughout neurons, and it is presumed that this distribution reflects local demands for energy production, calcium homeostasis, or other key mitochondrial functions. As the half-life of mitochondria is likely to be considerably less than the life expectancy of the host neuron, there is an on-going need to replace mitochondria, which requires the biogenesis and delivery of new mitochondria, and the retrieval and destruction of old organelles. We are investigating the hypothesis that interruption of the normal mitochondrial life cycle is a direct cause of neuronal injury. We will present several examples of

pathogens that impact the life cycle. These include: glutamate, which alters trafficking and morphology, nitric oxide, ATP synthesis inhibitors, and zinc, which inhibit trafficking without changing morphology, and expanded poly-Q huntingtin which locally inhibits mitochondrial movement as the consequence of aggregate formation. We will also provide evidence that rotenone inhibits mtDNA replication, which may affect biogenesis. While these data do not unequivocally establish that interruptions in the mitochondria life cycle are a direct cause of neuronal injury, they nevertheless indicate that altered trafficking is at least one important consequence of the actions of neurotoxins on central neurons.

S6 Assembly and trafficking of NMDA receptors

S6.A
SYNTHESIS AND ASSEMBLY OF N-METHYL-D-ASPARTATE (NMDA) RECEPTORS

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The N-methyl-D aspartate receptor (NMDAR) requires both NR1 and NR2 subunits to form a functional ion channel and the focus of our research has been to elucidate the molecular determinants that determine subunit association and to examine when, and where, in the cell the subunits become associated. These studies have made use of our past finding that neither the NR1 nor the NR2A NMDA subunits can be transported to the cell surface when expressed alone. By combining measurements of cell surface expression of NR2A with co-immunoprecipitation experiments and the use of Blue Native gel electrophoresis to determine the oligomerization status of the subunits we have shown that regions of the N-terminus of NR1 are critical for subunit association whereas the truncation of the C-terminus of NR1 before the last transmembrane region has no effect on the association of the subunits. All the evidence from these studies suggests that NR1 subunits alone can form stable dimers. Subsequently, using a cell line, which can be induced to express the NMDA receptor following exposure to dexamethazone, we have found that NMDA receptors can be expressed at the cell surface within 5 hours of subunit induction and that there appears to be a delay between the first appearance of the subunits and their stable association. This has prompted an examination of the folding of the subunits when expressed alone using a combination of biochemical methods and live imaging of yellow fluorescent protein-tagged NR1 and NR2 subunits. The findings from all these studies will be discussed in view of our current understanding of the assembly of glutamate receptors.

S6.B
DELIVERY OF NMDA RECEPTORS TO NASCENT SYNAPSES

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One of the critical events in synapse formation is the recruitment of ionotropic glutamate receptors to new sites of contact between axons and dendrites. The trafficking of glutamate receptors in neurons is of the utmost importance for synapse formation and synaptic plasticity. We have shown that both NMDA and AMPA receptors reside in mobile transport packets that are recruited rapidly and independently to nascent synapses. Furthermore we have determined that NMDA receptors cycle with the plasma membrane during pauses of movement along microtubules while trafficking. We are now studying how the cell adhesion molecule Neuroligin 1 is involved in the trafficking of NMDA receptors during synapse formation. Neuroligin 1 may thus play a part in the recruitment of NMDA receptors to new synapses.

S6.C
NATIVE NMDA RECEPTOR SUBTYPES IN CEREBELLAR CELLS

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The cerebellum, with its simple architecture and easily identified cell types, is an ideal system for examining NMDA receptor channel diversity. In situ hybridization studies suggest that all four NR2 subunits (2A, 2B, 2C and 2D) occur in this brain region. There is compelling evidence for the existence of several distinct subtypes of native NMDA receptors, which vary in their response to endogenous ligands, and modulatory ions. This has important implications both for fast and long-term signalling at central glutamate synapses. Single-channel evidence supports the proposal that two functionally distinct classes of native NMDA receptors are present in central neurons. One of these corresponds to the 'conventional' 50pS type of NMDA receptor channel that has been widely described. Work on native and recombinant NMDA receptors has demonstrated that 50pS NMDA channel openings are associated with the expression of the NR2A or NR2B subunits. The other class, readily identified from its low channel conductance and reduced sensitivity to Mg^{2+} block, is associated with expression of NR2C or NR2D subunits. I will describe some of our evidence for the presence of two functionally distinct types of NMDAR channels in cerebellar cells – a low and a high conductance channel. Second, I will consider the evidence that low conductance NMDAR channels, with low sensitivity to block by Mg^{2+}, are present at mossy fibre to granule cell synapses in the cerebellum. Third, I will address the issue of whether or not the NR2C- or NR2D-containing class of NMDAR-channel is activated during synaptic transmission, as either a di- or triheteromeric assembly.

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S6.D
TRAFFICKING OF NMDA RECEPTORS REQUIRES AN INTERACTION WITH PDZ PROTEINS AND THE EXOCYST COMPLEX

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The delivery of functional NMDA receptors to the synapse requires the coordinated interaction of multiple proteins at several steps. Addressing the hypothesis that NMDA receptors interact with a PDZ protein early in the biosynthetic pathway, we have focused on the PDZ protein SAP102 and trafficking proteins with which it interacts. Using yeast two-hybrid screening, we have identified two proteins that interact with SAP102 and influence the trafficking of the NMDA receptor in neurons. The first is Sec8, which binds to the PDZ domains of SAP102. Sec8 is a member of the mammalian exocyst complex, which has been implicated in regulating trafficking of proteins to the plasma membrane. A dominant negative construct of Sec8, in which the PDZ binding domain is mutated, dramatically reduced delivery of NMDA receptors to the plasma membrane in both heterologous cells and neurons. A second protein, LGN (or mPins), interacts with the SH3/GK domains of SAP102. Mutations in LGN also reduce NMDA receptor delivery to

the plasma membrane. In brain, complexes containing Sec8/SAP102/NMDA receptor and LGN/SAP102/NMDA receptor can be identified by co-immunoprecipitation. For both Sec8 and LGN, interactions through SAP102 can occur in heterologous cells with NR2 alone (which is retained in the endoplasmic reticulum in the absence of NR1), suggesting that the complex forms at the level of the ER. Both Sec8 and LGN can interact with multiple other proteins implying that the trafficking of NMDA receptors in neurons depends on a large number of proteins that interact both directly and indirectly with the NMDA receptor.

S7 From cell–cell recognition to memory formation

S7.A
SYNAPTIC PLASTICITY AND NEURONAL CELL ADHESION MOLECULES

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Neuronal activity modulates synaptic function through induction of properties of synaptic plasticity such as long-term potentiation, but it also triggers remodelling of synaptic structures. In two models of synaptic activation, LTP induction by theta burst stimulation and application of transient anoxia/hypoglycemia, we found a lasting enhancement of AMPA receptor mediated transmission associated with various forms of synaptic structural remodelling. Using time lapse 2-photon confocal imaging we could observe enlargements in spine shape, growth of dendritic filopodia, formation of new spines as well as growth of presynaptic axonal protrusions. Using electron microscopic analyses, 3-dimensional reconstructions and stereological measurements we could detect spine size changes, a remodelling of postsynaptic densities and evidence for synaptogenesis taking place within 30–60 minutes after activation. The neural cell adhesion molecule (NCAM) and its polysialilated for PSA-NCAM contribute to these mechanisms as interference with these molecules prevents the synaptic enhancement. We found that they also participate in structural remodelling and in synaptogenesis. In dissociated hippocampal cultures prepared from wild type and NCAM deficient mice, synaptogenesis is significantly reduced on neurones deficient in NCAM or PSA-NCAM. Similarly, in slice cultures, activity induced structural remodelling of PSDs and synaptogenesis are prevented by elimination of PSA from NCAM. These effects could be mediated through interactions of NCAM with heparan sulphate proteoglycans as enzymatic removal of heparan sulphate reproduced these effects. It is proposed that PSA-NCAM in complex with heparan sulphate proteoglycans promotes synaptogenesis and activity-dependent structural plasticity.

S7.B
RAPID SYNAPTIC CROSSTALK IN THE HIPPOCAMPUS: A COMPONENT OF INFORMATION FLOW?

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Synaptically released glutamate can under some conditions exert long-range actions in the cortical microcircuitry, thus adding a diffuse component to the classical, point-to-point synaptic transmission. Extrasynaptic diffuse signalling is controlled by glutamate uptake systems and by the extracellular diffusivity, suggesting that use-dependent changes in the distribution of glutamate transporters and in the extracellular space architecture could affect information flow in the synaptic circuitry. We have found that rapid synaptic crosstalk in the hippocampus occurs through activation of high-affinity, NR2B subunit-containing NMDA receptors, whereas the lower-affinity, NR2A-containing (intrasynaptic) NMDA receptors sense glutamate released only at the immediate active synapse. It has recently emerged that similar dichotomy separates induction mechanisms of long-term depression and long-term potentiation: these two forms of plasticity appear to rely on, respectively, NR2B and NR2A-containing receptors. We therefore propose that diffuse depression, together with other, newly emerging non-Hebbian mechanisms of synaptic modification, could contribute to the optimization of memory storage in the hippocampal network.

S7.C
ROLE OF NEURONAL CELL ADHESION MOLECULES IN STRESS-INDUCED COGNITIVE IMPAIRMENT

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Chronic exposure to stress can have deleterious effects on brain structure and function, which might be manifested either immediately after stress exposure, or as a long-term vulnerability to cognitive deficits in later life. In rodents, stress-induced alterations in spatial learning and fear conditioning are particularly apparent in a subset of individuals. Our work aims to understand how the effects of stress can eventually translate into changes in neural circuits, and consequently in behaviour and cognition. In line with reports in humans indicating that prolonged stressful situations are associated with structural atrophy in specific brain regions, chronic stress in rats was found to alter synaptic structures in CA3 and CA1 hippocampal subfields. In the search for cellular and molecular mechanisms that mediate the effects of stress on brain structure and function, we have implicated the neural cell adhesion molecules of the immunoglobulin superfamily, NCAM and L1, on the effects of stress on brain and cognitive function. The pattern of changes observed on the hippocampal expression of these molecules in chronically stressed rats fits with specific roles for these adhesion molecules in stress-induced neuronal damage and neuroprotective mechanisms. These findings underscore cell adhesion molecules as potential therapeutic targets in stress-related cognitive disturbances. New developments are likely to come from recent findings showing beneficial effects, on synaptic function and memory formation, of small peptides that mimic specific functional aspects of cell adhesion molecules.

S7.D
NEW STRATEGIES FOR TREATMENT OF DYSFUNCTIONAL PLASTICITY, LEARNING AND MEMORY USING NCAM MIMETIC PEPTIDES

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NCAM plays a pivotal role during development of the nervous system, and in the adult brain it regulates synaptic plasticity, including learning and memory consolidation. NCAM is a multiligand receptor involved in homophilic binding to itself and in heterophilic interactions with a variety of components of the extracellular matrix, other cell adhesion molecules, GDNF and its receptor, GFRα, and fibroblast growth factor (FGF) receptor. Recent advances in the understanding of the structural basis for the homophilic binding and activation of the FGF-receptor, followed by identification of a number of NCAM mimetic peptides constitute a breakthrough in pharmacological approaches both as regards the study of the role of NCAM in synaptic plasticity and neuroprotection and as regards development of com-

pounds for treatment for neurodegenerative disorders. Whereas a traditional approach is based on antibody intervention and the use of transgenic or knockout animals, the NCAM mimetic peptides provide compounds that mimic various aspects of NCAM functions. One of these peptides – a part of an NCAM binding site for the FGF receptor – was found to bind to and activate the receptor, to induce neuritogenesis and synaptogenesis, to enhance presynaptic function, and promote survival in vitro. In vivo, the peptide was found to facilitate learning and memory consolidation and to protect against neurotoxic effect of the amyloid-beta peptide.

S8 Cell transplants and gene therapy: insights in neural repair

S8.A
TRANSPLANTATION OF HUMAN NEURAL STEM CELLS PROMOTE AXONAL GROWTH AND FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY IN PRIMATES

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Neural progenitor cells, including neural stem cells (NSCs), are an important potential graft material for cell therapeutics of damaged spinal cord. Here we used as a source of graft material a NSC-enriched population derived from human fetal spinal cord (Embryonic week 8–9) and expanded in vitro by neurosphere formation. NSCs labeled with BrdU (TP, n = 5) or culture medium (CON, n = 5) were transplanted into the adult marmoset spinal cord after contusion injury at C5 level. Grafted NSCs survived and migrated up to 7 mm far from the lesion epicenter. Double-staining with TuJ1 for neuron, GFAP for astrocyte, or CNPase for oligodendrocyte and BrdU revealed that grafted NSCs differentiated into neurons and oligodendrocytes 8 weeks after transplantation. More neurofilaments were observed in TP than those of CON. Furthermore, behavioral assessment of forelimb muscle strength using bar grip test and amount of spontaneous motor activity using infrared-rays monitoring revealed that the grafted NSCs significantly increased both of them compared to those of CON. These results indicate that in vitro expanded NSCs derived from human fetal spinal cord are useful sources for the therapeutics of spinal cord injury in primates.

S8.B
AN EFFECTIVE COMBINED THERAPY FOR REMYELINATION AND RESTORATION OF CNS FUNCTION

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Oligodendrocytes (OL) are the cells that form and maintain myelin in the central nervous system (CNS). Demyelination due to disease like multiple sclerosis or trauma is debilitating and it is associated with OL impairment, CNS dysfunction and poor quality of life. Neural transplantation holds promise for CNS repair and remyelination. We previously used the demyelinating 4e mouse characterized by the overexpression of the proteolipid gene. Heterozygous mice develop and myelinate normally. But progressive demyelination of the CNS begins around 4 months of age. We previously showed that CG4 cells when grafted into 4e mice partially remyelinate their CNS. Moreover, cografts of CG4 cells with the trophic factor secreting cell line B104 enhanced the remyelination process. Since B104 cells are malignant, they are unsuitable for therapeutic application. In the present study our aim was to determine if CG4 cells in combination with a cocktail of specific trophic factors "TS1"(UCLA case no.2004-488) grafted into the corpus callosum of 4e mice would support and maintain remyelination of 4e axons. We found that a single administration of CG4-TS1 was enough to support extensive remyelination with a concomitant increase of function of the hind limbs in 4e hosts. The treatment led to a reduction of cold induced seizures and increased grooming, balance and motor function that were sustained for at least three months. Thus, this combined therapy is effective to remyelinate naked axons and restore neurological function.

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S8.C
NEURAL STEM CELLS: FACTS AND MISFACTS

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The finding that the central nervous system (CNS) embodies neurogenetic regions enriched of neural stem cells (NSCs) has spurred a flurry of studies on both their basic biology and their perspective application for the therapy of many neurological disorders. NSCs are multipotential precursors that grow and self-renew for extensive time in culture. It has recently been argued that NSCs undergo rapid, spontaneous transformation, which would represent an obstacle to their use both in therapeutic applications for neurological disorders as well as in basic research. I shall be discussing recent data in our hand, which describe the systematic investigation of the functional properties of NSCs upon long-term culturing and show their lack of transformation. NSCs do not display any sign of transformations neither at early nor at late culture stages. The self-renewal capacity of NSCs – i.e. their ability to generate new stem cells – does not change over time and no chromosomal abnormalities are observed up to passage 30 in human NSCs. Following removal of mitogens, mouse and human NSCs stop dividing and differentiate into neurons and glia with highly reproducible frequency. I will illustrate the lack of any tumorigenic potential of NSCs and their striking capacity for engraftment in the CNS and their utmost clinical efficacy in the context of multiple sclerosis (MS) or Spinal Cord Injury (SCI) as animal model of human pathologies. The peculiarity of these protocol arises from the fact that the NSCs are not transplanted directly into the CNS damage parenchyma, but are rather injected into the cerebrospinal fluid (i.c.) (MS) or intravenously (i.v.) (MS and SCI). By this technique, NSCs enter and distribute in the CNS parenchyma at the site of the lesions.

S8.D
THE DEVELOPMENTAL ROLE OF NEURAL STEM CELLS MAY SUGGEST NOVEL THERAPEUTIC APPLICATIONS

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An intriguing, novel phenomenon with possible therapeutic dividends has begun to emerge from our observations of the behavior of neural stem cells (NSCs) in various mouse models of CNS injury & degeneration. During phases of active neurodegeneration, factors seem to be transiently elaborated to which NSCs may respond by migrating (even long distances) to degenerating regions & differentiating specifically towards replacement of dying neural cells. In other words, NSCs may

'attempt' to emulate in the brain what hematopoietic stem cells do in the periphery: repopulate & reconstitute ablated regions. These 'repair mechanism' may actually reflect the re-expression of basic developmental principles (particularly during particular temporal 'windows' following injury) that may be harnessed for therapeutic ends. In addition, NSCs may serve as vehicles for gene delivery and appear capable of simultaneous neural cell replacement & gene therapy (e.g., with factors that might enhance neuronal differentiation, neurite outgrowth, proper connectivity, and/or neuroprotection). Intriguingly, many of these factors are produced spontaneously by the stem cells based on their state of differentiation and do not require ex vivo genetic engineering (though that technique can be used to enhance the expression of certain molecules). An intricate meshwork of many highly-arborized neurites of both host- and donor-derived neurons emerges, and some anatomical connections appear to be reconstituted. The NSCs altered the trajectory and complexity of host cortical neurites promoting their entrance into the lesion. In a reciprocal manner, tract tracing demonstrated donor-derived neurons extending processes into host parenchyma as far as the opposite hemisphere.

C6 Free radicals as neurochemical signals

C6.A
SYNTHESIS AND ACTION OF NITRIC OXIDE SYNTHASE-DERIVED FREE RADICALS

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The synthesis of nitric oxide (NO) is catalyzed by tightly regulated and highly complex enzymes, termed NO synthases (NOS). In two consecutive reaction steps the amino acid substrate L-arginine is converted to L-citrilline and NO. Oxidation of L-arginine requires molecular oxygen which is reductively activated at a cytochrome P450-type heme group by NADPH-derived reducing equivalents. Unlike classical P450s, NOS requires the pterin cofactor tetrahydrobiopterin (BH4) as 1-electron donor for efficient coupling of reductive O_2 activation to product formation. At subsaturating concentrations of BH4, the uncoupled reaction results in the generation of superoxide instead of NO. The highly anticooperative binding of BH4 to NOS dimers implicates that the enzymes will generate roughly balanced amounts of NO and superoxide over a wide range of BH4 concentrations. Although NO and superoxide react rapidly with each other to form the potent cytotoxin peroxynitrite, the biological chemistry of NO/superoxide differs significantly from that of authentic peroxynitrite. Cellular scavengers, such as superoxide dismutase, GSH and ascorbate can shift the outcome of the reaction towards net formation of free NO or S-nitrosation. Accordingly, depending on the redox-state of the cell, co-generation of NO and superoxide may give rise to a variety of cellular reactions, including oxidation, nitration, nitrosation and glutathiolation of proteins and DNA. The functional consequences of selected protein modifications will be discussed.

C6.B
INTERACTION OF NO AND TRANSCRIPTION FACTORS IN NEURONAL CELLS

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Nitric oxide (NO) is involved in neurogenesis, synaptic plasticity and memory. One of the mechanisms of action of NO is the transcriptional activation of CREB (Ciani *et al.*, J. Neurochem., 82, 1282, 2002; J. Biol. Chem., 277, 49896, 2002). In neuronal cells, NO also negatively regulates proliferation by downregulating N-MYC (Ciani *et al.*, J. Cell Sci., 117, 4727, 2004). During rat cerebellar development, pharmacological block that reduced by 95% nitric oxide synthase (NOS) activity from PD1 to PD3, increased proliferation rate, determined through 3H-thymidine incorporation into DNA, and neurogenesis, revealed through BrdU labeling of granule neurons. Some of these effects were replicated by inhibition of soluble guanylate cyclase, indicating that NO deprivation was mediated by decreased cGMP. Increased proliferation was accompanied by increased expression of N-MYC, a transcription factor essential for the neurogenesis of granule neurons, as well as of cyclin D1. Treatments covering different developmental windows (e.g. PD3–PD5 or PD5–PD7) did not affect neurogenesis nor N-MYC or cyclin D1 expression. To get more information concerning the neurochemical milieu of the effect of NO deprivation on cerebellar neurogenesis, we started to investigate the expression of proteins related to the NO-cGMP cascade. So far, we have noticed a marked increase in the expression of the beta-1 guanylate cyclase subunit from PD3 to PD5. Studies have also been undertaken on effects of neonatal NO deprivation on neurogenesis in the hippocampal dentate gyrus. These results point at the importance of transcription factor activation in NO-regulated processes of brain development.

C6.C
METABOLIC CONTROL ANALYSIS: CONNECTING MOLECULAR MECHANISMS TO CELLULAR RESPONSES

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Metabolic Control Analysis began in exploration of the dependence of the rate of cellular metabolism on the activity of a single enzyme. Generally, this dependence varies continuously with the activity of the enzyme, and is also affected by the activities of other enzymes in the system, so it is truly a system property that must be characterised quantitatively. For most enzymes, small variations in enzyme activity have little impact on metabolic rate. In fact, even large increases in a single enzyme activity from normal will tend not to have much effect. Responses to reductions in enzyme activity behave differently though, even here, more than 50% reduction is usually necessary for a significant impact. Enzymes differ in the reduction in activity needed to produce a significant response at the cellular level, which means that the effectiveness of drugs that inhibit them will vary for reasons apart from the strength of the drug-target interaction. This can be illustrated for different inhibitors of mitochondrial oxidative phosphorylation, and these system-dependent effects need to be considered in the selection of potential drug targets. Similar considerations can be applied to the effects of changes in activity of an enzyme on concentrations of a metabolite, but generally metabolites are more sensitive to variations in enzyme activity. In fact, much of the machinery of metabolic regulation is probably directed to control of metabolite concentrations rather than metabolic rates. Although these analyses have originated in metabolism, similar considerations can be shown to apply to other cell processes, such as signal transduction and the cell cycle, so that cellular responses depend not only on the change in activity of a component, but also the systemic sensitivity of the process to that component.

C6.D
EFFECTS OF FREE RADICAL QUENCHERS IN FOODS: CHEMISTRY AND FANTASY

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Free radical metabolism is altered in aging and in a number of neurodegenerative diseases, including Alzheimer (AD), Parkinson (PD), and motor neuron (ALS) diseases, but unhappily the results of applying this neurochemical knowledge to therapy in humans have been equivocal at best. Over fifty 'free radical quenchers' have been suggested as potentially therapeutic, many on theoretical grounds. Treatment with free radical quenchers such as vitamin E has had notable successes in cellular and animal models of neurodegenerations, but none of these compounds has proven reliably useful in patients. A possible explanation is chemical. Free radicals differ significantly among themselves, as do quenchers – for instance, in their electrochemical potentials. Another possible explanation is quantitative. The amounts of free radical quenchers that can be ingested safely by humans are

relatively small compared to the amounts of endogenous radicals and quenchers generated in normal humans. Both these considerations point to the importance of the cellular 'redox pitch' as a control mechanism for the ratios of oxidized to reduced forms of compounds that can act as electron donors or receptors. In a small, double-blind, placebo-controlled, prospective trial of a mixture of food constituents designed to affect mitochondrial function and therefore free radical metabolism, the progression of AD appeared to be slowed. More extensive investigations are necessary before conclusions about the efficacy of this approach can be drawn. (The preparation described above has been patented.)

C7 Significance of intracellular Aβ peptides in neuronal function and dysfunction

C7.A
CELLULAR GENERATION OF Aβ PEPTIDES

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When amyloid β (Aβ) started to come into vision it was, unlike tau, little more than strange deposits observed in brains of patients suffering from what later became known as Alzheimer's disease (AD). In time focus shifted and Aβ42 is now implicated as the causative factor for AD. Sequencing of Aβ lead to the identification of the amyloid precursor protein (APP) and of γ-secretase, the proteolytic complex generating Aβ. Little is known about the mechanism by which Aβ42 may cause neurodegeneration, even less so its physiological function. Aβ is produced by all cells, however the sub cellular compartments differ. 'Extracellular' Aβ production occurs at the plasma membrane and predominates in non-neuronal cells. Intracellular Aβ production in neurons is complex because not only several compartments are involved, but these differ for Aβ40 and Aβ42. Unlike the secreted Aβ pool, the intracellular Aβ pool contains significant amounts of Aβ42. Typically Aβ42, but not Aβ40, is produced in the endoplasmic reticulum. Aβ42 is additionally produced in the TGN, but here Aβ40 predominates. Production inside the Golgi apparatus has infrequently been observed and while endosomes are critical for the initial β-cleavage and Aβ is usually not produced there. No rule without exception, certain late endosomal compartments may participate in Aβ or Aβ42 production, these include multi lamellar, multi vesicular and the cholesterol sorting compartments. Aβ40 eventually becomes secreted, but the Aβ42 produced in the ER accumulates and secretion has not been observed. An emerging picture is that of different γ-secretase complex functionality for intracellular and secretory Aβ.

C7.B
SUBCELLULAR Aβ ACCUMULATION INDUCED SYNAPTIC ALTERATIONS IN APP MUTANT NEURONS

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Aβ accumulates within neurons of familial Alzheimer's disease mutant APP mice (Tg2576) with time in culture and with aging *in vivo* (Takahashi *et al.*, 2002; 2004). By immuno-gold electron microscopy this Aβ42 accumulation occurs in multivesicular bodies (MVBs) and smaller vesicles. Aβ42 accumulation, and especially oligomerization, in Tg2576 neurons with aging *in vivo* or with time in culture in vitro is associated with ultrastructural pathological alterations within neurites and synaptic compartments. We have further characterized the localization and cellular consequences of Aβ42 accumulation in Tg2576 primary neurons in culture. By immuno-fluorescence microscopy, Aβ42 co-localizes especially with markers of late-endosomes/MVBs. Following internalization of fluorescent-labeled transferrin (Tf) or EGF, we observe that Aβ42 shares more compartments with EGF-EGFR that traffic to late endosomes than with Tf-TfR that traffic to early endosomes. We hypothesize that Aβ accumulation in the late endosomal/MVB pathway promotes early synaptic dysfunction in AD. To evaluate for synaptic alterations in Tg2576 neurons accumulating Aβ we have compared levels of pre- and post-synaptic proteins in Tg2576 compared to wild type neurons with time in culture. Compared to 12 DIV (days in vitro), Tg2576 neurons at 19 DIV demonstrate selective alterations in levels of pre- and post-synaptic proteins. Reductions in synaptic PSD-95 and surface levels of GluR1 occur already at 12 DIV. γ-secretase inhibition in Tg2576 neurons reverses these synaptic alterations.

C7.C
MISFRAMING OF THE APP MESSAGE AND ITS INTRACELLULAR CONSEQUENCES

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Apart from genomic mutations there is evidence that frameshift (+1) proteins resulting from mutations in the mRNA of amyloid precursor protein (APP) and ubiquitin (UBB) are linked to neurodegeneration in Alzheimer disease (AD). The aberrant +1 proteins (APP + 1 and UBB + 1) accumulate intraneuronally in the hallmarks of AD in sporadic forms of AD (Van Leeuwen *et al.*, Science 279, 242ff, 1998). In addition, UBB + 1 accumulates specifically in other tauopathies (FASEB J 17, 2014ff, 2003), in polyglutamine diseases (Hum.Mol.Genet., 13, 1803ff, 2004) and in non-neuronal cells (e.g. Gastroenterology 122, 1878ff, 2002). UBB + 1 is an inhibitor of the ubiquitin-proteasome system and it leads to neuronal apoptosis (e.g. FASEB J. 15, 2680ff, 2001). APP + 1 is present in beaded fibres of young Down syndrome individuals, devoid of neuropathology, and contributes in cell lines to amyloid β (Aβ)40 formation (e.g. Van Dijk *et al.*, J. Neurochem. 90:712ff, 2004), that is the prominent Aβ form in the Flemish type of APP mutation (Kumar-Singh *et al.*, Am.J.Pathol. 161, 507ff, 2002). To understand the role of APP + 1 we have generated transgenic APP + 1 mice. They accumulate APP + 1 in the hippocampal pyramidal cells in the absence of neuropathology and a behavioural phenotype. In agreement with studies on postmortem CSF of individuals lacking neuropathology (Hol *et al.*, J.Biol.Chem 278, 39637ff, 2003), APP + 1 is readily released into the CSF of these transgenic mice. Since APP + 1 has been shown to promote Aβ40 formation in cell lines, APP + 1 transgenic mice could elucidate whether this protein contributes to intraneuronal accumulation of Aβ forms.

C7.D
POSSIBLE ROLES OF ABETA PEPTIDES IN NEURONAL FUNCTION AND DYSFUNCTION

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Intracellular accumulation of A-beta peptides has been observed in CNS neurons in Down Syndrome and early phases of Alzheimer's Disease. We have developed transgenic rats with a phenotype of intracellular A-beta accumulation in the cerebral cortex and hippocampus. We observed that elevated intracellular A-beta disrupts subcellular organelles, notably Golgi cisterns and lysosomes. In the hippocampus we also observed a robust up-regulation of the MAP kinase ERK2 accompanied by an augmented phosphorylation of tau. Persistent ERK2 activation diminished the degree of phosphorylation of p90RSK,

which should have a negative impact in synaptic plasticity. Consistent with the above observation these transgenic rats display moderate behavioural impairments at 16-months of age. In order to ascertain if small, physiological, amounts of endogenous A-beta had an impact on ERK-mediated pathways we investigated in PC12 cells the effects of APP transient transfections on the activity-dependent stimulation of the CRE-directed gene expression. The results obtained would indicate that small levels of endogenous A-beta peptides can facilitate CRE-directed gene expression, a mechanism linked to synaptic plasticity and memory formation. In conclusion, the above observations favor the notion that small amounts of endogenous A-beta facilitates the positive regulation of CRE-directed gene expression while abnormally elevated intracellular A-beta would disrupt neuronal cell function, independently of the postulated toxic effects of extracellular A-beta peptides.

Acknowledgement: Supported by grants from the Canadian Institutes for Health Research.

C8 Unraveling molecular targets for physiological reinforcers and drugs of abuse

C8.A
PSEUDORABIES VIRUS-ASSISTED IDENTIFICATION OF NEURONAL CIRCUITS INVOLVED IN FOOD INTAKE REGULATION

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Despite our understanding of molecular events associated to energy homeostasis, higher-order brain centers responsible for food intake are not yet fully identified. We sought to discover brain areas that project toward peripheral organs involved in the food intake process and to characterize the neurochemical properties of neurons in those areas. To achieve this we have identified neurons belonging to multiple neuronal circuits by means of retrograde neuronal tracing with recombinant pseudorabies viruses (PRVs) encoding the reporter proteins green fluorescent protein (GFP) or red fluorescent protein. PRV strains were injected in the following peripheral organs of male CB57BL/6J or Fvbn mice: submandibular salivary gland, masseter muscle, and circumvallate taste papillae. Infected brains were analyzed by immunofluorescence microscopy. Brain areas belonging to multiple neuronal circuits were defined as putative food intake control centers (FICCs). Our results indicate that discrete amygdaloid nuclei as well as the insular and rhinal cortices are putative FICCs. A further neurochemical classification of specific neuronal subtypes in FICCs has been performed by detecting PRV-infected neurons in FICCs from several GFP transgenic mice lines from 'The Gene Expression Nervous System Atlas (GENSAT) Project' (http://www.gensat.org);/ our results indicate the presence of heterogeneous neuronal populations within the FICCs. Identification of markers for FICC neurons and their genes will provide the basis of studies aimed to probe their contribution to mechanisms involved in the food intake process. Ultimately this should provide new insights into imbalances associated to eating disorders such as anorexia and obesity.

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C8.B
INFLUENCE OF ESTROUS CYCLE ON THE BASAL AND ETHANOL-STIMULATED ACTIVITY OF CORTICAL DOPAMINERGIC NEURONS IN FEMALE RATS

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Recent experimental evidences have shown that progesterone and its metabolites can modulate basal and Ethanol (EtOH)-stimulated cortical dopaminergic (DAergic) neurons activity in male rats. Using microdialysis we now evaluated the influence of estrous cylce on basal and EtOH-stimulated DA extracellular concentrations in the prefrontal cortex (PFC) of female rats. Our results show marked differences in DA extracellular concentrations during the different phases of the estrous cycle. The highest concentration of DA (14.05 ± 0.11 fmol/sample) was observed during the estrous phase; on diestrous it decreased to 6.98 ± 0.06 fmol/sample, reaching the lowest value during the proestrous (3.02 ± 0.06 fmol/sample). The difference in basal extracellular concentration of DA reflected also a different sensitivity of DAergic neurons to a challenge administration of EtOH (0.5 g/kg, i.p.). In fact, while during the estrous phase this dose of EtOH was able to induce a significant increase in DA output (+60%), the same dose was completely ineffective in all the other cycle stages. Ovariectomy completely antagonized the EtOH-induced increase in DA output which was restored by treatment with estrogen but not progesterone. Finasteride and clomiphene pre-treatment antagonized the increase in DA output elicited by EtOH during estrous phase. Our results indicate for the first time that DA basal extracellular concentration in the PFC fluctuate during the estrous cycle and that these fluctuation reflect a different sensitivity of cortical DAergic neurons to EtOH. The differences in basal and EtOH-stimulated activity of these neurons might be a physiologically relevant mechanism underlying the cycle- and gender-related differences in drug sensitivity and in mood states.

C8.C
CANNABINOID–OPIOID BEHAVIOURAL INTERACTIONS IN DRUG ADDICTION AND RELAPSE

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We first demonstrated the existence of a functional specific interaction between the endogenous cannabinoid and opioid systems in reward-related events and drug-taking behaviour. Recently, we reported that acute priming with either heroin or cannabinoid (CB1) receptor agonists readily reinstates extinguished responding for the drug in rats with a previous history of heroin self-administration (SA), thus extending the cross-talk between cannabinoid and opioid receptors to the neural processes underlying relapse phenomena. Accordingly, heroin but not cocaine primings elicits relapse to cannabinoid-seeking behaviour following a long period of withdrawal. Ultimately, we found that cannabinoid-induced reinstatement of heroin-seeking behaviour is significantly attenuated by either the CB1 receptor antagonist SR 141716A (SR) or the opioid antagonist naloxone (NX). Moreover, as trained rats are switched from heroin to CB1 receptor agonist WIN 55,212-2 (WIN) SA, responding rapidly declines on the first day of drug substitution. However, when rats are given access to WIN after 7, 14 or 21 days of extinction, they promptly resume operant behaviour and do self-administer WIN in a manner dependent upon the length of withdrawal period. Notably, cannabinoid intake in abstinent rats is fully prevented by SR but only attenuated by NX pre-treatment. Taken together, our findings strengthen previous hypothesis of a strict functional interaction between cannabinoid and opioid receptors in the modulation of central mechanisms regulating relapsing phenomena, suggesting that the neural substrates triggering relapse to drug-seeking after chronic drug intake may be different from those underlying drug-taking behaviour.

C8.D

INVERSE AGONISTS OF THE $GABA_A$ RECEPTOR AS A NOVEL APPROACH TO TREAT COGNITIVE DYSFUNCTION

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The observation that classical benzodiazepine (BZ) agonists are amnesic, coupled with the preservation of GABA-A receptors in brain areas most affected by Alzheimer's disease (AD), highlighted the GABA-A receptor as a potential target for the treatment of cognitive dysfunction in this neurodegenerative disease. In contrast to the amnesic BZ agonists, the BZ inverse agonists, which attenuate GABA-A receptor function, have been shown to improve performance in animal models of learning and memory. Unfortunately, such non-selective ligands also induce anxiety and convulsions and therefore cannot be used therapeutically. GABA-A receptors containing the α5-subunit account for less than 5% of the total GABA-A receptor population in the brain, but in the hippocampus, a region of the brain associated with learning and memory, they represent 20% of all GABA-A receptors. We have been able to develop selective α5 inverse agonists which significantly enhance performance in two rodent tests of learning and memory (spatial learning in the Morris water-maze and contextual conditioning) but are devoid of the adverse effects associated with activity at other GABA-A receptor subtypes in other brain regions. Symptomatic treatment of AD is currently achieved via cholinesterase inhibitors, which only provide minimal improvement and are associated with adverse effects. Preclinical results with these GABA receptor modulators are encouraging, and if the efficacy and safety profiles of α5 inverse agonists in humans prove to be similar, such compounds would offer significant improvement over existing therapies.

W4 Transgenics and knock-outs in neurochemistry

W4.A
RNAi STRATEGIES FOR CREATING KNOCK-OUT ANIMALS

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Mice transgenic for shRNA expression vectors exhibit bodywide gene silencing, comparable to knockout mice. We aim to develop RNAi into a standardised procedure for conditional gene knockdown in mice to produce mutants with less time and effort as compared to gene knock-out technology. We developed an approach to activate shRNA vectors through Cre recombinase mediated deletion of a loxP-flanked transcriptional stop cassette. Hairpin RNA expression cassettes are inserted into the Rosa26 locus of hybrid ES cells via recombinase mediated cassette exchange (RMCE). An universal acceptor ES cell line harbouring recognition (attP) sites for C31-Integrase allows the efficient insertion of one or more shRNA expression units flanked by recombinase attB donor sites. Engineered ES clones are then injected into tetraploid blastocysts and transferred into pseudopregnant foster females. Through the use of F1 hybrid ES cells completely ES cell-derived males can be obtained at an efficiency of 5% upon tetraploid complementation. These mice can be directly used for an initial phenotype assessment; for follow up studies larger numbers of male and female knockdown mice can be raised through breeding. Following this scheme the production of the first generation of adult RNAi knockdown mice requires 5 months. To study the biochemical regulation of anxiety and depression in mice we apply this technology to knockdown key molecules of the MAPK and Wnt signal transduction pathways for which condtional knockout mice are not available. Presently we derived and tested hairpin expression constructs specific for B-Raf, MEK1, MEK2, GSK3beta, Wnt1, and CRHR1. These lines will be crossed to strains expressing Cre recombinase in neurons and analysed in a behavioural test programme.

W4.B
GENETICALLY-ENGINEERED MOUSE MODELS OF CEREBRAL AMYLOIDOSIS

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Cerebral proteopathy is a unifying term for cerebral neurodegenerative diseases in which aggregated proteins are abnormally deposited in the brain. The hallmark proteopathy is Alzheimer's disease (AD) in which fibrillar amyloid-beta (Abeta) peptide is deposited extracellularly in the form of parenchymal plaques, as well as in the vasculature resulting in cerebral amyloid angiopathy (CAA). To understand how abnormal protein processing and aggregation leads to cerebral amyloidosis, cellular dysfunction, and dementia, several amyloid precursor protein (APP) transgenic mouse models have been generated that develop, to varying degrees, the age-related deposition of parenchymal vs. vascular amyloid. Results reveal that shifting the ratio of neuronally-produced Abeta40:42 towards Abeta42 favors the production of parenchymal amyloid plaques, while shifting the ratio towards Abeta40 favors Abeta deposition in the form of cerebral amyloid angiopathy. Cerebral amyloidosis can be initiated and accelerated in a time- and concentration-dependent manner by the intracerebral injection of AD brain extracts into young transgenic mice. However, injections of various synthetic amyloid preparations do not induce amyloidosis, suggesting that additional factors such as chaperones, or different Abeta conformations, are necessary for the initiation of cerebral amyloidosis *in vivo*. The further understanding of the mechanism of cerebral amyloidosis and its relationship to neuronal dysfunction is crucial for current developments in anti-Abeta therapeutic strategies.

W4.C
DEVELOPMENT OF DOPAMINERGIC NEURONS – LESSONS FROM KNOCK-INS, KNOCK-OUTS AND CONDITIONAL MUTAGENESIS

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Midbrain dopaminergic (mid-DA) neurons play a pivotal role in the control and modulation of different brain functions and their degeneration or dysfunction leads to severe neurological and psychiatric disorders, among them Parkinson's Disease (PD). Therapeutic strategies for PD include the replenishment of the degenerating by healthy mid-DA neurons that were generated either in vitro or *in vivo*. Therefore, understanding the normal development of this cell population is also of high clinical interest. Despite the importance of mid-DA neurons, the molecular mechanisms controlling their development are still poorly understood. During embryogenesis, midbrain dopaminergic neurons are specified rostral to the mid/hindbrain boundary (MHB). We have previously shown that the position of the MHB determines the location and size of the midbrain dopaminergic cell population *in vivo*. Furthermore, it was shown in explant cultures that Fgf8, secreted from the rostral hindbrain at the MHB, and Shh, secreted from the floor plate of the neural tube, are necessary and sufficient for ectopic induction of mid-DA neurons (Ye *et al.*, 1998). The secreted glycoprotein Wnt1 is expressed in close vicinity to developing mid-DA neurons in the cephalic flexure of the mouse embryo. Using different gain-of-function and loss-of-function mouse mutants, we now provide evidence for the first time that Wnt1 controls a genetic network including Otx2 and Nkx2-2 which leads to the establishment of the mid-DA progenitor domain during early neural development. In addition, Wnt1 is directly required for the terminal differentiation of mid-DA neurons *in vivo*. Furthermore, in the absence of Wnt1, neither Fgf8 nor Shh are sufficient to induce ectopic mid-DA neurons in explant cultures.

W4.D
STEM CELLS, REPROGRAMMING AND CANCER

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Nuclear transfer experiments allow studying the role of epigenetic modifications in cellular differentiation and transformation. Moreover, nuclear transfer provides a tool to derive autologous embryonic stem cells for a potential use in cell therapy. We have shown that nuclear transfer of mature lymphocytes produces monoclonal mice that carry a single antigen receptor in all tissues, thus demonstrating that the nucleus of a terminally differentiated cell can be reprogrammed to

totipotency. Likewise, we have shown that the genomes of certain cancer cells are amenable to epigenetic reprogramming and can support partial development, indicating that the epigenetic state of at least some cancers may be reversible. To demonstrate the potential use of nuclear transfer for cell therapy, we have established a mouse model of 'therapeutic cloning' by combining nuclear transfer with gene and cell therapy to treat a genetic disorder in a mouse model of disease. We will discuss the potential factors involved in epigenetic reprogramming with an emphasis on the homeobox transcription factor Oct-4 and its role in differentiation and tumorigenesis. These experiments exemplify the advantage of the mouse model as a system to study mammalian development, differentiation and disease.

C9 Transglutaminases in neuronal function and dysfunction

C9.A
TRANSGLUTAMINASES IN PERSPECTIVE

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Transglutaminases are a large, enigmatic family of Ca^{2+}-dependent acyl transferase-enzymes that mediate a myriad of biological reactions, including those involved in important processes, such as blood coagulation, skin-barrier formation, bone mineralization, extracellular matrix assembly and wound healing. These reactions are all dependent on the rapid generation of covalent crosslinks within proteins, or the post-translational modification of proteins by primary amines or lipids. Some TGs function as molecular switches in signal transduction, in cytoskeletal scaffolding and in modulating protein–protein interactions. Of these, the generation of oligomeric or polymeric protein assemblies by the formation of intrachain isopeptide bonds between a Glu acceptor and deprotonated Lys donor, is the best studied. However, amine incorporation into a Glu acceptor residue, acylation of a Lys side chain in a donor protein, and even esterification or deamidation of a Glu residue are well-recognized TG-catalyzed reactions. Three structurally-characterized families have been identified – the papain-like TGs (including the eukaryotic enzymes, fXIIIA, human TG2 and TG3), those with both disulfide isomerase and TG activity, and those of bacterial toxins. Only the first of these, which are members of the papain-like superfamily of cysteine proteinases and include also papain, calpain, foot and mouth virus protease, deubiquitylating enzymes and N-acetyl transferases, will be considered here in detail. This family is encoded by nine genes in the human genome. Of these, one codes for a catalytically inactive homologue, band 4.2, that acts as an important structural component of the erythrocyte cytoskeleton. The structure, functions and catalytic activity of the eukaryotic TGs, and their potential relevance to neuropathology will be considered in this presentation.

C9.B
CROSSLINKS IN THE CORE AND PERIPHERY OF LEWY BODIES: DISTINCT ROLES AND DIFFERENT TRANSGLUTAMINASES?

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α Synuclein immunopositive particles from brains of Lewy body dementia patients were subjected to increasing times of trypsin digestion. The amount and sequence location of γ-glutamyl-ε-lysine (GGEL) crosslinks was assessed by mass spectrometry in the time fractions. Early digestion products from the periphery of the bodies contained mainly HSP27-Ubiquitin and some α Synuclein Gln^{99}-Ubiquitin Lys^{29}-crosslinks, similarly to Alzheimer's neurofibrillary lesions. Prolonged digestion eventually produced an α Synuclein-rich, trypsin-resistant core, which was soluble in guanidine-HCl. The relative abundance of GGEL was ≈1 crosslink in 270 α Synuclein molecules. Mass analysis of the crosslinked proteins showed that Gln^{79} and Lys^{58} residues of α Synuclein were transglutaminated to form an intramolecular bond. Transfection studies with α Synuclein and Transglutaminases (TGases) 1, 2, 3, and 5 showed that overexpression of α Synuclein results in the formation of ubiquitylated cytoplasmic aggregates, which were rapidly cleared in the absence of TGases 2 or 3. *In vitro*, the latter TGases formed chiefly intermolecular crosslinks and showed a promiscuous utilization of all glutamines and lysines of α Synuclein. Only TGase1 formed specifically intra-synuclein Gln^{79}–Lys^{58} bonds if α Synuclein was allowed to attach to membranes. We hypothesize that the formation of an intramolecular crosslink in α Synuclein by TGase1 may affect the attainment of the propagative conformation, which recruits the protein into fibrils and suprafibrillar assemblies. Whereas, the crosslinking of peripheral ubiquitin moieties by the soluble TGases may contribute to the blockade of cellular elimination mechanisms, such as proteasomal breakdown and autophagy.

C9.C
TRANSGLUTAMINASES IN HUNTINGTON DISEASE (HD)

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Transglutaminases (TGases) catalyze the Ca^{2+}-dependent linkage of a K to a Q residue in proteins/peptide substrates forming an N^{ε}-(γ-L-glutamyl)-L-lysine (GGEL) isopeptide bridge. Protein cross-linking is increased in HD brain as evidenced by a 3-10-fold increase of GGEL levels in the CSF and autopsied brain of HD patients. Excess TGase activity may contribute to the pathogenesis of HD. Two groups have shown that the *in vitro* TGase inhibitor, cystamine, increases survival in R6/2 mice (an HD model). Cystamine in cell culture models inactivates caspase 3 (implicated in HD cell death) and increases levels of the anti-oxidant glutathione (GSH). Mechanisms other than TGase and caspase inhibition may afford protection *in vivo*. Recent studies show that cystamine-treated R6/2 mice exhibit no change in brain GSH, but exhibit an increase in cysteine, another antioxidant. We have extended these results using cystamine-treated YAC128 mice (another HD model) and their WT littermates. Our studies show that levels of cystamine and the potential cystamine metabolites, cysteamine and hypotaurine, were below the limit of detection in the plasma and brains of cystamine-treated YAC128 mice (≤0.2 nmol/mg of protein). Taurine, a potential product of cystamine metabolism was not increased in brains of these mice. Influx of cysteine-cysteamine mixed disulfide followed by reduction/disulfide exchange may account for increased cysteine. The released cysteamine is probably rapidly converted to as yet unidentified metabolites. Identification of these metabolites may offer clues as to the protective effect of cystamine and suggest better treatment for HD whether or not TGase inhibition is involved.

C9.D
TISSUE TRANSGLUTAMINASE: A DOUBLE-EDGED SWORD

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Tissue transglutaminase (tTG) is a multifunctional protein that plays a modulatory role in numerous cellular processes. tTG has transamidating and GTPase activities, and is also likely to function as a scaffold protein. One critically important process that is regulated by tTG is

apoptosis. Early studies clearly demonstrated that tTG could act as a pro-apoptotic protein, and although the exact mechanisms involved have not been elucidated, these findings have been thoroughly substantiated. In addition, there is increasing evidence that tTG may contribute to neuronal dysfunction and death in several neurodegenerative diseases including Huntington's disease and Alzheimer's disease. Although the data strongly indicates that tTG is not facilitating the formation of the insoluble, fibrillar aggregates that occur in these diseases, recent findings suggest that tTG could contribute to the stabilization of toxic oligomers of the disease-specific proteins. Even though it is evident that tTG can be detrimental to neuronal cell survival, recent findings have demonstrated that depending on the apoptotic signal and the intracellular location of tTG, tTG can also protect cells against cell death. Further, tTG can also facilitate pro-survival processes. The protective functions of tTG may be due to its ability to enhance the production of cAMP and also regulate nuclear events that result in activation of pro-survival processes. Overall, it is clear that tTG is capable of facilitating neuronal dysfunction and death. However, depending on the situation tTG also can contribute to neuronal survival. Therefore, tTG can be viewed as a 'molecular switch' that plays a role in determining how a cell responds to its environment.

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C10 Molecular mechanisms of cell signaling

C10.A
A POSITIVE FEEDBACK LOOP BETWEEN GSK3β AND PP1 AFTER STIMULATION OF NR2B NMDA RECEPTORS

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N-methyl-D-aspartate receptors (NMDARs) are critical for neuronal plasticity and survival, while their excessive activation produces excitotoxicity and may accelerate neurodegeneration. Here, we report that stimulation of NMDARs in cultured rat hippocampal or cortical neurons and in the adult mouse brain *in vivo* disinhibited Glycogen Synthase Kinase 3β (GSK3β) by protein phosphatase-1(PP1)-mediated dephosphorylation of the serine 9 residue. NMDA-triggered GSK3β activation was mediated by NMDAR that contained the NR2B subunit. Interestingly, GSK3β inhibition reduced inhibitory phosphorylation of the PP1 Inhibitor 2 (I2) and attenuated serine 9 dephosphorylation by PP1. These data suggest existence of a feedback loop between GSK3β and PP1 that results in amplification of PP1 activation by GSK3β. In addition, GSK3β inhibition decreased PP1-mediated dephosphorylation of the cAMP-response element binding protein (CREB) at the serine 133 residue in NMDA-stimulated neurons. Conversely, overexpression of GSK3β abolished non-NR2B-mediated activation of CRE-driven transcription. These data suggest that crosstalk between GSK3β and PP1 contributes to NR2B NMDAR-induced inhibition of CREB signaling by non-NR2B NMDAR. The excessive activation of NR2B-PP1-GSK3β-PP1 circuitry may contribute to the deficits of CREB-dependent neuronal plasticity in neurodegenerative diseases.

C10.B
A NOVEL INTERCELLULAR COMMUNICATION SYSTEM, THE CD47-SHPS-1 SYSTEM, IN NEURONAL NETWORK

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SHPS-1 (SHP substrate-1) and its ligand CD47 are immunoglobulin superfamily membrane proteins. The cytoplasmic region of SHPS-1 is tyrosine phosphorylated in response to growth factor and binds to protein tyrosine phosphatase, SHP-2. CD47 is a penta-transmembrane protein, which is cis-associated with the integrins. Both are highly expressed in the brain and constitute an cell–cell communication system (the CD47-SHPS-1 system), while its function in the CNS is unknown yet. Here, we analyzed expression patterns of these two molecules by using primary cultured hippocampal neurons. We found that SHPS-1 and CD47 were localized in a different manner; the former was predominantly on axons and dendrites and the latter on dendrites, respectively. Exogenous expression of SHPS-1 and CD47 in cultured neurons also confirmed this observation. We have also found that forced expression of CD47 induced neurite extension in N1E-115 neuroblastoma cells. Furthermore, an Fc fusion protein containing the extracellular region of the CD47 ligand SHPS-1 induced filopodium formation, and this effect was enhanced by CD47 overexpression. Inhibition of Rac or Cdc42 preferentially blocked CD47-induced neurite and filopodium formation, respectively. Furthermore, a monoclonal antibody to the integrin beta3 subunit also inhibited neurite extension induced by CD47. These results suggest that differential localization of SHPS-1 and CD47 at axons and dendrites generates a directional cell-cell communication system in the CNS, and that the interaction of SHPS-1 with CD47 regulates morphological changes of neuronal cells through the function of Rac, Cdc42, and integrins containing the beta3 subunit at downstream of CD47.

C10.C
TROPHIC FACTORS, TYROSINE RECEPTOR KINASES AND MEDIATED SIGNAL TRANSDUCTION MECHANISMS: IMPLICATIONS IN SUICIDAL BEHAVIOR

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Accumulating evidence suggests structural abnormalities in the brain of affective disorder patients, who constitute a significant subpopulation of suicide victims, including loss of neurons and decreased glia numbers. In addition, stress, which is closely associated with suicidal behavior, causes atrophy and loss of neurons. The molecular and cellular nature of the events that may lead to such abnormalities, however, is not known. One of the epigenetic factors that regulate cell survival is neurotrophic factors and their functional response mediated by tyrosine receptor kinases (Trk) and cell survival pathways, including extracellular signal-regulated kinases (ERK). To examine whether abnormalities occur in neurotrophic factors and/or in their signaling mechanisms, we investigated expression of BDNF, TrkB and activation and expression of ERK1 and ERK2, MAP kinase phosphatase (MKP)-2, responsible for deactivation of ERK1/2, in postmortem brain of suicide victims. We observed that expression level of BDNF was significantly decreased in PFC and hippocampus of suicide victims. We also observed reduced expression of Trk B, which was associated with only full-length, but not truncated Trk B. In the ERK pathway, activation of ERK1/2 was significantly decreased in both PFC and hippocampus of suicide victims, which was associated with decrease in mRNA and protein expression of both ERK1 and ERK2. On the other hand, the expression of MKP-2 was significantly increased. Our results demonstrate decreased trophic support, functionality, and synaptic plasticity in brain of suicide victims. These abnormalities may serve as important vulnerability factor in the pathophysiology of suicide.
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C10.D
THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN THE PERIPHERAL NERVOUS SYTEM

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Basic fibroblast growth factor (FGF-2) is found in low- and high-molecular weight isoforms that are strongly up-regulated in response

to peripheral nerve lesion. The present study investigates the subcellular localization, regulation and functions of FGF-2 isoforms in peripheral ganglia after axotomy. In postganglionic sympathetic neurons obtained from the neonatal rat superior cervical ganglion FGF-2 is detected in the nucleus and in perinuclear Golgi fields. Biolistic transfection of plasmids encoding FGF-2 isoforms fused to fluorescent proteins demonstrates nuclear targeting of 18kDa FGF-2 and 23kDa FGF-2 with prominent accumulation in the neuronal nucleolus. A similar distribution of FGF-2 and FGF receptor type 1 is detected in sensory neurons derived from adult lumbar dorsal root ganglia. In this model, FGF-2 isoforms only marginally promote axon elongation but significantly increase the number of axonal branch points in dissociated cultures. In response to a preconditioning lesion, i.e., transection of the sciatic nerve one week before culture, the axonal length of ipsilateral lumbar sensory neurons increases twofold when compared to non-lesioned control rats, and this response is significantly enhanced by FGF-2 isoforms but not by NGF. The stimulating effects of FGF-2 isoforms on axon growth are fully blocked, and the enhanced regeneration of pre-lesioned neurons is reduced by the FGFR inhibitor SU5402 suggesting an involvement of endogenous FGF signalling in peripheral nerve lesion models.

W5 New approaches in neurodegenerative disease research and diagnosis

W5.A

CORRELATING MICROARRAY DATA WITH FUNCTIONAL/BEHAVIORAL MARKERS REVEALS NEW CLUES TO ALZHEIMER'S DISEASE AND BRAIN AGING

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Gene microarrays provide a powerful new approach for addressing the complexity of neurodegenerative processes, in that they allow more comprehensive overviews of the interplay among processes and of the context in which any specific molecule or pathway may be operating. However, the other side of the coin is that microarray analyses are costly and generate such massive amounts of data that it is extremely difficult either 1) to analyze the results using appropriate statistical power and methods or 2) to draw inferences regarding the functional relevance of any of the hundreds of changes that may be detected. In our studies of aging-related neurodegenerative conditions, we have attempted to counter the first problem by using large samples of independent subjects and a separate microarray chip for each. This allows application of formal statistical approaches, largely obviating questions of validity specific to microarrays. For the second problem, we have developed a strategy of correlating microarray changes with behavioral/functional markers of the condition being studied. In our study of Alzheimer's disease (AD) (Blalock *et al.*, 04), we correlated hippocampal expression of each of thousands of genes with two established markers of AD, the Mini-Mental Status Exam and a neurofibrillary tangle index, and in a study of normal aging in rats (Blalock *et al.*, 03), we correlated gene expression with maze learning performance. These approaches revealed involvement of pathways not previously suspected and illuminated the complexity of processes already implicated. Together, the results have provided new clues and generated new models of the pathogenesis of AD and brain aging.

W5.B

SURFACE PLASMON RESONANCE SPECTROSCOPY FOR THE ANALYSIS OF β-AMYLOID INTERACTIONS IN ALZHEIMER'S DISEASE RESEARCH

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Alzheimer's disease (AD) is characterised by the accumulation of amyloid deposits, the major component of which is the 4 kDa β-amyloid protein (Aβ). Aβ has been shown to interact with a number of intracellular and extracellular molecules, but the relative contribution of these interactions to the toxicity of Aβ is not well understood. A critical step in characterising the importance of these interactions is to measure both the affinity and kinetics of these interactions. Our studies have shown that Aβ is toxic to vascular smooth muscle cells in culture and one mechanism underlying this toxicity may be that Aβ can bind to membranes. Surface plasmon resonance (SPR) spectroscopy has become a widely used optical biosensor-based technique to study molecular interactions and we have used SPR to study Aβ binding to membranes. The results showed a very good correlation between membrane binding and toxicity. Furthermore, binding was strongly influenced by the concentration of cholesterol in the membrane. Moreover, cholesterol biosynthesis inhibitors (statins) decreased membrane binding and also decreased toxicity. Ageing the peptides by incubation for 5 days increased the proportion of oligomeric species and also increased toxicity and membrane binding. The results strongly suggest that Aβ toxicity is a direct consequence of binding to lipids in the membrane. The application of SPR to the study of the molecular interactions associated with AD and how this information enhances our molecular understanding of Aβ-mediated toxicity will also be discussed.

W5.C

ATOMIC FORCE MICROSCOPY OF AMYLOID PROTEINS: EXAMINING REAL-TIME 3D CONFORMATIONS AND ROLE IN PROTEIN CONFORMATIONAL DISEASES

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Protein conformational diseases, including neurodegenerative (Alzheimer's, Parkinson, Huntington, prion encephalopathies, familial British & Danish dementias), systemic (type II diabetes, light chain amyloidosis) and other (cystic fibrosis) diseases result from protein misfolding that alters their 3D conformation from native (often soluble) to non-native (often insoluble) folded structures. Prevailing dogma suggests that misfolded proteins assume fibrillar feature termed amyloid that results into a gain-of-function and induce pathophysiologic cell response by altering cell membrane composition and destabilizing ionic homeostasis. Recent studies, however, indicate that only their globular conformation is sufficient to induce pathophysiology. Thus, understanding such misfolding and the resulting 3D conformations is the most important and yet challenging area of research. We have used atomic force microscopy to examine native 3D structure of single amyloid molecules, their real-time oligomerization/fibrillization, and their interaction with lipidic membrane. We will present our results from work on various amyloids and their physiological roles. For example, we show, for the first time, that these peptides when reconstituted in vesicles or reconstituted in lipid bilayers, form ion-channels that induce ionic currents are selective to cations (e.g., Ca). The channel activity is regulated by appropriate antagonists and blockers and induce nanoscale, fast and dose-dependent cell degeneration.

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W5.D

TERTIARY STRUCTURE OF AMYLOID-BETA OF ALZHEIMER'S DISEASE VISUALIZED BY SCANNING PROBE MICROSCOPY

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Accumulation of the β-amyloid protein (Aβ) in the brain is generally considered to be the cause of Alzheimer's disease. Aβ can aggregate to form toxic protofibrils and fibrils. However, the orientation of Aβ monomers within Aβ protofibrils and fibrils is unknown. In this study, we examined the structure of Aβ1-40 by atomic force microscopy (AFM) and scanning tunnelling microscopy (STM). As seen by AFM, low concentrations of freshly preparared Aβ were deposited on a surface of highly oriented pyrolytic graphite (HOPG) and atomically flat gold as distinct globular structures. However, after incubation of the Aβ1-40 for 5 days at 37°C (ageing) short protofibrils composed of linear sequences of globular units were observed. To examine the structure of the globular units, STM was employed. On a surface of atomically flat gold, we achieved unprecedented sub-nanometer resolution of the tertiary folding of the globular structures. Each structure was 3–4 nm wide and consisted of a single molecule of Aβ. The polypeptide chain of each Aβ monomer was highly folded into 4 domains. Protofibrils were formed by the end-to-end association of monomers in which the polypeptide backbone was oriented at approximately 90° to the longitudinal axis of the protofibril. This study provides the first report of a protein tertiary structure visualized by STM and it suggests a mechanism for Aβ protofibril assembly.

W6 Survival skills for career development in neurochemistry

W6.A
SURVIVAL SKILLS FOR CAREER DEVELOPMENT IN NEUROCHEMISTRY

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A career is difficult to define in broad terms, yet most young professionals know what they desire from their careers in terms of achievement, satisfaction, and lifestyle. At the outset of a career, perhaps the most important question will be, "where do I want to be 10, 20, and 30 years from now?" Prioritizing individual goals, such as financial success, notoriety, good science, and altruism are important steps towards "knowing what you want." Once answers are framed for these "where and what" questions, exploring options for the "how" becomes appropriate. In other words, you will never arrive *where* you want to be unless you know where you are going. This workshop focuses on the equally important question, "how do I get there" once the "what and where" questions have been settled. We will particularly emphasize careers in academic/industrial research. As with any endeavor, success starts by listening to and engaging with experienced and successful people who have gone before us. Panelists in this workshop where chosen on the basis of their highly successful careers – even though no two paths are alike. The "whys" and "wherefores" of their individual successes will be discussed in depth with special attention to the "how." Analyzing strengths and weaknesses, seeking out quality training environments, and developing confidence, will be explored for each individual. Tremendous benefit can be gleaned by understanding the actions and skills employed by these individuals even though each person has diverse starting points and different end points. As we will see, there are many paths to a successful career and choosing the right one is both an individual choice and a stochastic process. Our goal will be to provide a compass and useful tools that will help guide scientists along their individually mapped career itinerary.

W6.B
SUCCESS ON THE CAREER PATH FROM POSTDOC TO JUNIOR FACULTY

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Postdoctoral research overseas or in your own country is tortuous, but the next career step requires calculated and continued planning. Decisions on the choice of a second postdoc or junior faculty position should consider the following: will the move advance your career in terms of research quality, opportunities for improving your skills, facilities and funding, the lab size, and the entrepreneurial or dictatorial style of the lab/department head. Will you grow as a scientist, teacher and person? Alternatively, the choice may be "getting a foot in the door" to gain vital research experience, or to initiate a career in research and/or teaching. As the "new kid on the block" you must create a good impression always, be it your first seminar, opportunity to give a few lectures or taking up the offer to supervise small or large student projects (lab- or non-lab-based). Helping to supervise research students generally means you get to be a co-author on subsequent publications. However, make sure you leave a few days to work in the lab yourself. Remember it's "publish or perish", so any science you are involved with collaboratively or personally (especially stuff from your first postdoc) should be written up quickly and professionally! Many countries and institutions have start-up funds, often small in value, and you should be positive in writing 1–2 per year for research support, equipment and travel to conferences. Remember to survive in research you need to attend overseas conferences, especially if not based in North America. At every opportunity assist in the organization of events associated with local neuroscience meetings. Being professional and producing good neurochemical data will get a good reference for the next step or the opportunity for promotion.

W6.C
COMING TO AMERICA: THE CHALLENGES FACING TRAINEES IN THE USA

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Graduate and post-doctoral training in the United States is a highly valued experience. Ideally, laboratories in the USA can offer a high quality research experience in an environment that is intellectually rich, well supported in financial terms, well endowed with the most recent equipment and resources, and all contained within a cultural and political environment that should be considered unique. However, trainees arriving in the United States also face a number of important hurdles that must be surmounted in order to allow the training process to occur. These include the difficulties in finding a laboratory that can offer financial support to foreign trainees, and an ever-growing burden associated with obtaining visas. Beyond that, the nature of the scientific experience can range from the truly outstanding, under the guidance of the caring and nurturing mentor, to something that more closely resembles slave labor in the hands of the over-achieving but unscrupulous advisor.

In this presentation I will discuss effective approaches to securing training positions in American laboratories, and describe the steps that have to be completed for such training to occur. I will also discuss strategies that will increase the chance that the training experience will be an enjoyable and fruitful one, and approaches to managing situations that are less than ideal. The presentation will mostly be focused on post-doctoral training, although I will address Ph.D. education as well.

Acetylcholine

P.138

EVALUATION OF THE COGNITIVE ENHANCING PROPERTIES AND ANTICHOLINESTERASE ACTIVITY OF RAPHIASAPONIN 1 IN ADULT MICE

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In this study, we evaluated *in vivo* the effect of raphiasaponin 1, isolated from raphia hookeri fruit mesocarp, on the hippocampal acetylcholinesterase activity, and cognitive impairment in adult mice. Mice (four months) were administered raphiasaponin1 (5, 10, 20 mg/kg, i.p) For seven days and controlled mice received daily i.p injections of saline followed by injection of scopolamine (1 mg/kg body weight i.p). After the final treatment, passive avoidance tests and performance in the Morris water maze were assessed while the activities of hippocampal AChE and ChAT were measured. Raphiasaponin1 significantly decreased hippocampal AChE activity in a dose dependent manner, as well as ameliorate, the learning and memory deficits induced by scopolamine. The results suggest that raphiasaponin1 could be a useful agent for the treatment of memory deficit related to CNS disorders.

Key words: Raphiasaponin 1, Acetylcholinesterase, Scopolamine, Avoidance Learning, Raphia hookeri, adult mice.

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EFFECT OF INTERLEUKIN-1 BETA AND AMYLOID-BETA ON MURINE CHOLINERGIC NEUROBLASTOMA T17 CELLS

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Amyloid-beta accumulation in brains of Alzheimer's disease patients is accompanied by glial inflammatory reactions and preferential loss of cholinergic neurons. The aim of this study was to find out whether interleukin 1beta (IL1b) modifies A-b effects on septum derived cholinergic neuroblastoma cells. Dibutyryl-cAMP and all-trans-retinoic acid resulted in differentiation of T17 cells (DC) evidenced by over two-fold increase of choline acetyltransferase (ChAT) activity and acetylcholine (ACh) content and four-fold increase of its release. In nondifferentiated cells (NC) A-b (1 μg/ml) alone brought about no changes of ChAT activity, ACh release and acetyl-CoA intracellular distribution. IL1b (10 ng/ml) had no effect on ChAT activity but activated ACh release in NC. Joint addition of A-b and IL1b increased activity of ChAT, had no effect on ACh release but elevated the fraction of non-viable NC. In DC A-b inhibited both ChAT activity and ACh release and decreased the content of acetyl-CoA in cell cytoplasmic compartment. IL1b elevated ChAT activity (54%), with no significant effect on remaining parameters. A-b abolished activatory effect of IL1b along with increase of cell mortality. On the other hand, IL1b fully reversed the inhibitory effects of A-b on cytoplasmic acetyl-CoA and ACh release, but did not decrease high rate of DC mortality. These data provide evidence for the existence of phenotype-dependent variable reciprocal interactions between IL1b and A-b in their cholinotrophic or cholinotoxic effects on cholinergic cells. They also indicate that IL1b in low concentrations may help to maintain proper level of cholinergic transmission despite increased cholinergic cell damage in neurotoxic conditions.

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MODULATION OF CHOLINERGIC TRANSMISSION BY BRAIN-DERIVED NEUROTROPHIC FACTOR AND A_{2A} ADENOSINE RECEPTORS IN THE RAT HIPPOCAMPUS

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Modulation of glutamatergic transmission by brain-derived neurotrophic factor (BDNF) is facilitated by the activation of A_{2A} adenosine receptors (Diógenes *et al.*, J Neurosci 24:2905). We now investigated if BDNF modulates hippocampal cholinergic transmission at the pre and postsynaptic levels and if its action is influenced by previous activation of A_{2A} receptors. Release of [^{3}H]acetylcholine (ACh) from hippocampal slices was evoked by electrical stimulation (240 pulses, 0.1 ms, 2 Hz). Uptake of [^{3}H]choline (Ch) by hippocampal synaptosomes was initiated adding [^{3}H]Ch (0.3 μM) and stopped by filtration. Whole-cell patch clamp was used to record cholinergic postsynaptic currents (PSCs) from interneurons activated with pressure application of ACh (500 μM). BDNF (20 ng/ml) increased the evoked release of [^{3}H]ACh by 18.7 ± 4.58% ($P < 0.05$, $n = 4$), an effect prevented by a previous blockade of A_{2A} receptors with SCH 58261 (100 nM) and potentiated (47.6 ± 8.89%, $P < 0.05$, $n = 3$) by the activation of A_{2A} receptors with CGS 21680 (30 nM). Neither BDNF (20 ng/ml) nor CGS 21680 (30 nM) appreciably modified the uptake of [3H]Ch by synaptosomes. However, their simultaneous application slightly enhanced the choline uptake by 9.4 ± 0.82% ($P < 0.05$, $n = 3$). Cholinergic PSCs were decreased by BDNF (20 ng/ml) (−14.4 ± 4.24%, $P < 0.05$, $n = 3$) and CGS 21680 (30 nM) (−20.4 ± 1.48%, $P < 0.05$, $n = 5$), but these drugs did not act synergistically when applied together. Our results suggest that BDNF facilitates hippocampal cholinergic transmission at both pre and postsynaptic levels but only its presynaptic action is dependent on adenosine A_{2A} receptors activation.

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SUBSTANCES FROM PLANTS WITH ACETYLCHOLINE ESTERASE INHIBITORY (ACHEI) ACTIVITY IMPROVE MEMORY IN CHOLINERGICALLY IMPAIRED RODENTS

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In efforts to develop novel AChE-Is with better pharmacological properties, Scopoletin (S) emerged as active substance from virtual screen-

ing of natural products. Screening of crude extracts from a plant X revealed compounds with AChEI activity. S and some of the isolated compounds of X showed in vitro and *in vivo* AChEI potencies comparable to galantamine. Micro-injection of S or substance I (isolated from X) into the lateral ventricle of the anaesthetized rat increased the extracellular level of acetylcholine in the nucleus accumbens (NA). We also tested in object recognition and T-maze alternation task whether these compounds (icv) can ameliorate scopolamine (SC)-induced amnesia. Object memory was nearly abolished by SC. This inhibition was almost restored by S and I. Both drugs increased the alternation rate of untreated mice and reversed the SC-induced decrease in the T-maze, suggesting facilitation of spatial working memory. Object recognition in rats was tested with intra-accumbally injected S in untreated rats and in rats pretreated intra-accumbally with MT7 an irreversible selective M1 ACh receptor antagonist which impairs visual memory in this test. Administration of S into the rat NA improved recognition and abolished the MT7 induced deficit. Our results demonstrate the ability of the compounds to ameliorate SC-induced deficits through their AChEI properties. S and I and derivatives may be useful in the treatment of disorders with cholinergic hypofunction.

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ESTABLISHMENT OF POSITIVE COOPERATIVITY BETWEEN N-METHYLSCOPOLAMINE AND WIELAND-GUMLICH ALDEHYDE BINDING TO M3 MUSCARINIC RECEPTOR

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We have investigated molecular determinants of allosteric interactions between Wieland-Gumlich aldehyde (WGA) and the classical antagonist N-methylscopolamine (NMS) at muscarinic M_3 receptors. The entire third extracellular loop (o3) of the M_3 muscarinic receptor was replaced with the corresponding sequence of M_2 receptor, or individual amino acids in the o3 loop were mutated to corresponding residues of M_2 receptor. Mutated receptors were expressed in COS-7 cells and their binding properties were determined in binding studies. Despite both parental subtypes (M_3wt and M_2wt) display negative cooperativity between NMS and WGA, exchange of the o3 loop of M_3wt switches original negative cooperativity at both native receptor subtypes to the positive cooperativity at chimeric receptor. This is the first demonstration that transposition of a sequence derived from receptor subtypes displaying negative cooperativity for a given pair of ligands to an analogous place of another receptor subtype that also exhibits negative cooperativity, constitutes the positive cooperativity of these ligands. Gradual replacement of individual amino acids in the o3 loop revealed that only three residues (N419, V421, and T423 of M_2 sequence) are involved in this effect. These observations are important for understanding mechanisms of conformation changes induced by allosteric modulator binding and for targeted design of chemical structure of allosteric ligands with specific properties.

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XANOMELINE QUASI-IRREVERSIBLY BOUND TO AN ECTOPIC SITE CAN STIMULATE PRESYNAPTIC M2 RECEPTORS VIA THE ORTHOSTERIC BINDING SITE

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Xanomeline is a muscarinic agonist which binds to all subtypes of muscarinic receptors with similar affinity, but in functional tests displays selectivity for the M1 and M4 subtypes. We observed previously that xanomeline binds reversibly to the orthosteric site and also to an ectopic site in a wash-resistant manner. In the present experiments, we investigated whether wash-resistant binding of xanomeline affects M2-receptor-mediated presynaptic regulation of acetylcholine (ACh) release from rat cortex slices. To this end we incubated cortical slices loaded with labelled ACh for 15 min in the presence of 100 μM xanomeline and then after extensive washing measured electrical stimulation-evoked release of ACh. Preincubation with xanomeline strongly decreased evoked ACh release to a level comparable with the full agonist carbachol present during stimulation and these inhibitory effects were not additive. Presence of N-methylscopolamine during preincubation with xanomeline did not counteract xanomeline inhibition of ACh release, while the effect of carbachol was abolished. This observation provides evidence that the orthosteric site is not involved in docking of xanomeline. In contrast, presence of N-methylscopolamine during electrical stimulation after xanomeline treatment and washing partially prevented the inhibition of ACh release. These observations are consistent with a notion that the free part of irreversibly bound xanomeline molecule can interact with the orthosteric binding site and stimulate M2 receptor.

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THE ROLE OF FUNCTIONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN CULTURED RAT CORTICAL ASTROCYTES

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This study was aimed at investigating the mechanisms underlying signaling mediated by neuronal nicotinic acetylcholine receptors (nAChRs) composed of an assembly between seven alpha and three beta subunits in cultured rat cortical astrocytes. Cortical astrocytes were incubated with fluo-3 AM for determination of intracellular free Ca^{2+} levels on fluorescence image analysis. Astrocytes were exposed to nicotine at different concentrations from 10 to 1000 μM, followed by determination of the fluorescence intensity in a single responsive cell. The exposure to nicotine increased the fluorescence intensity in a concentration-dependent manner in a single astrocyte. This increase by nicotine was significantly prevented by the addition of the α4β2 heteromeric subtype specific antagonist dihydro-β-erythroidine and the α7 homomeric subtype specific antagonist methyllycaconitine, respectively. Moreover, cortical neurons were treated or not treated with 10 μM cytosine-β-D-arabino-furanoside (Ara-C) for 24 h during a period of 2 to 3 days *in vitro* (DIV), followed by additional cultivation up to 12 DIV and subsequent exposure to nicotine for 8 h toward determination of cell survivability on MTT colorimetric assays. Cell survivability was significantly increased by nicotine in astrocyte-rich cultures not treated with Ara-C, but not in astrocyte-poor cultures treated with Ara-C. These results suggest that nicotine may increase intracellular free Ca^{2+} levels through the influx of extracellular Ca^{2+} ions across α4β2 and α7 channels functionally expressed by cultured rat cortical

astrocytes. The possibility that activation of nAChRs in astrocytes may protect and/or activate neurons is also conceivable.

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MODULATION OF ACH AND 5-HT RELEASE IN HIPPOCAMPAL AND CORTICAL SLICES OF 5-HT1B-KNOCKOUT MICE

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Hippocampal ACh release seems to be negatively correlated with hippocampal 5-HT release (Birthelmer *et al.*, 2002, Eur J Neurosci, 16:1839). Hippocampal 5-HT depletion attenuates cognitive deficits produced by cholinergic denervations (Lehmann *et al.*, 2002, Eur J Neurosci, 15:1991). Besides other possibilities (Buhot *et al.*, 2003, Learning & Memory 10:466), improved spatial memory observed in 5-HT1B-knockout (KO) mice (e.g. Malleret *et al.*, 1999, J Neurosci 19:6157) might be due to reduced presynaptic influence of 5-HT on ACh release. The latter hypothesis was tested using electrically-evoked release of ACh and 5-HT in brain slices of wild-type (WT) and 5-HT1B-KO mice. Superfused slices preincubated with [3H]choline or [3H]5-HT were stimulated electrically in absence or presence of 5-HT1B-agonists/-antagonists (CP-93,129/GR-55,562). Confirming previous findings, evoked release of 5-HT was increased in cortical and hippocampal slices of KO mice; 5-HT1B ligands had no effect. In hippocampal slices of WT mice, CP-93,129 (as well as 5-HT) weakly inhibited evoked release of ACh, but had almost no direct effect on cholinergic terminals in the cortex (which confirms data by Consolo *et al.*, 1996, JPET 277:823). CP 93,129 had similar effects in the rat, although inhibition of hippocampal ACh release was larger than in mice. In KO mice the evoked release of ACh was unexpectedly reduced, but ChAT activity was increased in the hippocampus, indicating possible compensations. Taken together, these observations suggest that the absence of 5-HT1B receptors has only weak direct effects on the modulation of acetylcholine release in the CNS.

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PHENOTYPE DEPENDENT EFFECTS OF ACETYL-L-CARNITINE AND NERVE GROWTH FACTOR ON CHOLINERGIC CELL FUNCTION AND SUSCEPTIBILITY TO NEUROTOXIC INPUTS

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The aim of this work was to investigate whether functional capacity of cholinergic neurons and their susceptibility to neurotoxic inputs depend on acetyl-CoA distribution in their mitochondrial and cytoplasmic compartments. Three day culture of nondifferentiated cholinergic T17 neuroblastoma cells (NC) with retinoic acid (RA) and cAMP caused their morphologic differentiation (DC) and the increase of choline acetyltransferase (ChAT) activity, acetylcholine (ACh) content and release, Ca++ accumulation in mitochondria and cytoplasm and p75 receptor expression. Simultaneously, the increase of cytoplasmic and decrease of mitochondrial acetyl-CoA levels took place. Inverse correlation was found between cholinergic activity and mitochondrial acetyl-CoA content in the cells. In NC NGF increased expression of the cholinergic phenotype along with decrease of mitochondrial acetyl-CoA. DC displayed increased sensitivity to amyloid-beta (A-b), which decreased ChAT activity, ACh release and mitochondrial acetyl-CoA level. In DC NGF suppressed cholinergic phenotype, and increased mitochondrial acetyl-CoA level and did not change cell mortality. It also aggravated toxic effects of A-b and NO in DC. In NC A-b exerted none or much smaller detrimental effects than in DC. Inhibitory effects of NGF in DC were reversed by anti-p75 receptor antibodies. In DC acetyl-L-carnitine reversed suppressing effects of A-b on ChAT activity, acetyl-CoA distribution but did not alleviate increased cell mortality. The data indicate that decreased level of mitochondrial acetyl-CoA and increased expression of p75 receptors may augment vulnerability of DC cholinergic cells to cytotoxic inputs.

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Addiction and drugs of abuse

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DIFFERENTIAL PROTEIN EXPRESSION IN THE WHITE MATTER OF THE DORSOLATERAL PREFRONTAL REGION (BA9) IN HUMAN ALCOHOLICS

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Neuroimaging and postmortem studies indicate that chronic alcohol use induces global changes in brain morphology, such as cortical and subcortical atrophy. Recent studies have shown that frontal lobe structures are specifically susceptible to alcohol-related brain damage and shrinkage in this area is largely due to a loss of white matter. This may explain the high incidence of cognitive dysfunction observed in alcoholics. The aim of this study was to compare the white matter from the dorsolateral prefrontal region (BA9) from human alcoholic and normal brains using proteomics-based methodology. Protein extracts from the BA9 white matter of 25 human brains (10 controls; 8 uncomplicated alcoholics; 6 alcoholics complicated with hepatic cirrhosis; 1 reformed alcoholic) were separated using 2D gel electrophoresis. The resultant 50 gels were fixed, Coomassie stained and scanned for image analysis. Overall, changes in the relative expression of 60 proteins were identified ($p < 0.05$, ANOVA) in the alcoholic BA9 white matter. Interestingly, many of the expression differences seen in the uncomplicated alcoholic group were exacerbated in the alcoholics with cirrhosis group. Eighteen protein spots have been identified using MALDI-TOF; including hNP22, α-internexin, transketolase, creatine kinase chain B, ubiquitin carboxy-terminal hydrolase L1 and glyceraldehyde-3-phosphate dehydrogenase. Some of these proteins have been previously implicated in alcohol-related disorders and brain damage. By identifying protein expression changes in this region from alcoholics, hypotheses may draw upon more mechanistic explanations as to how chronic ethanol consumption causes white matter damage.

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PATERNAL MORPHINE ADDICTION REDUCED LTP INDUCTION IN PERFORANT PATH TO DENTATE GYRUS OF HIPPOCAMPUS IN RAT OFFSPRING

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Background: Addiction is a major problem for society and evidences have shown several side effects of maternal morphine addiction, but unfortunately focus on the singular role of the mother in the health of the fetus and neonate has tended to minimize concern for possible male-mediated effects. However experimental animal and human studies reported adverse effects of paternal opioids exposure in offspring including low birth weight, increased congenital anomalies and learning and memory deficiencies. LTP is a cellular mechanism of learning and memory. In this study the effects of paternal morphine addiction on LTP induction were assessed by electrical stimulation of perforant path.
Material and methods: In this study 8 male rats (120–140 days old) were addicted by oral administration (32 mg/kg twice daily) of morphine 5 days prior to mating. Addicted male rats were exposed to non addicted female rats ($n = 20$). In pubertal offsprings, LTP was induced in dentate gyrus by high frequency stimulation (HFS) of perforant path.
Results: Amplitude of population spike (PS) and slope of excitatory post synaptic potential(EPSP) 60 and 120 minute after HFS in male and female offsprings of test group were less than control group ($p < 0.01$).
Conclusion: with respect to results of this study the side effects of paternal morphine addiction on offsprings must be considered.

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DRUG INDUCED RELEASE OF DOPAMINE AND NORADRENALINE IN THE FOREBRAIN OF THE PRENATALLY STRESSED RATS

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Stress applied to the gestant dam was shown to produce long-term effects on the adult offsprings. Among other effects, prenatal stress (PS) increases the levels of D2-type dopamine (DA) receptors and the vulnerability to drugs of abuse such as amphetamine and nicotine in the adult offspring. In this study we measured the levels of DA and noradrenaline (NA) in brain by microdialysis following nicotine or amphetamine injections to freely moving rats. Due to the possible influence of pubertal hormones, we performed this study in prepubertal (4 weeks) and adult (8 weeks) prenatally stressed male offsprings. The results show that the levels of DA and NA both in PFC and Nucleus Accumbens Shell (NAcS) of prenatally stressed rats were not significantly different to those of control rats. Amphetamine stimulation of DA and NA concentration in dialysates was lower in PFC of prenatally stressed rats of 4 weeks old as compared with controls. Nicotine stimulation of DA output in the nucleus accumbens shell (NAcS) was not different at both 4 and 8 weeks, whereas nicotine stimulation of NA was lower in 4 but higher in 8 weeks old rats. These data might suggest that changes in the responsiveness of the monoaminergic system in both the NacS and in the PFC may be related to prenatal stress experience. In particular the reduced effect of amphetamine in the PFC of prepubertal rats might be related to the elevated levels of dopamine receptor observed in the same region in the adult prenatally stressed offspring.

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IN VIVO MICRODIALYSIS REVEALS SLOW ELIMINATION OF REMIFENTANIL FROM NUCLEUS ACCUMBENS

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The ultra-short acting mu opioid agonist analgesic/anesthetic remifentanil (RMF) is extremely rapidly eliminated from blood plasma (half-life in rats, 0.7 min; Haidar *et al.* 1997). This extremely fast elimination is thought to be the main reason why RMF possess such a high reinforcing strength in animal operant conditioning models of drug addiction (Panlilio and Schindler 2000; Woods, personal communication 2005). We investigated if this fast elimination of RMF also occurs in the brain, i.e., in the pharmacokinetic compartment that is thought to be mediating the reinforcing effect, and hence, abuse liability of drugs.

To that end, rats were implanted with *in vivo* microdialysis probes aimed at the nucleus accumbens (NAC) and with intrajugular catheters. Two days later, they were administered 5 intravenous injections of 0.032 mg/kg RMF spaced 40 min apart, and the dialysate was analysed for neurotransmitters and remifentanil by tandem mass spectrometry. Intra-accumbens RMF peaked in the first 10-min sample and decreased exponentially with an elimination half-life of 9.7 ± 2.4 min ($N = 20$ determination in a total of 5 rats). Thus, RMF was eliminated 14 times more slowly from brain tissue than from plasma. To extrapolate, the decision to press a lever for the next infusion of RMF is made by the rat long before the brain level of RMF falls appreciably. Therefore, our data indicate that the fall in the brain concentration of RMF is not driving responding for RMF in the lever-press-based operant conditioning paradigm.

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EFFECT OF ALCOHOLISM AND SMOKING ON SELECTED GENES IN THE HUMAN PREFRONTAL CORTEX

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In view of the high coincidence of smoking with alcoholism (80–98%), we are investigating the gene and protein expression profiles in regions of the mesocorticolimbic system of chronic alcoholics and non-alcoholic smokers. Our goal is to elucidate changes in gene expression specific to long-term alcoholism, long-term smoking, and those common to both. An understanding of these mechanisms might lead to new treatment regimes. We have chosen 4 genes from the prefrontal cortex involved in regeneration (midkine), degeneration (apolipoprotein D, metalloproteinase inhibitor 3) and excitotoxicity (excitatory aminoacid transporter 1). We utilized semi-quantitative realtime PCR and established the relative expression of these target genes to the housekeeping gene ACTB. One-way ANOVA and Student's t-test were applied to reveal changes in individual cases of alcoholics with smoking comorbidity, non-alcoholic smokers and controls. The results identify genes that are particularly responsive to alcohol exposure and others that are sensitive to both smoking and alcoholism. In summary, our data shows that smoking makes a significant and variable contribution to the change in gene expression of specific target genes in the prefrontal cortex of the alcoholic brain. We are in the process of further investigating the changes of 3 target genes at the protein level via western blotting and immunohistochemistry.

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IDENTIFICATION OF ETHANOL-RESPONSIVE GENES IN RAT CORTEX

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Complex behaviours such as tolerance, dependence and sensitisation are induced by chronic ethanol exposure and thought to be mediated by different pathways in the brain. Transient or permanent alterations in patterns of gene expression are believed to underlie the neuroadaption responsible for the physiological and behavioural changes that occur during the development of ethanol dependence and withdrawal. We conducted comparative expression profile analysis of the cortex of ethanol-treated and control rats using cDNA microarrays. Using strict criteria 38 and 177 annotated genes were found to be down and up regulated respectively. We have selected several genes for further analysis. Real-time PCR, using glyceraldehyde-3-phoshphate dehydrogenase and actin beta as housekeeping genes, has confirmed the differential expression of Timp3, Mbp and Sgk in rat cortex. Sgk plays an important role in cellular stress responses and the regulation of processes such as cell survival. Thus increased expression of Sgk implies that it is a key enzyme for the survival of neurons. Mbp has an important role in remyelination of axons and in the structural organization of the myelin sheath. Increased expression of this protein may counteract demyelination that can occur with excessive ethanol use. Timp3 may be down regulated in response to ethanol to promote the cell's survival as it is involved in the induction of apoptosis. To determine whether these changes are a result of acute ethanol exposure or neuro-adaptation to ethanol dependence, we have extended these studies in a cell culture system using PC12 cells, which exhibit a neuronal phenotype after induction with nerve-growth factor.

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A NEUROTOXIC METHAMPHETAMINE REGIMEN REDUCES DOPAMINE, BUT NOT SEROTONIN, TERMINAL FUNCTION

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Fast scan cyclic voltammetry (FSCV) uses carbon fiber microelectrodes to monitor monoamine dynamics such as evoked release, uptake and diffusion. In the mouse brain, FSCV can be used to record dopamine (DA) and serotonin (5-HT) in the terminal field regions, the striatum [caudate-putamen (CPu) and nucleus accumbens (NAc)] and the substantia nigra pars reticulata (SNr), respectively. Acutely, amphetamine and its derivatives compete with monoamines for uptake into presynaptic terminals via biogenic amine transporters, resulting in slowed kinetic profiles and transporter-mediated release. It is also well known that amphetamines are neurotoxic. However, the effects of repeated administration of these drugs on presynaptic terminal function are not well understood. Here, we use FSCV as an assay of terminal function to evaluate the effects of a neurotoxic methamphetamine regimen (METH, 10 mg/kg × 4, 2 hr apart) throughout a time course of 21 days after treatment. This regimen is expected to deplete markers of DA and 5-HT 50–70% in terminal fields but not cell body regions. We find that release and uptake of DA and 5-HT in cell body regions are unaltered after this regimen. At DA terminals, we find the greatest functional deficits in release and uptake dynamics in the CPu (90–99%), with lesser deficits seen in the NAc core (80–85%) and NAc shell (30–70%). However, CPu function recovers more quickly and to a greater extent than in either subregion of the NAc over a 21 day period. Surprisingly, no differences in 5-HT terminal dynamics were seen between METH and saline treated mice. Future studies will further investigate these functional differences in METH induced DA and 5-HT toxicity as well as behavioral correlates of neurotoxicity.

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Abstract has been withdrawn.

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ALCOHOL-RESPONSIVE GENES IN THE HUMAN AMYGDALA

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The study of alcohol-induced alterations in brain gene expression may improve our understanding of the adaptive changes which result from long-term exposure to alcohol. The amygdala is a key structure in the brain's emotional system and plays an important role in the acquisition and expression of conditioning that is crucial to continued drug-seeking and relapse. For a better understanding of the role of the amygdala in alcoholism we compared gene expression in the amygdala of six alcoholics and six matched control cases using microarrays. RNA was amplified by linear amplification and hybridised to 19K human OCI

chips. Individual cases were compared to a common reference RNA. The results were analyzed with Imagene and Gene Spring software, an analysis of variance (ANOVA, p-value <0.05) was performed and the resultant genes were classified into functional groups using the program 'GO'. There was a predominance of down-regulated genes in the alcoholic cases, and almost half of the differentially expressed genes were uncharacterised or 'hypothetical proteins'. Differentially expressed genes included those involved in metabolism, protein trafficking, cell signalling, immune response, transcription and translation regulation, transport, stress response and neurotransmission. A very broad range of genes is affected by alcohol, many reflecting the neurotoxic effects of alcohol. Other alcohol-responsive genes included protocadherins gamma, PKC delta and L-type voltage-dependent calcium channel subunits, the glutamate receptor 2, cannabinoid receptor 1 and glutamate dehydrogenase 1. Some of these genes have a role in neurotransmission, LTP, LTD and synaptic plasticity and may also underlie the neuroadaptation and tolerance to alcohol.

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DOLICHYL PHOSPHATE DEFICIENCY AS A POSSIBLE MECHANISM OF ALCOHOL DEPENDENCE

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Background: Chronic alcoholics have more than 10-fold higher urinary dolichol (Dol) concentration than normal subjects. Dolichyl phosphate N-acetylglucosamine-1-phosphate transferase (GPT) is the first enzyme in the Dol pathway of protein N-glycosylation. In the present study we describe the effect of chronic alcohol consumption on the levels of GPT and on the transcription rate of the GPT gene.
Methods: The brain tissues obtained at autopsy from 36 chronic alcoholics and 28 normal subjects served as a valuable material to investigate the elements of dolichyl phosphate deficiency. Dol and dolichyl phosphate (DP) were assayed by HPLC, dolichol kinase (DolK) by the Adair and Cafmeyer method.
Results: The results indicate, that DP content differs significantly ($P = 0.001$) from a normal level (24.5 + 1.6 mg/g wet weight) and makes up 6.6 + mg/g for chronic alcoholics respectively. The enzymatic activities paralleled GPT gene expression.
Conclusion: The data present evidence that decrease of GPT gene activity also resulted in DP deficiency and provide additional insights into the possible mechanism of alcohol dependence.

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CHARACTERISATION OF GAMMA-HYDROYBUTIRATE BINDING SITE IN THE NUCLEUS ACCUMBENS

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The nucleus accumbens (NA) plays an important role in the development of drug abuse. Underlying pathomechanisms involve alteration of synaptic strengths and the number of gap junctions. The presence of the target for an illicit drug, gamma-hydroxybutirate (GHB), has been shown in the NA, but the pharmacological profile of binding was not characterised yet. In many brain areas GHB, a metabolite of succinate semialdehyde (SSA), acts on its own receptor and also binds to GABAB receptors. Screening of GHB, the antagonist NCS-382, the other SSA metabolite succinate, GABAB agonist, baclofen and antagonist CGP-55845 were performed in [3H]GHB displacement measurements. Competition and saturation experiments showed only one GHB binding site (KD = 0.9 μM and BMAX = 8.5 pmoles/mg protein) in rat rostral telencephalon membranes. GHB, succinate and NCS-382 inhibited [3H]GHB binding (KI,GHB = 3 μM, KI,SUC = 172 μM and KI,NCS = 1 μM), but baclofen and CGP-55845 (<1 mM) did not. A similar profile, inhibition by GHB and NCS-382, but not by GABAB ligands was found in human NA membranes. The gap junction inhibitor, carbenoxolone (CBX), but not quinine and 18β-glycyrrhetinic acid, displaced [3H]GHB binding in rat (KI,CBX = 38 μM) and human NA membranes. This unique feature indicates the presence of NCS-382-sensitive GHB receptors in the NA and overlapping binding sites for GHB and CBX.
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ETHANOL DIFFERENTIALLY STIMULATES THE *IN VIVO* RELEASE OF METHIONINE-ENKEPHALIN FROM THE RAT NUCLEUS ACCUMBENS

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The dopaminergic mesolimbic system plays a key role in the reinforcement mechanisms elicited by drugs of abuse. Ethanol reinforcing properties and high alcohol drinking behavior may be due, at least in part, to the ethanol-induced activation of the endogenous opiod system. Ethanol may alter opioid transmission at different levels, including the synthesis, release and/or receptor binding of opioid peptides. The aim of this work was to investigate the effects of different doses of ethanol on the *in vivo* release of Methionine-enkephalin (Met-enk) from the rat nucleus accumbens. Met-enk release was studied by microdialysis in anesthetized rats and peptide concentrations were quantitated by radioimmunoassay. Ethanol was given by intraperitoneal injection after a 2 h basal release period. Ethanol at doses of 0.5 and 2.5 g/kg induced a two fold increase in Met-enk release, similar to that produced by 100 mM KCl. An ethanol dose of 1 g/kg induced a three fold increase in Met-enk release. In contrast, low doses of ethanol (0.25 g/kg) did not alter peptide release. The ethanol-induced release of Met-enk from the nucleus accumbens might represent a key event in the reinforcement mechanisms elicited by the drug. Released Met-enk might participate in modulation of the dopaminergic mesolimbic activity in response to ethanol. The observed ethanol differential effects might be related to the drug-induced behavioral actions.
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ABILITY OF SUBSTANCE P TO INFLUENCE ALCOHOL INTAKE IS RELATED TO DOPAMINE AND NEUROKININ REINFORCING MECHANISMS

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Substance P (SP) and their receptors participate in control of dopaminergic activity and were reported to affect alcohol consumption. We tried to elucidate ability of SP to influence alcohol intake in chronic experiment and how its effects are related to dopamine. During 6 month Wistar male rats were subjected to consumption of 15% ethanol followed by testing alcohol preference in free choice between alcohol and water. Administration of SP whether ip (200 mcg/kg) or icv (1 mcg) decreased intake of ethanol. This effect was proved to be dopaminergic because pretreatment with ip D1 (SCH-23390) or D2 (sulpiride) antagonists diminished alcohol-inhibiting activity of SP. In the conditioned place preference test (CPP) the same rats preferred compartment paired with icv SP while intact animals did not. Thus long-term alcohol exposure leads to changes in reactivity of reward system to SP. We established by microdialisys that icv SP do facilitate release of dopamine in the nucleus accumbens of intact rats. So negative results obtained in CPP may be due to possible aversive properties of SP, which were regressing during ethanol exposure. To identify their putative neurochemical substrate we studied expression of neurokinin receptors by PCR and found that NK-3 receptors mRNA decreased in the nucleus accumbens of ethanol-treated animals. So though SP is known to be a direct activator of dopamine cells and dopamine is a crucial component of ethanol motivation, other neurokinin receptor mediated pathways may be involved in ethanol dependence.

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MATERNAL MORPHINE ADDICTION INCREASE PAIN TOLERANCE IN RAT OFFSPRINGS: TAIL FLICK METHOD

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Background: Consumption of narcotic drugs such as cocaine and opioids during pregnancy cause brain growth retardation, decrease neuronal excitability and synaptic transmission. Since maternal morphine addiction may disturb pain transmission in offsprings, in this study the effects of maternal morphine addiction on tail flick latency in rat offsprings have been investigated.

Material and methods: In this study 20 female rats (120–140 days old) were addicted by oral administration (32 mg/kg twice daily) of morphine 5 days prior to mating. Then addicted female rats were exposed to non addicted male rats ($n = 5$). In male and female pubert offsprings pain tolerance was assessed by the tail flick test.

Results: In pubert offsprings ($n = 10$) tail flick latency in maternal addicted male (3.2 ± 0.34 s) and female (2.97 ± 0.2) offsprings was significantly longer than male (2.27 ± 0.19) and female (2.07 ± 0.23) offsprings in control groups. So maternal morphine addiction increase pain tolerance.

Conclusion: With respect to results of this study the side effects of maternal addiction on offsprings must be considered.

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PHARMACOLOGICAL STUDIES OF AN INDIGENOUS CNS-ACTIVE DRUG DELPHINIUM DENUDATUM WALL. IN VIEW OF ITS ROLE IN MORPHINE ADDICTION

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Delphinium denudatum Wall (Dd) is one of the drugs of Unani Medicine. Its roots are used in variety of ailments. Classical literature mentioned its use in opium addiction esp. in attenuation of craving. A number of studies have been done on its phytochemical and pharmacological properties; no work on its morphine (M) addiction has ever been done. The present study was undertaken to investigate if Dd could attenuate withdrawal symptom in moderately and severely induced M dependence. For exploring its mech. of action, Dd has also been studied for analgesic, antipyretic and CNS activity including Open Field Behaviour (OFB) and Pentobarbitone Induced Hypnosis (PIH) Potentiation. Charles foster male albino rats weighing 100–150 g were selected for all above studies. They were made M dependent of both moderate and severe intensity by injecting M Sulph. 10 mg/kg ip BD for 4 d and in increasing doses for 7 d respectively. The Dd was administered po in different regimens: (a) single dose 10 h before first dose of M, (b) single dose 10 h after last dose of M, (c) multiple doses along with M for 4 and 7 d depending upon the study. The spontaneous withdrawal signs observed 12 h after last dose were quantified by the counted and checked signs. Maximum reduction was observed in regimen b followed by regimen c and a. In OFB, count for rearing was significantly increased while counts for ambulation, preening and defecation were decreased. Sleeping time during PIH was found to be increased significantly from 40.6 to 57 min. Similarly significant increase in reaction time was observed in group pretreated with extract in both analgesic tests, but no antipyretic activity.

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RAT BRAIN SEROTONIN NEURONES THAT EXPRESS NEURONAL NITRIC OXIDE SYNTHASE HAVE INCREASED SENSITIVITY TO THE SUBSTITUTED AMPHETAMINE SEROTONIN TOXINS 3,4-METHYLENEDIOXYMETHAMPHETAMINE AND P-CHLOROAMPHETAMINE

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Substituted amphetamines cause selective destruction of serotonin axons by unknown mechanisms. Since some serotonin neurones also express neuronal nitric oxide synthase (nNOS), the study was to determine whether NOS expressing serotonin neurones are selectively vulnerable to MDMA (ecstasy) and p-chloroamphetamine (PCA). Using double-labelling immunocytochemistry and double in situ hybridisation for nNOS and the serotonin transporter (SERT), it was confirmed that about two thirds of serotonin cell bodies in the dorsal raphé nucleus expressed nNOS, however few if any SERT immunoreactive axons in striatum expressed nNOS at detectable levels. Western blotting revealed that MDMA (30 mg/kg) or PCA (2×10 mg/kg) administration to Sprague-Dawley rats caused modest decreases in the levels of SERT protein in frontal cortex and striatum seven days after drug administration, even though axonal loss could be clearly seen by immunostaining. PCA or MDMA administration did not alter the level of nNOS

in striatum or frontal cortex. Analysis of serotonin neuronal cell bodies seven days after PCA treatment, revealed a net down-regulation of SERT mRNA levels, and a profound change in expression of nNOS, with 33% of SERT mRNA positive cells containing nNOS mRNA, compared to 65% in control animals. Altogether these results support the hypothesis that serotonin neurones which express nNOS are most vulnerable to substituted amphetamine toxicity, supporting the concept that the selective vulnerability of serotonin neurones has a molecular basis.

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ASSOCIATION STUDY OF CATECHOL-O-METHYLTRANSFERASE AND MONOAMINE OXIDASE A POLYMORPHISMS AND PERSONALITY TRAITS IN CHINESE CLUB DRUG USERS

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This study investigated a possible association of catechol-O-methyltransferase (COMT) and monoamine oxidase A (MAO-A) Val158Met and T941G polymorphisms respectively in 354 Chinese club drug users and 303 controls. Their association with personality traits was also examined. We found that club drug users had a significantly higher frequency of the high-activity (Val) COMT variant (79%) than controls (73%; $P = 0.013$). For MAO-A, which is X-linked, males ($N = 184$) and females ($N = 86$) were analysed separately. Female subjects have higher frequency of T allele (49% vs 37% in controls; $P = 0.012$); no significant difference was found in male users. Interactions between genotypes and phenotypes (subscales of Zuckerman Sensation Seeking Scale (SSS) and Behavioral Inhibition System and Behavioral Activation System Scale (BIS/BAS) personality traits) were also examined. Individuals with high-activity COMT gene variant (val) had higher scores for 'boredom susceptibility' ($P = 0.02$) and BAS 'drive' ($P = 0.01$). For MAO-A gene variants, female subjects with a T allele have a lower mean score (16.5 ± 2.3 vs 17.6 ± 1.8) in 'reward responsiveness' subscale of the BAS ($P = 0.02$) and a lower mean BIS score (19.7 ± 2.9 vs 20.4 ± 2.5) than those without a T allele ($P = 0.03$). Our results indicate that individuals with COMT and MAO-A gene variants are associated with sensation seeking and harm avoidance personality traits respectively, perhaps rendering these individuals more predisposed to their experimenting of club drugs use.

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THE INFLUENCE OF MORPHINE TREATMENT ON ALPHA-SYNUCLEIN PROTEIN AND mRNA LEVELS IN THE MOUSE BRAIN

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Opioid dependence and addiction are underlain, in part, by neuroadaptations in the brain dopaminergic system produced by chronic exposure to opioids. The presynaptic protein α-synuclein has recently been ascribed a role of a negative regulator of dopaminergic neurotransmission. It could thus mediate some effects of opioids. The influence of acute and chronic morphine treatment on α-synuclein protein and mRNA levels was studied in the mouse brain. Chronic (but not acute) administration of morphine produced 2- to 3-fold increases in the α-synuclein protein levels in the striatum / n. accumbens and amygdala (the forebrain regions receiving dopaminergic input), but not in the ventral mesencephalon, where dopaminergic neuron somata are located. The increases were observed only after longer periods of withdrawal from chronic morphine (48 h, but not 4 h) and were still significant after 2 weeks of withdrawal. In contrast, levels of α-synuclein mRNA were either decreased (in the forebrain) or unchanged (in the dopaminergic cell body fields) during the opioid withdrawal. This suggests that the accumulation of α-synuclein was due to changes in the protein stability rather than to increased transcription of the α-synuclein gene. Almost exclusively presynaptic localization of α-synuclein was confirmed by immunohistochemistry. Thus, we have demonstrated that chronic administration of morphine leads to a long-lasting accumulation of α-synuclein in the striatum / n. accumbens and amygdala, which may take place, at least in part, in dopaminergic terminals. Elevated levels of α-synuclein may inhibit dopaminergic neurotransmission, whose deficiency is typical of opioid withdrawal.
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Astrocytes

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TRANSGLUTAMINASE AND CYCLIN D1 IN ASTROCYTE CULTURES: EFFECTS OF CHOLINERGIC PRECURSORS

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Aim of investigation was to study the effects of cholinergic precursors (choline, CDP-choline, Acetyl-choline Ach, α-glyceril-phosphoryl-choline α-GPC) on transglutaminase (TG) and Cyclin D1 expression in primary astrocyte cultures at 14, 21 and 35 DIV. Confocal laser scanning microscopy analysis evaluated by dansylcadaverine (DC) incorporated into living astrocytes showed a dose-dependent increase of TG activity in CDP-choline-treated cells in comparison to untreated ones; specifically, 1 μM CDP-choline exposure in 14 DIV astrocyte cultures increased the fluorescence. In 0.1 μM choline-treated astrocytes an intense fluorescence pattern was observed. 1 μM α-GPC 24 h-treated astrocyte cultures showed enhanced fluorescence in cytosol and nuclei. Western blot analysis showed in 24 h 1 μM α-GPC and choline-treated astrocytes cultures increased TG, whereas in 24 h 1 μM CDP-choline and Ach-treated astrocytes no effect was observed. In 1 μM αGPC, CDP-choline and choline-treated astrocytes TG expression at 21 and 35 DIV slightly decreased. 1 μM ACh treatment reduced it at 21 DIV. In astrocytes cultures at 14 and 39 DIV cholinergic precursors 24 h treatment showed a marked down-regulation of Cyclin D1 expression, with reduced Cyclin D1 expression in 1 μM α-GPC-treated astrocytes cultures. In conclusion, our data suggest a crucial role of choline precursors during different stages of cultured astrocyte proliferation and differentiation.

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EFFECTS OF DEHYDRATION ON AQUAPORIN-4 DISTRIBUTION IN SUPRAOPTIC NUCLEUS AND IN NEUROHYPOPHYSIS OF MICE

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Studies in central nervous system demonstrate that Aquaporin water channels are widely distributed in perivascular astroglial cells of mouse, rat and human. The cytoskeletal protein dystrophin and it's associated protein complex (DAPs) has been shown to be essential for localization of AQP4 protein in the astroglial end-feet surrounding brain capillaries. Moreover, the hypothalamic magnocellular neuron cell bodies located in supraoptic nucleus and their neurohypophysial ending nerves are both associated to astroglial cells and pituicytes, respectively. In the present work we compare the Aquaporin distribution in the HNHa of euhydrated and salt loading mice. In euhydrated mice, immunohistochemistry analysis using Aquaporin-4 antibodies, revealed strong labelling of perivascular membranes of astrocytes in hypothalamic supraoptic nucleus (SON) and in neurohypophysial pituicyte expansions. In dehydrated mice, Aquaporin-4 is more expressed in glial cells, principally in the astroglial end-feets surrounding capillaries and in the ventral glial limitans of SON, as well as, in pituicytes of the neurohypophysis. Finally, the increasing of Aquaporin-4 expression in dehydrated mice in glial and probably neuronal cells could be involved in important water movement to restore the hydromineral homeostasis.

Key words: Aquaporin-4, astrocytes, DAPs, dehydration, dystrophies, hydromineral homeostasis, immunohistochemistry, mice, neurohypophysis, supraoptic nucleus.

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INVOLVEMENT OF THE UBIQUITIN-MEDIATED PROTEOLYTIC SYSTEM IN THE SIGNALING PATHWAY INDUCED BY CERAMIDE IN PRIMARY ASTROCYTE CULTURES

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The selective degradation of abnormal or short half-life proteins in eucaryotic cells proceeds through the ubiquitin (Ub) dependent proteolytic system. The signals that tag the proteins for their ubiquitination are not well known. In the present study, our aim was to investigate the relationship between the action of ceramide and the changes in the expression of certain Ub pathway proteins mRNAs and in the activation of the Ub-dependent system in cultured astrocytes. Changes in the expression of components which are known to be substrates of the Ub mediated pathway and that participate in the regulation of cell death process were also studied. Addition of different concentrations of ceramide to cultured astrocytes produced an increase in the expression of the Ub gene and in the gene that codifies for E1, one of the enzymes involved in the ubiquitination process, without any changes on cell viability. Immunocytochemical studies showed an increase in the expression of bcl-2, a decrease in IKB and in its phosphorylated form as well as a translocation of NFK-B to the nucleus. All of these compounds appear to be acting as possible modulators of astrocyte antiapoptotic responses to ceramide. Our results seem to indicate that ceramide, at the concentrations used in this study, does not produce apoptosis in astrocyte primary cultures. However, it induces an activation of the Ub-dependent proteolytic pathway, probably as a consequence of the activation of phosphatases and kinases, or through the generation of reactive oxygen species (ROS), which act as triggering signals of the Ub-dependent pathway.

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THE MULTIDRUG RESISTANCE PROTEIN 1 (MRP 1) MEDIATES EXPORT OF GSH AND GSSG FROM ASTROCYTES

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Astrocytes play an important role in the antioxidative defense and the glutathione (GSH) metabolism of the brain. These cells are able to

export both GSH and glutathione disulfide (GSSG) which are known substrates of multidrug resistance proteins (Mrps). To test for an involvement of Mrps in the release of GSH and GSSG from astrocytes, we used astrocyte primary cultures from wild type (wt), Mrp1-deficient (Mrp1–/–) and Mrp5-deficient (Mrp–/–) mice. During incubation of wt astrocytes GSH accumulated in the medium with a rate of 2.8 ± 0.7 nmol/(h × mg). A similar GSH efflux rate was observed for Mrp5–/– cells, whereas the export of GSH from Mrp1–/– astrocytes was only one third of that of wt cells. Presence of the Mrp-inhibitor MK571 at a concentration of 50 μM inhibited the rate of GSH release from wt and Mrp5–/– astrocytes by 60%. In contrast, MK571 did not affect GSH release from Mrp1–/– astrocytes. During exposure to hydrogen peroxide stress, wt and Mrp5–/– astrocytes exported substantial amounts of GSSG, whereas no extracellular GSSG accumulation was observed for astrocytes from Mrp1–/– mice although the intracellular ratio of GSSG to GSH was even higher in these cells than in wt astrocytes. These data demonstrate that in astrocytes Mrp1 participates strongly in the release of GSH and that Mrp1 is responsible for GSSG export during oxidative stress.

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EXPRESSION OF THE IEGS IN CULTURED ASTROGLIAL CELLS AFTER ACUTE TREATMENT WITH ANTIDEPRESSANT DRUGS

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Numerous recent studies indicate that glial cells participate in rapid synaptic transmission. Many papers present, that glia can express different transmitter receptors including: glutamate, serotonin, dopamine and norepinephrine. All the above mentioned receptors and neurotransmitters play crucial role in etiology of depression and in the mechanisms of antidepressant action. In the present study the effect of 24 h exposure of amitrypyline, fluoxetine and moclobemide on striatal astrocytes cultures was investigated. The antidepressants was added to the culture medium at the concentration of 1 and 10 microM. In addition we studied the IEGs mRNA expression in cultured cells, exposed to the condition of simulated ischemia and following reoxyganation. Astrocytes cultures were prepared according to the method of Hertz *et al.* RT-PCR analysis of c-fos and c-jun (IEGs) expression was used after total RNA isolation. The densitometry of IEG mRNAs products were quantified with LabWorks UV program and were normalized with the expression of the GAPDH constitutive gene. In our experiment we observed that, three antidepressant drugs with different mechanism of action, caused discrete but significant increase of c-fos and c-jun expression, proportionally to the two concentration used. Our results demonstrate the significant increase of IEGs expression after process of reoxygenation. These data confirm the participation of astroglial cells in the molecular mechanism of adaptive changes in the rat brain after antidepressants administration. These findings suggest that astroglial cells may modulate the activity of neural transmission by the IEG mRNAs expression. The proposed model of simulated ischemia allows qualifying the influence of brain tissue oxygenation on the neuron adaptation after use of antidepressants.

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A TWO-STAGE RESPONSE OF THE NITRIC OXIDE/CYCLIC GMP PATHWAY IN CULTURED RAT ASTROCYTES TREATED WITH AMMONIA

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Neurotoxic effects of ammonia are mediated by increased accumulation of nitric oxide (NO), which is coupled to generation of cGMP. Previous reports indicated that activation of this pathway primarily occurs in neurons. Here we tested the possibility that ammonia may directly affect the NO/cGMP pathway in astrocytes. Neocortical rat astrocytes were treated for 1 h (acute treatment) and 24 h (chronic treatment), with 1 or 5 mM ammonium chloride ('ammonia') and then subjected to: i) cGMP measurement using an enzymeimmunoassay and ii) analysis of expression of soluble guanylate cyclase (sGC) mRNA and protein using real-time PCR and Western blot analysis, respectively. Treatment with 1 mM ammonia decreased the expression of the catalytically active β 1 sGC subunit upon 24 h incubation. A two-stage effect was noted following the treatment with 5 mM ammonia: increased accumulation of cGMP and β 1 sGC subunit mRNA expression, not accompanied by significant changes in the protein expression, was observed at 1 h incubation. This was followed by a dramatic decrease of cGMP level at 24 h treatment, associated with a decreased sGC expression at both the mRNA and protein levels. The results point to the astrocytic NO/cGMP pathway as a potential target of ammonia neurotoxicity. The different response noted following acute and chronic treatment reflects the pattern of changes observed in human patients and experimental animals *in vivo*.

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UPREGULATION OF GLUTAMATE TRANSPORTER ACTIVITY WITH CHANGES IN ASTROCYTIC PHENOTYPE

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Homeostasis of extracellular L-glutamate is maintained by excitatory amino acid transporters (EAAT1-5) of which the astrocytic EAAT1/2 are functionally the most important. Altered expression or function of EAATs occurs in both acute and chronic neurological conditions, although little is known about EAATs in reactive astrocytes found in neuropathologies. These studies investigated the functional regulation of astrocytic EAATs in response to experimental manipulations which alter phenotype and cytoskeletal mechanisms. Pure astrocytes cultures (prepared from C57black6 mice forebrain, postnatal day 1.5) were maintained until confluent (d 10) and formed a layer of flattened, polygonal, GFAP-positive cells. After re-plating in 24-well plates, astrocytes were treated (d 22, 2–72 h) with dibutyryl cyclic AMP (dbcAMP, 1 mM), lipopolysaccharide (LPS, 1 μg/ml) or the Rho kinase inhibitor HA1077 (100 μM). [^{3}H]D-Aspartate uptake and immunocytochemistry for EAAT1/2 provided indices of transporter function and expression, respectively. Astrocytic phenotypic was monitored by GFAP immunocytochemistry. All treatments altered morphology, with astrocytes becoming stellate: rapidity of changes HA1077 > dbcAMP > LPS. Redistribution of EAAT1 and EAAT2 within astrocytes was also observed. With LPS, and especially HA1077 treatment, there was evidence of punctate EAAT localization suggestive of EAAT clusters. [^{3}H]D-Aspartate uptake was significantly increased ($p < 0.001$) by 24 h with dbcAMP and HA1077, and LPS at 48 h. Stellate morphology was sustained during treatment, but GFAP and EAATs were not always

fully co-localized. These findings suggest an association of cytoskeletal changes in astrocytes with EAAT activity, a topic being investigated further.

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TNF-ALPHA MEDIATED SHORT-TIME REGULATION OF A_{2B} ADENOSINE RECEPTOR FUNCTIONING IN HUMAN ASTROGLIAL CELLS

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A_{2B} adenosine receptors (A_{2B} ARs), low affinity adenosine binding sites, are mainly activated during pathological conditions, when a large amount of adenosine and other mediators, including cytokines are released in the brain. Since receptor regulation plays an important role in the cellular responses during pathological conditions, the aim of this work was to investigate cytokine-adenosine system cross-talk during the acute phase of cerebral damage in human astroglial cells (ADF). In a previous work [Trincavelli ML *et al.*, J Neurochem. 2004 91(5):1180–90] we have demonstrated a significant increase of A_{2B}-mediated functional response without any significant changes in receptor expression levels following ADF cell exposure to TNF-alpha for long time (24 hours). In this work A_{2B} AR functional responsiveness was evaluated following short-time ADF cell exposure to TNF-alpha. By the means of functional assay and GTPγS binding we demonstrated that cytokine decreased A_{2B}AR-mediated cAMP accumulation and G protein coupling, with a maximal effect after 3 h cell treatment. The involvement of intracellular kinases in TNF-alpha mediated effects was evaluated using selective PKC, PKA and PI3K inhibitors. The obtained results demonstrated that PKA and PKC were involved in the heterologous A_{2B} AR desensitization evoked by the cytokine. Moreover, TNF-alpha-mediated A_{2B} AR desensitization involved receptor phosphorylation on threonine residues. These data suggest that TNF-alpha plays an important role in the heterologous control of A_{2B} AR regulatory processes in ADF cells resulting in different behaviors under acute or chronic damage.

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MODULATION OF Jagged1 EXPRESSION ON RAT ASTROCYTES

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It has been shown that the Jagged-Notch-Hes pathways are implicated in many aspects of the CNS development and functions, including differentiation and myelination. During development Jagged1 is down-regulated, oligodendrocyte precursors mature and myelination starts. Human oligodendrocyte maturation is blocked in the presence of cells transfected with Jagged1. In inflamed MS tissue, Jagged1 is expressed by reactive astrocytes whereas astroglial cells in myelinated areas do not show Jagged1 immunoreactivity. First, in order to characterize the different elements of the Jagged-Notch-Hes pathways, we studied the expression of these different elements after activation of the astrocytes. To induce the activation of these cells, we used LPS or a simultaneous incubation of IFN-γ and TNF-α. Then, in an attempt to modulate the expression of Jagged1 on astrocytes, we have studied the characterization of pharmacological properties of hybrid compounds combining an omega -alkanol structure and an antioxidant moiety. The compound, TFA12, is able to modulate the expression of Jagged1 on resting and activated astrocytes. Interestingly, this compound is also able to down-regulate the NO synthesis, the iNOS expression as well as the TNF-α expression observed on activated astrocytes. These results indicate that this compound is able to induce a modulation of Jagged1 and simultaneously also induces an inhibition of the inflammatory response of the astrocytes. Taken together these preliminary results could have interesting issues on demyelinating diseases like MS.

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EXPRESSION OF OCTN2 (ORGANIC CATION/CARNITINE TRANSPORTER) IN BRAIN CELLS

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Carnitine (4-N-trimethylammonium-3-hydroxybutyric acid), necessary in the peripheral tissues for a transfer of fatty acids for their oxidation within the cell, accumulates in the brain despite low β-oxidation and stimulates acetylcholine synthesis, neuronal differentiation and expression of GAP-43 (B-50, F1, neuromodulin) – a protein involved in neural development, neuroplasticity and neurotransmission. Since 1998 several carnitine transporters have been known to be present in peripheral tissues, they comprise family of organic transporters (OCTN1, OCTN2, OCTN3), carnitine transporter CT2 and amino acid transporter $ATB^{0,+}$. OCTN2 and $B^{0,+}$ are expressed in the brain endothelial capillary cells forming the blood–brain barrier. Carnitine transport in neurons is Na-dependent and inhibited by GABA, although the involvement of GABA transporters (GAT1, GAT3, GAT4) has been excluded. The expression of OCTN2 in brain capillary endothelial cells, astrocytes and cerebral cortex neurons was studied by immunoblotting and immunocytochemistry with use of antibodies generated to the carboxy-terminal (537–553) region of rat OCTN2, kindly supplied by Prof. B. Hinton (Virginia, USA). OCTN2 was found in rat astrocytes, brain capillary endothelial cells and in neurons, although the amount of this transporter was much higher in neurons isolated from suckling animals than in neurons from adults. Polarity of OCTN2 was further studied in an *in vitro* model of the blood–brain barrier, with brain capillary endothelial cells grown on filter inserts in the presence of glial cells. The Z-axis analysis resulting in three-dimentional images in confocal microscope pictures was applied with anti-glycoprotein-P antibodies as the marker of apical membrane.

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Abstract has been withdrawn.

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Abstract has been withdrawn.

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THE GLUTAMINE TRANSPORTER EXPRESSED BY CULTURED RAT CORTICAL ASTROCYTES

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The system A transporter glutamine transporter (GlnT; SAT1) has been proposed to mediate the incorporation of extracellular glutamine (Gln) into neurons to fuel the excitatory neurotransmitter glutamate (Glu) through the Glu/Gln cycle in the mammalian central nervous system. In this study, we have attempted to elucidate possible functional expression of GlnT in cultured rat cortical astrocytes outside neurons. Semi-quantitative RT-PCR analysis revealed that similarly high expression of mRNA was seen for GlnT between cultured cortical astrocytes and neurons, with significantly lower expression in cultured astrocytes prepared from hippocampus and cerebellum than in those from neocortex. Cultured microglia also expressed mRNA for GlnT to the level lower

than those found in all cultured neuronal and glial cells examined. In cultured cortical astrocytes, [^{3}H]Gln was accumulated in a temperature-dependent manner, with a plateau within 30 min at 37°C, as seen in cortical neurons. In cultured cortical astrocytes, the accumulation of [^{3}H]Gln was saturable with increasing concentrations of [^{3}H]Gln up to 750 μM, whereas Lineweaver-Burk plot analysis revealed that [^{3}H]Gln accumulation consisted of a single component with a Km value of 219 ± 59 μM and a Vmax value of 5.8 ± 1.6 nmol/min/mg protein, respectively. Moreover, the accumulation of [^{3}H]Gln was significantly inhibited by the inhibitor of system A neutral amino acid transporter MeAIB at 10 to 20 mM to a lesser extent in cortical astrocytes than in cortical neurons. These results suggest that GlnT may be functionally expressed by astrocytes with hitherto unidentified functions in mechanisms associated with the Gln/Glu cycle required for recycling of Glu in neurons.

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CELLULAR DRINKING IN REACTIVE ASTROCYTES IS ESSENTIAL FOR CELL MIGRATION AND IS MEDIATED BY C-Raf AND ACTIN

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The plasma membrane separates the inside of the cell from the outside and is constantly being remodeled by endocytosis allowing cells to control how they respond to external stimuli. Here we report a real time visualization of cellular drinking in living reactive astrocytes induced by scratch injury. Based on the measurement of diameter of pinosomes and their dynamics, we demonstrated that these activities in reactive astrocytes were macropinocytosis. Electron microscopy (both Transmission and Scanning Electron Microcopy) and atomic force microscopy captured the cellular spreading in the different stages of macropinocytosis. LY294002, one of PI-3 kinase inhibitor, could reduce cytoplasmic spreading in the reactive astrocytes and, at the same time, inhibit macropinocytosis. This indicated that the process of macropinocytosis is closely associated with cellular spreading of reactive astrocytes. We confirmed that the macropinocytosis in these reactive astrocytes involved with C-Raf activation and also the translocation of these C-Raf to bind with F-actin in the membrane.

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ROLE OF GLIAL CELL IN 6-OHDA INDUCED NEURONAL DYSFUNCTIONING

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Glial cells co exists with neurons in brain and plays important part in maintenance of proper neuronal functioning. In the conditions of neuronal injury or death they may actively take part and have been shown to protect the neuron. But, still it is not clear in what manner they get affected themselves as any impairment in their functioning may lead to the imbalance of ionic environment around the neurons thus further deteriorating the situation and probably this is of importance in progressive degenerative disorders like Parkinson's disease. In the present study the status of glial cells in 6-hydroxy dopamine induced degeneration of neuronal (dopaminergic) cells was evaluated using primary neuron and glial cultures and co-cultures. For this 6-OHDA (10–14 to 10–19 M) exposure was given to glial cells or to neurons in absence and presence of glial cells / glial cells pre exposed to 6-OHDA.

6-OHDA was not shown to exert any significant astrocytic cell death at 24 hours, however at 48 and 72 hours a significant mitochondrial impairment was observed following 6-OHDA (10–4 and 10–5 M) exposure. Activation of glial cells was also evident on exposure to 6-OHDA when co cultured with neurons or when cultured alone, where they showed changed in appearance as becoming fibrous. This is further supported by increase in GFAP/S-100 expression as done using immunocytochemistry. 6-OHDA can directly affect the astrocytic function as evident from impaired 3H Glutamate uptake and glutamine synthase activity. The neurons co cultured with astrocytes (control cultures) have shown less impairment in TH expression when compared to co-cultured with pre treated astrocytes on exposure to 6-OHDA. The results suggets that 6-OHDA at can impair glial functioning which eventually may effect neurons making them more vulnerable to toxic insults and thus may contribute in conditions of progressive DA loss.

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EXPRESSION OF GCPII IN HUMAN ASTROCYTOMA

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Glutamate carboxypeptidase II (GCPII), also known as NAALADase I, folylpolyglutamate hydrolase (FOLH) or prostate specific membrane antigen (PSMA) is localized in number of tissues. In brain astrocytes, it regulates neurotransmission by cleaving neurotransmitter N-acetylaspatylglutamate (NAAG) into N-acetylaspartate and most common excitatory neurotransmitter glutamate. GCPII is identical to prostate-specific membrane antigen (PSMA), a tumor marker in prostate cancer. GCPII is also found in the membrane brush border of the small intestine where it acts as a folate hydrolase. This reaction expedites intestinal uptake of folate through hydrolysis of folylpoly-gamma-glutamates to folates. GCPII inhibitors might thus be useful in the imaging and treatment of tumors where folate is required for their growth. Therefore it was of interest to investigate whether GCPII might be upregulated in brain tumours as well. In order to analyze this possibility, we took 57 samples from 49 patients with brain tumours treated in Faculty Hospital Motol during 1999–2004 and determined expression and activity of GCPII by Western blots and immunohistochemistry using monoclonal and polyclonal antibodies developed against extracellular epitopes of GCPII. Moreover, we characterized the enzymatic activity of the enzyme in human samples and correlated the expression of GCPII with the type and grade of the tumor.

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ROSENTHAL FIBER FORMATION IS DEPENDENT ON BOTH QUALITY AND QUANTITY OF GFAP

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Alexander disease is caused by heterozygous mutation in glial fibrillary acidic protein (GFAP). The pathological hallmark is the presence of astrocytic GFAP aggregation called Rosenthal fibers (RF). This indicates that RF formation is dependent on GFAP quality. On the other hand, overexpression of wild type GFAP caused the formation of aggregation: 1) In the case of lasting GFAP overexpression such as pilocytic astrocytoma, RF formation occurred without GFAP mutation. 2) Wild

type GFAP overexpressed mice exhibited fatal encephalopathy with RF. These reports suggest that RF formation is also dependent on GFAP quantity. We asked if the existence of mutant GFAP is necessary and sufficient for the formation of RF. To address this question we generated transgenic mice that express mouse GFAP driven human GFAP R239H mutant. We manipulated to insert single copy or triple copies of the transgene into an identical chromosomal locus, so that the transgenes receive the same chromosomal positional effect and their expressions were dependent on the copy number. We performed immunohistochemistry using human GFAP specific antibody, SMI21. Lines with single copy exhibited weak immunoreactivity in the astrocytes but did not exhibit GFAP aggregation despite of the existence of mutation, whereas lines with triple copies exhibited strong SMI21 immunoreactivity and aggregation. GFAP aggregation existed only in the hippocampus, olfactory bulb, white matter, glia limitans, and rostral migratory stream. We concluded that 1) the existence of mutant GFAP is insufficient for the formation of aggregation and 2) the process of the formation needs a certain amount of mutant GFAP.

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OXIDATIVE AND PROLIFERATIVE ACTIONS OF LEPTIN ON RAT GLIAL CELLS *IN VITRO*

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The studies indicate that leptin has involved in not only energy expenditure and food intake, but also in protection against apoptosis, in inflammation and in stimulation of proliferation. Many human tissues including the brain have leptin receptors. Antioxidant defense elements in human and mouse are increased with the treatment of leptin. However, leptin treatment increases the oxidative stress in many cell culture studies. This contradiction evoked a question of whether leptin acts as an oxidant or antioxidant on glial cells. We have tested this conflict and also the possible proliferative effect of leptin on rat primary glial cell culture. Glial cells from 1–3 day old rat brain frontal lobe were first treated with 10–1000 μM hydrogen peroxide (H_2O_2) for 3 hours at 37°C and 5% CO_2 with 100% humidity. The dose of H_2O_2 that killed 75% of the cells was determined as 100 μM. Combined with this dose, leptin or well known antioxidant glutathione (GSH) at 100–8000 μM were applied to the cells for three hours. After 24 hours, we determined the survival rate of the cells by using 3-(4,5-D-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thyazolyl blue method. Our preliminary results indicate that leptin could not eliminate the toxicity of H_2O_2. However, GSH from 100 μM started to show protective effect against H_2O_2 and the GSH doses between 500–8000 μM completely eliminated toxic effect of H_2O_2 ($p < 0.001$). We also investigated the effects of 1, 10, 100 and 1000 ng/ml leptin doses on proliferation of the glial cells. The treatment of glial cells with respective leptin doses for 24 hours did not show any negative or positive effect on cell proliferation. Further studies are underway.

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GLUTAMATE-STIMULATED ATP RELEASE FROM SPINAL CORD ASTROCYTES IS POTENTIATED BY SUBSTANCE P

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The importance of glutamate-stimulated ATP release from astrocytes has recently been revealed by studies that have shown its contribution to intercellular communication between networks of astrocytes and to the modulation of synaptic activity in a feedback manner. However glutamate is not released onto astrocytes in isolation at spinal cord dorsal horn pain synapses, but is co-released with the neuropeptide substance P. Astrocytes possess all three kinds of substance P receptor, so the opportunity exists for substance P to modulate the process of glutamate-stimulated ATP release. The aim of this study, then, was to examine whether substance P modulated glutamate-stimulated ATP release from spinal cord astrocytes and if found to do so, to examine the mechanisms involved. ATP release was measured by online-bioluminescence of cultured spinal cord astrocytes. Substance P (100 μM) did not cause ATP release from spinal cord astrocytes. However when it was co-applied with 1 mM glutamate it caused a 336% increase in ATP release as compared to that stimulated solely by glutamate. Substance P was found to act through neurokinin-1 receptors. When glutamate was applied on its own it acted exclusively through AMPA receptors to stimulate ATP release. However when substance P was co-applied with glutamate, ATP release could be elicited by activation of NMDA and metabotropic glutamate receptors. ATP release resulting from AMPA and kainate receptor stimulation was not affected by substance P. These results suggest that astrocytes surrounding glutamatergic pain synapses in the spinal cord may have a more dominant influence on neighbouring glia and neurons than was previously proposed.

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GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) CONTROLS MIGRATION AND HYPERTROPHY OF REACTIVE ASTROCYTES

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We had observed reactivation of astrocytes with hypertrophy, migration, hyperplasia and increased GFAP content in pure astrocytic culture after scratch injury. We adopted length of process (hypertrophy) and rate of wound closure (migration) as parameters for quantifying the degree of reactivation,. During a five-day observation in the same field, reactive astrocytes sent out hypertrophic process about 250 mm long and closed the 750 mm wound gap. To reveal the relationship of cytoskeletons with reactivation, we used antisense GFAP, beta-tubulin, beta-actin and vimentin to affect the hypertrophy and migration. After single treatment and 5-day observation, we found that only antisense GFAP mRNA had a significant effect to block the reactivation of astrocytes. We also treated astrocytes with FITC-probed antisense GFAP mRNA, located the transfected astrocytes, time lapse recorded them for 3 days. Result confirmed the effect of antisense on astrogliotic response to mechanical damage.

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TARGETING JAK2/Stat3 SIGNALING RESCUES ASTROCYTE CELL DEATH FROM OXIDATIVE STRESS

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Oxidative stress is involved in the pathogenesis of a number of neurodegenerative diseases. Elucidation of mechanisms underlying oxidative stress responses in the central nervous system is necessary for therapeutic development for as well as understanding pathogenesis of the neuropathologies. The signaling molecules activated in response to oxidative stress in brain astrocytes have yet to be fully understood. Hydrogen peroxide treatment has been known to activate ERK 1/2 and p38 MAP kinases in astrocytes. In this study, we examined whether

Janus kinase (Jak)/signal transducer and activator of transcription (Stat) signaling could be activated by oxidative stress in astrocytes. Tyrosine phosphorylation of Jak2 and Stat3 was rapidly induced by hydrogen peroxide in a dose-dependent manner in U87 human astrocytoma cells. Compared to the transient activation of Stat3 by interleukin-6, the kinetics of Stat3 activation by hydrogen peroxide demonstrated a more sustained pattern. We then examined the role of Jak2/Stat3 in oxidative stress-induced cell death in astrocytes. Pretreatment of U87 cells with Jak2 inhibitor AG490 or Stat3-SH2 domain-binding phosphopeptide blocked Stat3 activation with a concomitant reduction in hydrogen peroxide-induced cytotoxicity. These signaling inhibitors also prevented hydrogen peroxide-induced changes of the expression of apoptosis related genes. Our findings suggest Jak2/Stat3 as oxidative stress-regulated signal transducers that may promote cell death in astrocytes. Thus, targeting Jak2/Stat3 signaling may provide a protective strategy for rescuing astrocytes under various neuropathological conditions associated with oxidative stress.

Auditory

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THE EXISTENCE OF OPIOID PEPTIDES AND RECEPTORS IN THE INNERVATION OF HAIR CELLS OF GUINEA PIGS INNER EAR

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Opioid peptides existed in the efferent fibres which innervated inner and outer hair cells via differential types of peptides. However, the precise innervation pattern of opioid fibres remains to be investigated. Opioid receptors are mainly expressed in hair cells of the Organ of Corti, inner and outer spiral bundles with different characteristics in each type of receptor. Mu-, delta- and kappa-opioid receptors were identified by colocalization study with their peptides, β-endorphin, enkephalin and dynorphin, respectively. The positive immunoreactivity of synaptophysin was used in this study to indicate the presynaptic site. Enkephalin was colocalized with synaptophysin prominently in the inner spiral bundle. Dynorphin was colocalized with synaptophysin in both inner and outer spiral bundles. Delta-opioid receptor was the most eminently colocalized with its peptide in the inner spiral bundle. Kappa-opioid receptor was seemingly present with dynorphin in both inner and outer spiral bundles. The established presence of opioid peptides and their receptor sites in the inner ear can justify and provide the tools for an in-depth investigation of the role of opioid system in the auditory functions.

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VINPOCETINE PREVENTS THE AUDITORY EVOKED POTENTIAL CHANGES AND THE HEARING LOSS THAT ACCOMPANY EXPERIMENTAL EPILEPSY

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Epilepsy is accompanied by cognition deficits and alterations in brainstem auditory evoked potentials (BAEPs). Several of the most commonly used antiepileptic drugs produce adverse effects, including BAEP alterations and cognition deficits, that may aggravate those produced by the illness. Vinpocetine is a sodium channel blocker and memory enhancer with antiepileptic potential. The objective of this study was to explore the capability of vinpocetine to inhibit the epileptic cortical activity, the abnormalities in the later waves of the BAEPs and the hearing loss induced by the convulsing agents pentylenetetrazole (PTZ) and 4-aminopyridine (4-AP). For this purpose EEG and BAEP recordings before and at specific times within two hours after the injection of convulsing doses of PTZ (100 mg/kg) or 4-AP (2 mg/kg) were taken in guinea pigs pre-injected few hours before with vehicle or with vinpocetine (2 mg/kg). The amplitude and latency of the BAEP waves induced by a monoaural stimulus of high intensity (100 dB) at 4 and 8 kHz pure tone frequencies and the auditory threshold were determined in the animals exposed to the different experimental conditions. We found that vinpocetine abolishes the epileptic cortical activity, the alterations in amplitude and latency of the later BAEP waves and the increase in the auditory threshold induced by both convulsing agents for the ictal and post-ictal periods. Taken together these results indicate that vinpocetine is a promising alternative for the treatment of epilepsy, as it prevents the retro-cochlear alterations and the hearing decline that accompany the epileptic cortical activity.

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SEXUAL DIMORPHISM IN A POPULATION OF INHIBITORY NEURONS IN AN AUDITORY AREA INVOLVED IN THE PERCEPTUAL PROCESSING OF BIRDSONG

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Song is a learned behavior that relies on auditory input for its development and maintenance in songbirds. Males, but not females, have a well-developed system of interconnected brain nuclei, the song system, involved in key aspects of song production and learning. The song system represents one of the first and clearest examples of brain sexual dimorphism in a vertebrate. Here we present evidence for a sexual dimorphism in the caudomedial nidopallium (NCM), an auditory area outside of the song system involved in the perceptual processing of birdsong. We show that cells immunolabeled for calbindin, a calcium-binding protein expressed in a subset of inhibitory neurons, are primarily localized to a caudal subdomain in NCM and are twice as numerous in males than in females. This caudal domain coincides with the area that expresses the estrogen-generating enzyme aromatase and could represent a local site of estrogen production. The dimorphism observed is neither a function of higher neuronal cell density in NCM, nor a reflection of higher number of GABAergic neurons in this area. Our observations demonstrate that NCM contains neurochemically-defined subdomains, with possible distinct functional properties, as well as a sexually dimorphic neuronal population. They also suggest that auditory auditory processing of birdsong may differ between males and females, and that local sex steroid levels may play a role in the processing of auditory information.

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QUANTITATIVE ANALYSIS OF COLOCALIZATION OF OPIOID RECEPTORS AND GABAERGIC NEURONS IN THE RAT INFERIOR COLLICULUS

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GABAergic and glutamatergic are attributed to be a major signal that recorded in the inferior colliculus. Opioid peptides are also reported in this brain area. It may play a modulatory role on neuronal communication. We examined the relationship of opioid receptor subtypes (mu, delta and kappa) to GABAergic neurons in the inferior colliculus. GABA immunoreactivity was observed predominantly in the neurons of central nucleus and pericentral nucleus of inferior colliculus. The immunoreactivities of opioid receptor subtype were also distributed non-uniformly in inferior colliculus. The immunostaining of mu, delta and kappa were found in both disc-shaped cells and stellate cells. Colocalization of GABA and mu was observed in neurons and nerve fibers in central nucleus and pericentral nucleus of inferior colliculus. Moreover, there were some colocalization of GABA and delta, GABA and kappa. However, they were less than that of GABA and mu. The present results indicate that the mu-opioid receptors may modulate the activity of GABAergic neurons in the inferior colliculus more than delta- and kappa-opioid receptors.

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Cell surface–cell membrane

P.190

THE EFFECT OF DIBUCAINE.HCL ON THE PHYSICAL PROPERTIES OF NEURONAL MEMBRANES

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Fluorescent probe techniques were used to evaluate the effect of dibucaine.HCl on the physical properties (transbilayer asymmetric lateral and rotational mobility, annular lipid fluidity and protein distribution) of synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex. An experimental procedure was used based on selective quenching of 1,3-di(1-pyrenyl)propane (Py-3-Py) and 1,6-diphenyl-1,3,5-hexatriene (DPH) by trinitrophenyl groups, and radiationless energy transfer from the tryptophans of membrane proteins to Py-3-Py. Dibucaine.HCl increased the bulk lateral and rotational mobility, and annular lipid fluidity in SPMV lipid bilayers, and had a greater fluidizing effect on the inner monolayer than the outer monolayer. The magnitude of increasing effect on annular lipid fluidity in SPMV lipid bilayer induced by dibucaine.HCl was significantly far greater than magnitude of increasing effect of the drug on the lateral and rotational mobility of bulk SPMV lipid bilayer. It also caused membrane proteins to cluster. These effects of dibucaine.HCl on neuronal membranes may be responsible for some, though not all, of the local anesthetic actions of dibucaine.HCl.

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DEVELOPMENTAL CHANGES IN THE ASSOCIATION OF NMDA RECEPTORs WITH LIPID RAFTS IN RAT FOREBRAIN

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Lipid rafts (LRs) are lipid microdomains present in the cell surface membrane that are involved in protein trafficking and formation of cell signaling complexes. LRs from adult rat brain contain NMDA receptors (NMDARs) and receptor associated proteins such as Src family tyrosine kinases and membrane-associated guanylate kinases (PSD-95 and SAP102), but the association of NMDARs with LRs during development is not known. We therefore investigated the effect of development on the association of NMDARs with LRs prepared from rat forebrains of postnatal ages 1 to 35 days. LRs were prepared from P2 membranes by extraction with Tx-100 followed by sucrose density gradient centrifugation. The yield of LRs, as reflected by protein levels, Thy-1 and flotillin-1 increased several-fold during postnatal development. NR2A and NR2B levels in LRs increased during the first three postnatal weeks. NR2B levels in LRs then decreased between P20 and P35, whereas NR2A levels remained constant after P25. These changes in LR-associated NMDARs were paralleled by changes in the amounts of PSD-95 and SAP10 located in LRs. In contrast to the developmental increase in LR-associated NMDARs, levels of heat shock proteins in LRs remained constant (Hsc70, Hsp40) or decreased (Hsp60) over the developmental period studied. Tyrosine phosphorylation of NR2B in LRs was not detected during the first postnatal week and increased thereafter. Src and Fyn, were both present in LRs prepared at P1. Whereas LR-associated Src remained constant thereafter, LR-associated Fyn decreased during postnatal development. These results demonstrate that the association of NMDARs and of NMDAR-associated proteins with LRs is developmentally regulated.

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POLYSIALIC ACID REGULATES THE EFFECTS OF BRAIN-DERIVED NEUROTROPHIC FACTOR ON CHOLINERGIC NEURONS

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Polysialic acid (PSA) is a carbohydrate linked to neural cell adhesion molecule (NCAM). In the presence of nerve growth factor (NGF), the removal of PSA increases the activity of choline acetyltransferase (ChAT; enzyme synthesizing acetylcholine) in sympathetic ganglion cells . Recently, PSA was suggested to present brain-derived neurotrophic factor (BDNF) to its receptor tyrosine kinase (TrkB) in a manner which potentiates BDNF's biological actions. ChAT activity is increased by NGF and BDNF in neurons of the septal nuclei. The objective of this study is to evaluate the influence of PSA on ChAT activity in embryonic septal neurons in vitro. Remarkably, the removal of PSA drastically increased ChAT activity, only in presence of BDNF, and without affecting the number of cholinergic neurons. ChAT activity induced by BDNF and PSA removal was abolished with K252a, a Trk receptor inhibitor, implying that TrkB signaling is involved. Binding assays indicated the number of [125I]BDNF receptor sites increased in the absence of PSA. Current experiments are assessing the phosphorylation states of TrkB receptors in each culture condition. Taken together, these results demonstrate a novel role for PSA in the regulation of ChAT activity by BDNF. PSA may prevent maximal TrkB activation by masking the receptor sites, changing receptor conformations or sequestering BDNF away from TrkB receptors. Relating to the *in vivo* situation, as cholinergic neurons mature, the normal loss of PSA may coincide with greater TrkB activation and increases in ChAT activity.

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EXOGENOUS CYTIDINE-5′-DIPHOSPHOCHOLINE INCREASES BRAIN CYTIDINE-5′-DIPHOSPHOCHOLINE LEVELS IN GERBILS

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The objective of this study was to determine whether administering exogenous cytidine-5′-diphosphocholine (CDP-choline) which is completely metabolized to choline and cytidine, can increase brain CDP-choline levels in gerbils. We also wanted to compare pyrimidine nucleoside metabolism in gerbils with that in rats or humans. CDP-choline (56 mg/kg) was administered by gavage; after 30 min plasma and brain samples were obtained by dipping the gerbils' heads into liquid nitrogen and then decapitating them. Nucleosides, nucleotides

and CDP-choline were assayed using HPLC. Basal plasma levels of uridine (7–7.5 μM) were higher, and increased more after CDP-choline [to 16 ± 0.4 μM ($P < 0.001$)] than those of cytidine which started as 1.3–1.5 μM and rose to 2.4 ± 0.2 μM ($P < 0.05$). Brain uridine was also significantly elevated after oral CDP-choline, from 22 ± 8.1 to 110 ± 23.6 μM ($P < 0.05$), but brain cytidine failed to rise. Brain UTP and CTP levels also did not change significantly, however CTP tended to increase. Brain CDP-choline levels did rise significantly from 8 ± 0.7 to 12 ± 1 μM ($P < 0.05$). We conclude that exogenous CDP-choline increases brain CDP-choline levels in gerbils by providing more plasma uridine and perhaps cytidine. The changes in plasma uridine and cytidine provided by giving gerbils exogenous CDP-choline resembles that in humans but not that in rats.

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EFFECTS OF MAMMALIAN GTRAP3-18 ON ER-to-Golgi TRAFFICKING

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GTRAP3-18 has been identified as an interacting protein of the neuronal type of glutamate transporters EAAC1. GTRAP3-18 is homologous to the prenylated rab acceptor proteins that have been shown to be important for transport of cargo from endoplasmic reticulum (ER) to the Golgi apparatus. Co-localization studies using fluorescently labelled markers for endoplasmic reticulum and Golgi revealed that the localization of GTRAP is predominantly the ER. The goal of the present study was to elucidate the particular importance of GTRAP for protein transport from the ER to the Golgi apparatus. By applying Fluorescence Resonance Energy Transfer we showed that our fluorescently tagged GTRAP3-18 constructs are oligomeric as reported in the literature. Properly oligomerized GTRAP3-18 slows down ER-to-Golgi transport rate of the temperature-sensitive vesicular stomatitis virus glycoprotein. In the C-terminus of GTRAP3-18 we found an LXXLE-motif that is known to bind Sec24, a component of the COPII coat. A deletion mutant of GTRAP3-18 that lacks the C-terminal 23 amino acids (GT-d23) displayed punctuate intracellular staining in contrast to the diffuse reticular staining of the wild type protein. In addition, GT-d23 did not show any effect on ER-to-Golgi trafficking. In conclusion, we describe the importance of oligomeric GTRAP3-18 along the secretory pathway and map a putative interaction domain at its C-terminus.

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A_{2A} ADENOSINE RECEPTOR ACTIVATION FACILITATES ADENOSINE TRANSPORT IN THE RAT HIPPOCAMPUS

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Neuromodulation by adenosine (ADO) mostly results from A_1 and A_{2A} receptors activation. Bi-directional equilibrative nucleoside transporters control ADO synaptic levels. We studied if A_1 and A_{2A} receptors activation modified ADO transport and tested the efficiency of these receptors on the modulation of [^{3}H]acetylcholine ([^{3}H]ACh) release vis-à-vis nucleoside transporter activity under different stimulation paradigms. Uptake assays were performed for 15 s with [^{3}H]ADO (1 μM). In ADO release experiments, synaptosomes were stimulated with high-K^+ (28 mM) and ADO released was analysed by HPLC. [^{3}H]ACh release was evoked either with low-frequency (LFS:2 Hz) or high-frequency (HFS:50 Hz) stimulation of CA3 hippocampal subslices. A_{2A} receptor agonist, CGS 21680 (30 nM), increased [^{3}H]ADO uptake by 66 ± 17% ($P < 0.05$; n = 7), an effect prevented by the A_{2A} receptor antagonist SCH 58261 (250 nM) and the PKC inhibitor GF109203X (1 μM). A_1 receptor agonist, CPA (100 nM), did not significantly modify [^{3}H]ADO uptake. CGS 21680 (30 nM) also facilitated ADO release by 91 ± 12% ($P < 0.05$; n = 4), an effect significantly reduced by nucleoside transporters blockade ($P < 0.05$; n = 6). A_1 receptor antagonist, DPCPX (250 nM), facilitated the evoked release of [^{3}H]ACh under LFS (14.5 ± 2.8%, $n = 4$, $P < 0.05$) but not under HFS, unless there was a previous blockade of nucleoside transporters (15.6 ± 0.9%, $n = 5$) or A_{2A} receptors (33 ± 10%, $n = 4$, $P <$ 0.05). Our results suggest that A_{2A} receptors activation enhances nucleoside transporters activity, regulating ADO availability for A_1 receptor activation.

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Cytoskeleton, microtubules

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CYCLIC GMP REGULATES CYTOSKELETON DYNAMICS IN GLIAL CELLS

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Reactive gliosis is a prominent feature of CNS injury that involves dramatic changes in glial cell morphology. The bacterial wall lipopolysaccharide (LPS), a strong inflammatory agent, induces morphological changes in astroglia and microglia. In mixed glial cultures LPS also induces expression of NO synthase type 2 and NO-dependent increases in cGMP formation. The cGMP/protein kinase G (PKG) pathway has been implicated in the regulation of Rho GTPases, a subgroup of the Ras superfamily that regulates the organization and dynamics of the actin cytoskeleton. In this work we have investigated if cGMP is implicated in the cytoskeletal changes induced by LPS in glial cultures from rat cerebellum. Our results show that exposure to LPS (0.5–1.0 μg/ml, 24 h) increases F-actin (rodhamine-phalloidin labelled) content in mixed cultures, an effect that appears to occur in microglia. In astrocytes, LPS induces reorganization of actin filaments (loss of stress fibers and process elongation), as well as glial fibrillary acidic protein (GFAP) intermediate filaments. These effects are prevented by the soluble guanylyl cyclase (sGC) inhibitor ODQ (20 μM), implicating NO-stimulated cGMP formation in LPS-induced cytoskeletal changes. In agreement with this, dibutyril-cGMP (dbcGMP; 100 μM) and the NO-independent stimulator of sGC, BAY 41-2272 (5 μM), mimic the effect of LPS on F-actin and GFAP. Moreover, the cytoskeletal reorganization induced by LPS and dbcGMP in astrocytes is prevented by the PKG inhibitor, Rp-8-Br-PET-cGMPS (5 μM). In microglial cultures treated with LPS or dbcGMP numerous cells assumed large, round shape and showed microspike projections containing thick bundles of F-actin. Thus, cGMP-mediated pathways appear to regulate the cytoskeletal changes occurring in reactive astroglial and microglial cells.

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FLATTENING OF GLUCOCORTICOID CIRCADIAN RHYTHM, NEURONAL CYTOSKELETON AND IN VITRO GLUTAMATE RELEASE IN RAT HIPPOCAMPUS

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Flattening of the circadian rhythm obtained by implantation of corticosterone pellets shares with depressive disorders two characteristics: memory impairment and dendritic atrophy of hippocampal neurons. The purpose of this work was to investigate the effect of the flattening of the circadian rhythm of corticosterone on cytoskeleton proteins of hippocampal neurons. Two different plasma levels of corticosterone were achieved by subcutaneous pellets implantation of 100 or 200 mg of the drug (CORT 100 and CORT 200) in adult male Wistar rats. Twenty one days later, four cytoskeleton proteins were quantified by immunohystochemistry in CA3 and dentate gyrus (DG): 68, 160 and 200 kDa subunits of intermediate neurofilaments and the dendritic marker MAP2. We also counted the NeuN positive cells in the same areas and performed in vitro experiments to evaluate the release of glutamate in hippocampal slices. Both levels of corticosterone induced a significant decrement of 68, 160 and 200 kDa subunits in CA3 and DG, but only CORT 200 animals showed a diminution in MAP2 immunoreactivity. In these animals a significant decrease in the in vitro potassium stimulated glutamate release was also found. No effect of CORT was observed in NeuN positive cells. Our results provide a subcellular insight for the trophic changes found both in experimental models of depression or in patients suffering from the affective disorder. Coincidence between MAP2 and glutamate release decrements may further explain memory impairment observed in depressive patients.

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MOLECULAR DETERMINANTS OF THE SUBCELLULAR TARGETING OF STATHMIN FAMILY PROTEINS IN NEURONS

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The establishment of diverse functional compartments in neurons requires the accurate targeting of proteins. In the developing and mature nervous system, stathmin family phosphoproteins are regulators of microtubule dynamics. The specific neural members of the family (SCG10, SCLIP, RB3) are associated to the Golgi and punctuate structures along neurites and at the growth cone, where microtubules are highly dynamic. In Drosophila, a single gene codes for variants corresponding to the stathmin family in vertebrates. The subcellular, membrane-associated distribution of stathmin family proteins is mediated by the presence of an N-terminal targeting region. In vertebrates, this region is composed of up to three domains: A (57 to 70% identity), A′ (in RB3/RB3′/RB3″), and A″ (only in RB3″). Within domain A, a median palmitoylated sequence determines membrane binding, whereas an N-terminal conserved sequence contributes to the specific Golgi targeting. Here, we show with GFP fusion proteins that, contrary to domain A, domains A′ and A″ are not directly involved in Golgi targeting but rather contribute to the diversity of the stathmin family, possibly by interacting with specific proteins. Furthermore, we show by site directed mutagenesis, chimera constructions and cell fractionation of transfected neurons that, depending on the level of palmitoylation, GFP fusion proteins with domain A of RB3 may be adressed to other compartments than the Golgi complex. Altogether, our results give new insights into the regulation of the subcellular localization of stathmin family proteins, an essential feature of their physiological functions in differentiating and mature neural cells.

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MECHANISMS IN NEURITE INITIATION: DYNEIN-DEPENDENT INDUCTION OF LOCAL CELL PROTRUSIONS

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A key event in neurite initiation is the formation of a thin, microtubule based protrusion from the base of the neuronal soma. We showed earlier that expression of MAP2c induces neurites in neuroblastoma cells via the formation of microtubule bundles, which protrude the membrane beyond the original cell borders. We hypothesize that an alteration of forces acting on the neuronal cytoskeleton induces this change in cell

morphology. Here, we performed nocodazole washout experiments in neuroblastoma and non-neuronal cells to study the formation and dynamic behavior of MAP2c-induced microtubule bundles. Microtubule bundles form rapidly following microtubule repolymerization after nocodazole washout. Such bundles are transported unidirectional through the cell and accumulate at the cell margin, producing force that deforms the plasma membrane. In neuroblastoma cells, this force is sufficient to induce neurite-like protrusions. In non-neuronal cells, membrane deformation was increased after F-actin disruption suggesting that microfilament generated contractile forces counteract microtubule generated pushing forces. Movement was not dependent on either microtubule or F-actin based tracks, but was reversed by injection of a function blocking anti-dynein antibody. In addition, bundle-induced formation of neurite-like protrusions was strongly impaired by dynein inhibition. We propose a model in which MAP2c and related neuronal MAPs reorganize the microtubule cytoskeleton and alter the force equilibrium to promote neurite initiation via a dynein dependent mechanism.

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EFFECT OF VALPROIC ACID ON CYTOSKELETON PROTEINS IN AN EXPERIMENTAL MODEL OF DEPRESSION

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In a previous work (Neuroscience 129: 529–538, 2004) we observed that exposure to an experimental model of depression, the learned helplessness (LH) paradigm, induces a significant decrement in the light subunit of the intermediate neurofilament (NFL) in the hippocampus. In the present project we studied the potential behavioral and structural effect of two drugs used to treat major depression in humans: fluoxetine (FLX) and valproic acid (VPA). Male adult Wistar rats in which LH behavior was developed as a consequence of exposure to inescapable footshocks were treated for 21 days with fluoxetine (FLX, 10 mg/kg, i.p.), valproic acid (VPA 400 mg/kg, i.p.) or the association of both (FLX-VPA400). Two control groups treated with saline solution were included: one in which the LH behavior was induced (LH-S) and the other not exposed to inescapable footshocks (C). In all these groups we assessed by immunohystochemistry the immunoreactive area for NFL and the depressive behavior in two tests: an avoidance task and the forced swimming test (FST). FLX chronic treatment reverted the LH behavior but failed to reverse the decrement in NFL. VPA400 did modify neither the LH behavior nor the FST behavior, but reverted the diminution in the cytoskeleton protein, and FLX-VPA400 treated group showed both the normalization of the behavioral performance and the reversion of the decrement in NFL. Our results seem to give a structural support for the adjuvant action of VPA in antidepressant treatments.

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THE ACTION OF NMDA AND Y27632 ON RHO GTPASES AND PHOSPHOINOSITIDE-3-KINASE AFFECTS MEMBRANE DYNAMICS AND DENDRITIC OUTGROWTH

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During their development hippocampal neurons elaborate dendrites and axons. The small GTPases Rho, Rac and Cdc42 influence morphogenesis by regulating the actin cytoskeleton. Dendrite formation is a dynamic process consisting of outgrowth and retraction. Rac and Cdc42 facilitate outgrowth, whereas Rho attenuates it. Retraction is mediated via phosphoinositide-3-kinase (PI3-K). We investigated, whether the activation of glutamate receptors of the NMDA type affects dendrite development. NMDA activated Rac, but only minor morphological effects occurred. When NMDA was combined with Y-27632, a Rho-kinase inhibitor, a pronounced dendrite formation was observed. NMDA alone increased PI3-K activity measured as phophorylation of AKT. Y-27632 prevented the NMDA-induced increase. Membrane dynamics, outgrowth and retraction were studied in time-lapse experiments. NMDA alone increased membrane dynamics but did not induce new neurites. The combination of NMDA and Y-27632 enhanced membrane dynamics and the addition of neurites and retraction was reduced. To study whether this reduction was due to the inhibition of PI3-K, LY2940002 was used as an inhibitor. LY2940002 and NMDA together reduced retraction to the same extent as the combination of Y27632 and NMDA, indicating that dendrite formation caused by this treatment was due to a reduction of PI3-kinase activity. Our results show that dendrite formation is dependent on the interaction between small GTPases and PI3-Kinase. The activation of Rac leads to increased membrane activity but not to a dendritic elaboration. This requires an additional reduction in Rho activity and a reduced PI3-kinase activity.

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STATUS EPILEPTICUS-INDUCED CHANGES IN THE CYTOSKELETAL MICROTUBULE-ASSOCIATED PROTEIN 2 IN THE DEVELOPING RAT HIPPOCAMPUS

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Microtubule-associated protein-2 (MAP-2) is the most abundant cytoskeletal microtubule-associated protein (MAP) in the mammalian brain having an important role in cellular stability and plasticity. We have now studied developmental changes in the expression of MAP-2a, MAP-2b, MAP-2c and MAP-2d isoforms in the rat hippocampi at postnatal (P) days 1–21 by semiquantitative Western blotting, which was also used to study the temporal expression patterns (30 min–7 days) of MAP-2 isoforms after kainic-acid (KA)-induced status epilepticus (SE) in the hippocampi of P9 rats. Immunocytochemistry was used to localize MAP-2a and b in the rat hippocampus through P1 to P21. In Western blotting, the expression of MAP-2a and b significantly increased, whereas those of MAP-2c and d significantly decreased through P1–P21, the most prominent changes occurring after the first postnatal week. KA-induced SE significantly, rapidly, but reversibly increased the expression of MAP-2a and b. Immunocytochemical staining showed temporally and spatially heterogeneous expression of MAP-2a and b in different neuronal compartments, the staining intensity increasing through P1 to P21 in particular in the stratum moleculare of the dentate gyrus, and the stratum radiatum of CA1 and CA3 regions. Our results suggest that various MAP-2 isoforms could have a different functional role during the neuronal differentiation in the rat hippocampus. Moreover, the structural integrity of MAP-2 proteins could enhance neuronal viability by stabilizing cytoskeletal structure in neuronal damage.

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Epilepsy

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GLIAL-NEURONAL INTERACTIONS DURING THE LATENT AND CHRONIC PERIODS FOLLOWING KAINATE INDUCED SEIZURES IN RATS

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Kainic acid (KA), a glutamate analogue and a potent convulsant, is commonly used in an animal model of human temporal lobe epilepsy. Previous studies of human epileptogenic hippocampus have revealed a perturbed glutamate homeostasis, with an extracellular glutamate overflow during seizures and a reduced glutamate-glutamine cycling. In addition, key enzymes in glutamate metabolism, phosphate activated glutaminase (PAG) and glutamine syntethase (GS) are increased and decreased, respectively. In the present study limbic seizures were induced in rats by intraperitoneal injections of KA. The rats were sacrificed either in the latent period, defined as the period that precedes the onset of chronic epilepsy or in the chronic phase which was validated by video surveillance. To elucidate the mechanisms underlying epileptogenesis we investigated glial–neuronal interaction in the hippocampal formation using ^{13}C NMR spectroscopy and Western blot analysis. In the latent period glutamine syntethase (GS) was increased and this was also the case for glial fibrillary acidic protein (GFAP) indicating gliosis. In the chronic phase, however, GS was not different from control, whereas GFAP remained increased indicating altered astrocytic metabolism. The predominantly neuronal enzyme phosphate activated glutaminase (PAG) was unchanged both in the latent and the chronic phase.

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LOSS OF Glut-CELLS IN THE HIPPOCAMPUS OF EPILEPSY PATIENTS WITH AMMON'S HORN SCLEROSIS

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We have recently identified two subpopulations of astrocytes coexisting in postnatal mouse hippocampus with distinct morphological and functional properties. In detail the cells show a non-overlapping expression of either AMPA-type glutamate receptors (GluR-cells) or glutamate transporters (GluT-cells) (Matthias *et al.*, 2003) and differences in their gap-junction coupling. While GluT-cells are coupled with more than 100 neighbouring cells, GluR-cells are always uncoupled (Wallraff *et al.*, 2004). To find out whether these two different astroglial cell types are also present in human tissue, astrocytes were investigated in hippocampal slices (CA1 region) obtained from surgical specimens of patients with pharmaco-resistant temporal lobe epilepsy. Both GluR- and GluT-cells were identified in patients with lesion-associated epilepsy with these cells displaying the same characteristics as previously observed in mouse. Strikingly, an almost complete loss of GluT-cells was noted in slices from patients with Ammon's horn sclerosis (AHS). To find out if this was due to death of GluT-cells or alterations of GluT-cell´s properties, we used a pilocarpine mouse-epilepsy model and found astrocytes in a transitional stage with both, glutamate-receptors and glutamate-transporters. In human sclerosis, the loss of GluT-cells forming the astroglial syncytium will impair the clearance of the extracellular space from neuronally released glutamate und K^+, resulting in enhanced depolarization of glial cells and neurons. We conclude that in AHS, altered astrocyte functioning contributes to the generation and/or spread of seizure activity.

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EXPRESSION AND SUBCELLULAR LOCALIZATION OF MATRIX METALLOPROTEINASE 9 (MMP-9) AND ITS mRNA IN RAT BRAIN AFTER EXPERIMENTAL STATUS

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Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults. It results in part from aberrant neuronal plasticity, that occurs in the hippocampal dentate gyrus (DG). Recently it has been demonstrated that matrix metalloproteinase 9 (MMP-9) is upregulated within DG in response to kainate-induced status epilepticus (a rat model of TLE induction). To address the question of the presumable role of MMP-9 in TLE pathogenesis, we studied the distribution of MMP-9 and its mRNA in the hippocampus, in both control and kainate treated rats, using high resolution immunohistochemistry and fluorescent in situ hybridization at both light-(confocal microscopy) and electron microscopic levels. In control hippocampi, we found both MMP-9 immunoreactivity and in situ hybridization in the form of small (submicrometer), discrete, weakly-to-moderately positive foci, distributed sparsely over the neuronal cell-bodies, and within the neuropil. Double labelling experiments showed them to colocalize with synaptic and dendritic markers. At 24 hours after kainate treatment, a dramatic increase occurred in the numbers of MMP-9- and MMP-9 mRNA-positive dendrites/synapses in the DG hippocampal subfield. As an outcome, our studies show that after status epilepticus, MMP-9 and its mRNA are increased within the DG neuronal dendritic/synaptic domains. Since those are the structures that undergo aberrant plastic changes during TLE progression, the results suggest a role for MMP-9 in the pathogenesis of this disease.

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METABOLISM IS NORMAL IN ASTROCYTES IN CHRONICALLY EPILEPTIC RATS: A ^{13}C NMR STUDY OF NEURONAL-GLIAL INTERACTIONS IN A MODEL OF TEMPORAL LOBE EPILEPSY

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The aim of the present work was to study potential disturbances in metabolism and interactions between neurons and glia in the lithium-pilocarpine model of temporal lobe epilepsy. Rats chronically epileptic for one month received [1-^{13}C]glucose, a substrate for neurons and astrocytes, and [1,2-^{13}C]acetate, a substrates for astrocytes only. Analyses of extracts from cerebral cortex, cerebellum and hippocampal formation (hippocampus, amygdala, entorhinal and piriform cortices) were performed using ^{13}C and ^{1}H nuclear magnetic resonance

spectroscopy and HPLC. In the hippocampal formation of epileptic rats, levels of glutamate, aspartate, N-acetyl aspartate, ATP plus ADP and glutathione were decreased. In all regions studied labeling from [1,2-^{13}C]acetate was similar in control and epileptic rats, indicating normal astrocytic metabolism. However, labeling of glutamate, GABA, aspartate and alanine from [1-^{13}C]glucose was decreased in all areas possibly reflecting neuronal loss. The labeling of glutamine from [1-^{13}C]glucose was decreased in cerebral cortex and cerebellum and unchanged in hippocampal formation. In conclusion, no changes were detected in glial-neuronal interactions in the hippocampal formation while in cortex and cerebellum the flow of glutamate to astrocytes was decreased, indicating a disturbed glutamate–glutamine cycle. This is, to our knowledge, the first study demonstrating that metabolic disturbances are confined to neurons inside the epileptic circuit.

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INVOLVEMENT OF PROTEIN KINASE C IN THE INHIBITION OF GLUTAMATE RELEASE MEDIATED BY NEUROPEPTIDE Y2 RECEPTORS IN THE HIPPOCAMPUS

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We investigated the intracellular mechanisms associated with NPY Y1, Y2 and Y5 receptors by looking at the modulation of glutamate release in control and epileptic rats, injected with kainic acid (KA; 10 mg/kg) and killed 6 h and 24 h post-injection. In hippocampal nerve terminals obtained from control rats, we observed that NPY (1 μM) inhibited the 15 mM KCl-evoked glutamate release to 66.0 ± 4.7% of the control. Moreover, the activation of Y1, Y2 or Y5 receptors with [Leu31, Pro34] NPY (1 μM), NPY 13–36 (300 nM) or NPY (19–23) (Gly1,Ser3,Gln4,Thr6,Ala32,Gln34)-PP (1 μM), respectively, inhibited glutamate release to 66.8 ± 2.2%, 71.0 ± 1.1% or 60.8 ± 3.5% of the control, respectively. However, in the presence of 300 nM PMA (Protein Kinase C activator) the release of glutamate was increased to 129.7 ± 3.0% of the control and the inhibitory effect induced by NPY or NPY13–36 was completely blocked. In epileptic rats, 6 h after KA injection, neither NPY or Y2 receptor agonist nor PMA had any effect on glutamate release. Conversely, 24 h after KA injection NPY or NPY13-36 inhibited glutamate release to 62.0 ± 1.4% or 68.6 ± 1.4% of the control, respectively, and this effect was once again blocked in the presence of PMA. Taken together, these results suggest a clear involvement of PKC in the signalling pathways through which NPY modulates glutamate release. Moreover, it seems that in epileptic rats (6 h post-injection) PKC is maximally active preventing the NPY receptor-mediated inhibition of glutamate release.

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VINPOCETINE INHIBITS THE RISE IN SODIUM AND CALCIUM INDUCED BY 4-AMINOPYRIDINE IN STRIATAL ISOLATED NERVE ENDINGS

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This study was directed to get a more understandable picture of the mechanism underlying the anticonvulsant action of vinpocetine (VPC). The question of how the cerebral excitability is affected was investigated by determining the effect of VPC on the changes on internal Na^+ and Ca^{2+} induced by the convulsing agent 4-aminopyridine (4-AP) in striatal synaptosomes. We found that VPC, like the Na^+ channel blocker, tetrodotoxin (TTX), abolishes the increase in Na^+ (determined by SBFI) induced by 0.1 mM 4-AP and partially inhibits the rise in Na^+ induced by 4-AP at higher concentrations. In contrast with the larger rise in Na^+ induced by 1 mM 4-AP than by 0.1 mM 4-AP, the rise in Ca^{2+} (determined by fura-2) induced by 0.1 and 1 mM 4-AP is similar, and markedly inhibited by VPC, as well as by TTX or by the blockade of calcium channels with w-agatoxin-IVA in combination with w-conotoxin-GVIA. These findings demonstrate that VPC only inhibits the fraction of the rise in Na^+ induced by 4-AP that involves the simultaneous activation of presynaptic voltage sensitive sodium and calcium channels. In agreement with this interpretation, the elevation of Ca^{2+} induced by 4-AP, that is VPC sensitive requires a physiological (out/in) Na^+ gradient. The elevation of Ca^{2+} induced by high K^+ (30 mM), that only involves activation of Ca^{2+} channels and is inhibited by the w-agatoxins IVA and TK, does not require a Na^+ gradient and is VPC and TTX insensitive. It is concluded that the negative modulation exerted by VPC on presynaptic sodium channels and the concomitant inhibition of calcium channels may underlie its anticonvulsant action *in vivo*.

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OMEPRAZOLE AFFECTS NEITHER ELECTROCONVULSIVE THRESHOLD NOR ANTICONVULSANT ACTIVITY OF DIPHENYLHYDANTOIN AND CARBAMAZEPINE IN MICE

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Omeprazole is a commonly prescribed drug for patients with peptic ulcerations. Its main mechanism of action is related to inhibition of H^+-K^+-ATP-ase, albeit it may also block carbonic anhydrase. This study evaluates the effects of acute and prolonged (3- or 7-day) intragastrical administration of omeprazole on the anticonvulsant activity of carbamazepine or diphenylhydantoin against maximal electroshock-induced seizures in mice. This test is used as an experimental model of generalized tonic-clonic epilepsy in humans. The experiments were carried out on male Swiss mice weighing 20–25 g. Electroconvulsions were produced using ear-clip electrodes (0.2 s stimulus duration of alternating current, tonic hindlimb extension taken as the endpoint). Omeprazole administered acutely (1-day), for 3-days or 7-days did not alter the electroconvulsive threshold in mice. Moreover, the drug did not affect the anticonvulsant activity of the tested antiepileptic drugs. Balakrishnan *et al.* (Epilepsy Res, 2001) showed that omeprazole effectively enhanced the threshold for electroconvulsions in rats and that the tested animals developed tolerance to the omeprazole-mediated anticonvulsant action when the drug was administered for 6 days. However presented results clearly indicate that omeprazole cannot be considered as a candidate for antiepileptic drug in further preclinical studies because of the lack of its efficacy against maximal electroconvulsions in mice and rapidly developed tolerance to the antiseizure properties in rats.

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VLGR1 IS RESPONSIBLE FOR AUDIOGENIC SEIZURE SUSCEPTIBILITY

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Susceptibility to audiogenic seizures, that are reflex seizures provoked by loud noise, can be induced in rodents by acoustic priming (expos-

ing animals to strong auditory stimuli at an early developmental stage). Some strains of mice and rats are susceptible to audiogenic seizures without priming, and they have been used as good experimental models with which to study epilepsies. Here we identified *Vlgr1d* and *Vlgr1e*, novel alternatively spliced variants of *Vlgr1b/MGR1*. *Vlgr1* (*Vlgr1b*, *Vlgr1d* and *Vlgr1e*) mRNA is expressed predominantly in the neuroepithelium of the developing mouse brain. Knockout mice lacking exons 2–4 of *Vlgr1* were susceptible to audiogenic seizures without priming, although there were not apparent histological abnormalities in their brain. Ninety-five percent of these knockout mice exhibited wild running, a feature typical of the preconvulsive phase of audiogenic seizures triggered by loud noise (11 kHz, 105 dB), and 68% exhibited tonic convulsions at 3 weeks after birth. To elucidate the influence of genetic background in the susceptibility of audiogenic seizures, we backcrossed the Vlgr1 knockout mice to C57Bl6/J mice that are known to be highly resistant to audiogenic seizures. C57BL/6J-backcrossed Vlgr1 knockout mice were surely susceptible to audiogenic seizures. Our monogenic mice, which have a unique genetic background, serve as a useful tool for further studies on seizures.

Ion channels

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PARADOXICAL NIFEDIPINE FACILITATION OF 45CA UPTAKE INTO RAT HIPPOCAMPAL SYNAPTOSOMES IS OPERATED BY NA+ LOADING THROUGH TTX-SENSITIVE CHANNELS

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Nifedipine, a drug used for treating hypertension and angina, exerts its effect by blocking Cav1 (L-type) channels. Compared with other dihydropyridines, nifedipine has a high incidence of neurologic adverse reactions. In the micromolar range (EC50 = 7.8 μM), nifedipine transiently increases epileptiform potentials and facilitate glutamate release, via a mechanism that is largely Ca^{2+}-independent. Thus, we investigated the action of nifedipine on 45Ca uptake into rat hippocampal synaptosomes depolarised directly, by high $[K^+]o$ (15–30 mM), or indirectly, by veratridine (VT, 1–10 μM). Synaptosomal loading was performed during 3 min with $45CaCl_2$ (0.32 μCi/μmol) in the presence of the depolarizing agent. 45Ca uptake was stopped with ice-cold EGTA followed by filtration through GF/C glass-fibre filters. In contrast to nitrendipine (0.001–10 μM), nifedipine (0.001–10 μM) caused a dual role on VT (10 μM)-induced 45Ca uptake. In the nanomolar range (<0.3 μM), nifedipine decreased 45Ca uptake in a $CdCl_2$ (500 μM)-sensitive manner. Nifedipine (0.3–10 μM) consistently facilitated 45Ca uptake even in the presence of $CdCl_2$ (500 μM). The facilitatory effect of nifedipine was not apparent upon depolarization with 30 mM $[K^+]o$. Extracellular Na^+ substitution by N-methyl-D-glucamine (132 mM) or blockade of Na^+ currents with tetrodotoxin (1 μM), prevented nifedipine-facilitation. Inhibition of the Na^+/Ca^{2+}-exchanger with KB-R7943 (3–50 μM) did not mimic facilitation by nifedipine. Data suggest that paradoxical nifedipine facilitation of 45Ca uptake into rat hippocampal synaptosomes is independent of calcium channels and may be operated by Na^+ loading through TTX-sensitive channels.

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PRIP IS INVOLVED IN THE RAPID DOWNREGULATION OF $GABA_A$ RECEPTOR SURFACE EXPRESSION CAUSED BY BDNF

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The brain-derived neurotrophic factor (BDNF) modulates several distinct aspects of synaptic transmission, including $GABA_A$ receptor. An exposure to BDNF rapidly down-regulates the $GABA_A$ receptor surface stability (or number) and changes the properties of $GABA_A$ receptor. We previously reported that the PRIP-1 (phospholipase C-related inactive protein type 1), an inositol 1,4,5-trisphosphate-binding protein, is an important molecule in the $GABA_A$ receptor signaling by analyzing the mice lacking PRIP-1 (Kanematsu *et al.*, EMBO J., 2002). In the present study, we studied the possible involvement of PRIP in the modulation of postsynaptic $GABA_A$ receptor by BDNF using the cultured hippocampal and cortical neurons prepared from PRIP-1 and -2 double knockout mice (PRIP-DKO mice). The exposure to BDNF reduced the GABA-evoked inhibitory current (I_{GABA}) in cultured hippocampal neuron of wild type (WT) mice, whereas a little potentiation was observed in the PRIP-DKO mice, corresponding to the surface expression of $GABA_A$ receptor number. As PRIP bound to β-subunits of $GABA_A$ receptor, we mapped the region in PRIP responsible for the interaction with the β-subunits, and the peptide mimicking that region blocked the co-localization of PRIP to $GABA_A$ receptor at the plasma membrane and the attenuation of I_{GABA} in WT hippocanpal neurons. These results indicate that the direct interaction between $GABA_A$ receptor β-subunits and PRIP plays a key role for the modulation of $GABA_A$ receptors by BDNF.

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MICROFLUIDIC CHIP SOLUTIONS FOR PATCH-CLAMP-BASED ION CHANNEL DRUG SCREENING AND CHARACTERIZATION

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We describe a method relying on microfluidic chip-based platform used together with conventional patch-clamp for high capacity dose-response analysis of substances affecting ion channels. Drug discovery and receptor characterization can be performed with high speed, high accuracy, low consumption of drugs (μL) and increased safety for the user. The microfluidic chips are comprised sample wells connected to an open volume chamber via tightly packed microchannels. By utilizing pressurized air, liquids are pumped from the reservoirs into the chamber where the liquid flow from each channel viscously couples creating a collimated, laminar flow. The liquid flow from each channel continues in discrete zones and creates an array of completely controlled liquid environments. A patch-clamped cell is used to scan the content of each zone for drugs affecting ion channels, basically like a bar code reader where each bar corresponds to the content of a channel. In this way, the drugs can be administered rapidly (>10 ms) with extremely rapid switch times (ms) to a patch-clamped cell with complete control over the liquid environment surrounding the cell. The microfluidic chip technology is scalable and applies to several areas in the drug discovery process such as target validation, later phases of lead identification, lead optimization, and preclinical studies as well as for safety assessment applications and biomedical research. The technology applies to most patch-clamp experiments using whole-cell or excised membrane patch-configurations. Data from characterization of agonists, antagonists and blockers acting voltage- and ligand-gated ion channels will be presented.

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TARGETING OF BK CHANNELS TO SPECIFIC DOMAINS OF DENTATE GYRUS GRANULE CELLS

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The precise subcellular localization of ion channels is of paramount importance in neuronal communication and integration. The physiological impact of each ion channel crucially depends on the site where it is localized. This study was undertaken to establish the complete subcellular distribution profile of big conductance calcium activated

potassium channels (BKs) in hippocampal granule cells of rodents at the ultrastructural level. Affinity purified, sequence specific rabbit antisera raised against the channel alpha subunit were used for immunodetection of these channels. The ultrastructural localization was studied by means of pre-embedding peroxidase and post-embedding immunogold electron microscopy. BK channels were found unevenly distributed over the cell surface membrane and targeted to specific subcellular domains. They were enriched in the presynaptic active zone of the granule cell axon terminals, the mossy fiber terminals. Besides the presynaptic localization, BK channels were detected in somatodendritic compartments. At dendritic spines, channels were localized to the plasma membrane of spine head and neck. Also at the granule cell soma, a low but consistent expression was seen. BK channels were absent from symmetric as well as asymmetric synaptic junctions but localized to the extrasynaptic plasma membrane. Clusters of BK channels were detected in areas of somatic subsurface membrane cisternae. In conclusion, we report specific targeting of BK channels to certain domains of hippocampal granule cells. This domain-specific localization of BK channels indicates physiological implications for several important functions ranging from spike processing to transmitter release.

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CALCIUM INFLUX INTO PURKINJE CELLS IS ESSENTIAL FOR ACTIVITY-DEPENDENT MATURATION OF CLIMBING FIBER INNERVATION

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To study possible involvement of calcium influx in the postnatal refinement of mammalian neural circuits, we investigated the morphological relationship between climbing fiber and Purkinje cell in the developing cerebellum lacking P/Q-type calcium channel α1A, constituting more than 90% of the total calcium current density into Purkinje cells. By injecting DiI into the inferior olivary nuclei of fixed brain, we succeeded to label some single climbing fibers. At postnatal day 7 (P7) and P9, a single-stem climbing fiber in the knock-out mouse branched a few times in the granular layer and its collaterals innervated Purkinje cell somata respectively, not forming a distinguishable nest. At P14, several distinct abnormalities in the climbing fiber trajectories were observed in the mutant, such as extensive arborization of afferent axons before reaching target cell layer and their impaired distal elongation along the dendritic trees of Purkinje cells. The expression level of α1A in Purkinje cells is significantly increased around P7 and sustained at later stages. Taken together, our anatomical observations suggest that two major processes of climbing fiber synaptogenesis, synaptic translocation and target convergence, may occur simultaneously during the second postnatal week and calcium influx into Purkinje cells in this phase triggered by climbing fiber activation may be an essential phenomenon for activity-dependent refinement of climbing fiber innervation.

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$Ca_v1.2$ SUBUNITS FORM ANOMALOUS L-TYPE CALCIUM CHANNELS IN MOUSE CEREBELLAR GRANULE CELLS

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Functionally different L-type Ca^{2+} channels (LTCCs) coexist in a variety of neurons including cerebellar granule cells (CGCs). Single channel recordings have previously shown that in addition to the classical neuronal 'cardiac-like' channel, LTCCs with 'anomalous' gating properties exist in CGCs. This anomalous LTCC gating is characterized by re-openings during negative voltages after a depolarizing prepulse. Their capability to produce a delayed Ca^{2+} influx after depolarization makes them ideally suited to enhance internal Ca^{2+} signalling following strong neuronal activity. The molecular basis for these anomalous currents is unknown, but they must be formed by either $Ca_v1.2$ or $Ca_v1.3$ Ca^{2+} channels. Absolute quantitation of mRNA levels showed that expression of $Ca_v1.3$ was 10% compared to $Ca_v1.2$. Single-channel patch-clamp recordings from CGCs of wildtype (wt), $Ca_v1.3^{-/-}$ and $Ca_v1.2DHP^{-/-}$ (DHP sensitivity of $Ca_v1.2$ was eliminated) mice were performed in the presence of the Ca^{2+} channel activator (+)-S202-791. Anomalous LTCCs were revealed in 38% (8 out of 21) of wt and in 48% (12 out of 25) of $Ca_v1.3^{-/-}$ mice by the presence of characteristic long re-openings. In $Ca_v1.2DHP^{-/-}$ mice only shorter re-openings were elicited in the presence of (+)-S202-791 ($n = 18$). Our results indicate that $Ca_v1.2$ LTCCs from anomalous Ca^{2+} currents in mouse CGCs. We suggest that in these cells the anomalous gating of $Ca_v1.2$ calcium channels plays an important role for differential neuronal response.

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BINDING STUDY OF HANATOXIN AND S3c SEGMENT IN HUMAN VOLTAGE-DEPENDENT POTASSIUM CHANNEL Kv2.1 WITH KINETIC ANALYSIS

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S4 segment is known as the voltage sensor in mammalian voltage-gated potassium channels, whereas the carboxyl terminus of S3 segment (S3c) bears its particular interests concerning the site for gating modifier toxins like hanatoxin. The thus derived helical secondary structural arrangement for S3c, as well as its surrounding environment, has since been intensively and vigorously debated. The most recently unraveled crystal structure of an archaebacterial K^+ channel, KvAP, has provided the possibility of advanced analyses. However, due to the possible distortion by monoclonal antibody used for crystallization, such porposed new model still remained controversial. In addition, biochemical data regarding this, especially for the interaction between S3c and its modifier toxins, are nevertheless so far absent. Therefore, in this study, to investigate the binding behavior between hanatoxin and drk1, we synthesized the FITC-labeled and unlabeled Kv2.1 S3c fragments, and performed kinetic competitions upon hanatoxin binding through stopped-flow analysis. After appropriate refolding treatment, a lower koff value indicating apparent binding between hanatoxin and S3c fragment was observed. In combination of the negative control with

shaker peptide, it is suggested a putatively very high kon value, subsequently reflecting a fairly low KD value and therefore fairly strong binding for the S3c-hanatoxin complex in human voltage-gated K^+-channel drk1.

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AGONIST BINDING, EFFICACY AND DOMAIN CLOSURE OF GluR2

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Ionotropic glutamate receptor involvement in neurological diseases has stimulated widespread interest in their structure and function. Despite a large number of agonists developed so far, few display selectivity among AMPA receptor subtypes. The present study provides X-ray structures of the GluR2-selective, partial agonist CPW399 in complex with a construct of the GluR2 ligand-binding core (GluR2-S1S2J) and with a (Y702F)GluR2-S1S2J mutant. In addition, the structure of the non-selective partial agonist kainate (KA) in complex with (Y702F)GluR2-S1S2J was determined. The selectivity of CPW399 towards GluR2 vs. -3 is reflected in the binding data on the two constructs, allowing the use of (Y702F)GluR2-S1S2J as a model system for studying GluR2/3 selectivity. Structural comparisons suggest that selectivity arises from disruption of a water-mediated network between ligand and receptor. CPW399 and KA induce greater domain closure in the Y702F mutant, indicating that these partial agonists act in a manner more like full agonists in this mutant. Both KA and CPW399 exhibited higher efficacy at (Y723F)GluR2$(Q)_i$ than at wild-type GluR2$(Q)_i$. Whereas an excellent correlation exists between domain closure and efficacy of a range of agonists at GluR2$(Q)_i$, determined by electrophysiology in X. laevis oocytes, a direct correlation between agonist-induced domain closure of (Y702F)GluR2-S1S2J and efficacy at GluR3 is not observed. While clearly controlling selectivity, mutation of this residue alone is insufficient to explain agonist-induced conformational rearrangements occurring in this variant.

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SERINES 890 AND 896 OF NMDA RECEPTOR SUBUNIT NR1 ARE DIFFERENTIALLY PHOSPHORYLATED BY PROTEIN KINASE C ISOFORMS

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The NR1 subunit of NMDA receptor has two serines (S890 and S896) which phosphorylation by protein kinase C (PKC) differentially modulates NMDA receptor trafficking and clustering. It is not known which PKC isoforms phosphorylate these serines. We studied, in primary cultures of cerebellar neurons, which PKC isoforms are responsible for the phosphorylation S890 and S896. We used specific inhibitors of PKC isoforms and antibodies recognizing specifically phosphorylated S890 or S896. The results show that PKC alpha phosphorylates preferentially S896 and PKC gamma preferentially S890. Activation of type I metabotropic glutamate receptors (mGluRs) with DHPG [(S)-3,5-Dihyidroxyphenylglycine] activates PKC gamma but not PKC alpha or beta. DHPG increases S890 but not S896 phosphorylation, supporting a role for PKC gamma in the physiological modulation of S890 phosphorylation. It is also shown that the pool of NR1 subunits present in the membrane surface contains phosphorylated S890 but not phosphorylated S896. This supports that differential phosphorylation of S890 and S896 by different PKC isoforms modulates cellular distribution of NMDA receptors and may also contribute to the selective modulation of NMDA receptor function and intracellular localization.

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AT LEAST THREE DIFFERENT L-TYPE Ca^{2+} CHANNELS CONTRIBUTE TO HIGH AFFINITY DHP-BINDING IN MOUSE BRAIN

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We recently developed Cav1.2DHP-mice in which high DHP sensitivity was removed from Cav1.2 voltage-gated L-type Ca^{2+} channels (LTCCs). In Cav1.2DHP-brains >80% of the high affinity binding for the LTCC-selective blocker (+)-[3H]isradipine (ISR) are absent and must represent Cav1.2. To determine the relative contribution of other LTCCs to ISR binding we generated Cav1.2DHP-mice also lacking Cav1.3 channels (double mutants, DM). Cav1.2DHP-mice exhibited $12.6 \pm 1.6\%$ ($n = 12$) and DM mice $3.7 \pm 0.6\%$ ($n = 10$) of WT ISR binding. This indicates that Cav1.3 accounts for 9% of the LTCCs in the brain. 3.7% must be associated with Cav1.1 which we identified on the mRNA level. We exploited Cav1.2DHP-mice to determine whether LTCC blockers show selectivity for Cav1.2 or the residual binding component by measuring IC50 values for ISR binding inhibition in WT and Cav1.2DHP-mice. Whereas isradipine, azidopine and amlodipine exhibited indistinguishable affinities a 3–5-fold Cav1.2 selectivity for the Cav1.2 component was observed for nitrendipine and nifedipine. This suggested that they bind with lower affinity to Cav1.3 (and Cav1.1). This lower affinity was directly confirmed in binding studies with Cav1.2 and Cav1.3 channels expressed in tsA-201 cells. We provide biochemical evidence that in addition to Cav1.2 and Cav1.3 another LTCC isoform, most likely Cav1.1, comprises about 4% of the LTCCs in mouse brain. We also demonstrate that some DHPs (e.g. nitrendipine, nifedipine) exhibit higher binding affinity for Cav1.2 than Cav1.3. This suggests that the development of selective modulators for these two major neuronal LTCCs may be feasible.

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THE $\alpha_2\delta$-1 SUBUNIT DIFFERENTIALLY REGULATES CURRENTS AND MEMBRANE EXPRESSION OF MUSCLE AND NEURONAL CALCIUM CHANNELS

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A variety of voltage-gated Ca^{2+} channels controls diverse functions of excitable cells. CaV1.1 is the voltage-sensor activating skeletal muscle contraction, CaV1.2 activates cardiac and smooth muscle contraction and is involved in activity-dependent modulation of synaptic strength in the brain, and CaV2.1 and CaV2.2 trigger neurotransmitter release. All of these channels contain an auxiliary $\alpha_2\delta$ subunit. However its role in the various cell functions is still poorly understood. Recently we reported that in skeletal muscle $\alpha_2\delta$-1 determines the characteristic slow L-type current kinetics, but it is non-essential for normal channel targeting and excitation-contraction coupling. siRNA depletion of $\alpha_2\delta$-1 in dysgenic (α 1S-null) myotubes reconstituted with the CaV1.2(α 1C) subunit shifted the voltage-dependence of the currents by +11 mV and altered the kinetics. However, activation kinetics were changed in the opposite direction as observed with CaV1.1(α 1S). This indicates that $\alpha_2\delta$-1 itself is not the determinant of either slow or fast current kinetics but that it enables each α 1 subunit to express its specific current characteristics. In contrast, depletion of $\alpha_2\delta$-1 showed no effects on voltage-dependence and kinetics of P/Q- and N-type currents, but current densities were reduced to 40% (CaV2.1) and 51% (CaV2.2) of control values. This was paralleled by reduced membrane expression observed in immunofluorescence analysis. Together these

results demonstrate that the $\alpha_2\delta$-1 subunit exerts specific effects on different calcium channel types.

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INHIBITION OF HIGH VOLTAGE-ACTIVATED CALCIUM CHANNELS BY SPIDER TOXIN, PnTx3-6

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Animal toxins have become powerful tools to study the structure-function relationships and physiological roles of voltage-activated calcium(Ca) channels. In the present study, we investigated the effects of PnTx3-6, a neurotoxin purified from the venom of the spider *Phoneutria nigriventer*, on cloned mammalian Ca channels expressed in HEK 293 cells. Patch clamp measurements indicate that PnTx3-6 reversibly inhibited P/Q-(α_{1A}/Ca_v2.1), L-(α_{1C}/Ca_v1.2), N-(α_{1B}/Ca_v2.2) and R-(α_{1E}/Ca_v2.3) type channels with the order of potency $\alpha_{1B} > \alpha_{1E} > \alpha_{1A} > \alpha_{1C}$ and IC_{50} values of 122 nM; 136 nM; 263 nM and 607 nM, respectively. Inhibition occurred without alteration of current kinetics or voltage-dependence of the exogenously expressed Ca channels. In contrast to its effects on HVA Ca channels subtypes, application of PnTx3-6 (1 μM) did not affect the T-type Ca channel. Based on our study, we suggest that PnTx3-6 may act as a ω-toxin that appears to target selectively high voltage-activated Ca channels with a preference for the Ca_v2 subfamily (N-, P/Q- and R-types).

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INFLUENCE OF OSMOTIC SHOCK ON SYNAPTOSOMAL FREE SODIUM CONCENTRATION

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Many diseases are accompanied with changing of the extracellular osmolarity. Osmotic shock leads to hyperexcitability, seizures. Mechanism of this phenomenon is still not clear. Sodium-dependent changing in neurotransmission can be background for these events. No direct measurements of sodium content in presynaptic endings were performed at changing of extracellular osmolarity. Intracellular sodium concentration was monitored by fluorescent dye SBFI. Media osmolarity varied from 230 mOsm/l to 810 mOsm/l. Basal concentration of intrasynaptosomal sodium was estimated as 10 ± 1.3 mM. When synaptosomes were treated with veratridine we observed an increase of Na^+ influx on over $30 \pm 0.2\%$. Ouabain leads to an increase of $[Na^+]i$ at 0.5 ± 0.01 mM. Sodium entry caused by veratrine was eliminated by tetrodotoxin. In synaptosomes exposed to hypotonic shock, no sodium entry was shown. These data suggest sodium influx doesn't play a key role in hypotonic swelling induced modulation of neurotransmitter transport. At hypertonic shrinkage of synaptosomes we detect massive Na^+ influx. Hypertonic induced Na^+ influx was not inhibited by tetrodotoxin, bumetanide, mechanosensitive channels blocker Gd^{3+}. Then we tested action of Na channels inhibitor amiloride, which blocks Na entry. We investigate effect of MIA and DMA, the specific blockers of Na^+/H^+ exchanger, these compounds didn't reduce hypertonic induced Na^+ influx. We suggest hypertonic induced Na^+ influx is mediated by amiloride sensitive degenerine/ENaC channels.

Neurodegenerative diseases Parkinson's and others

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H_2O_2 IS AN ENDOGENOUS MODULATOR OF MIDBRAIN DOPAMINE NEURON ACTIVITY VIA K_{ATP} CHANNELS

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We reported previously that endogenous as well as exogenous H_2O_2 can suppress dopamine (DA) release in substantia nigra pars compacta (SNc) in guinea-pig midbrain slices (Chen *et al.*, 2002). Here we assessed the physiological mechanism of this effect using whole-cell recording coupled with H_2O_2 imaging. Identified DA cells in SNc were loaded with an H_2O_2-sensitive dye, DCF, via a patch pipette. Basal DCF fluorescence in all recorded neurons indicated tonic H_2O_2 generation. Decreasing intracellular H_2O_2 by including the peroxidase enzyme catalase in the patch-pipette or blocking ATP-sensitive K^+ (K_{ATP}) channels by glibenclamide increased the spontaneous firing rate of all DA neurons. However, only 50% of DA cells showed K_{ATP}-channel dependent hyperpolarization when H_2O_2 was increased by exogenous application (53%; $n = 15/28$) or by inhibiting glutathione peroxidase with mercaptosuccinate (MCS; 53%; $n = 20/38$). Responders were also hyperpolarized with diazoxide, a SUR1-subunit selective K_{ATP}-channel opener, but not with cromakalim, a SUR2-subunit selective opener, indicating that SUR1-based K_{ATP} channels conveyed enhanced sensitivity to elevated H_2O_2. Conversely, increasing intracellular H_2O_2 by inhibiting catalase with 3-amino-1,2,4-triazole (ATZ) caused a glibenclamide-reversible hyperpolarization in all recorded cells ($n = 12$). Excitingly, an initial increase in DCF fluorescence with ATZ was significantly greater than with MCS, with a t50 (time of 50% maximal response) of 1.4 ± 0.1 min ($n = 5$) for ATZ vs. 2.1 ± 0.2 min ($n = 5$; $P < 0.01$) for MCS responders and 2.3 ± 0.2 min ($n = 5$; $P < 0.01$) for MCS non-responders. Thus, H_2O_2 acts in a graded manner to regulate both tonic DA-cell activity and responsiveness to oxidative challenge via K_{ATP} channels.

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MPTP EXPOSURE INDUCES ALTERNATIVE SPLICING-ASSOCIATED CHANGES IN STRIATAL GENE EXPRESSION

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To explore the possibility that alternative splicing modifications are involved in the progression of Parkinson's disease, we developed a spotted microarray carrying oligonucleotide probes for spliceosome components, splicing and alternative splicing regulators and selected target genes. In FVB/N mice exposed to the dopaminergic neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), 9.5% of the tested transcripts showed 30%–50% up-regulation (e.g. in helicase ddx41 and splicing factor phosphorylation proteins ppm1g, SRPK2 and CLK) or down-regulation (e.g. in spliceosomal assembly mediator RBM8 and snRNP protein SNRPN). Because exposure to acetylcholinesterase inhibitors (anti-AChEs) increases the risk of Parkinsonism (1), we further studied TgS and TgR transgenic mice over-expressing the synaptic AChE-S or the exposure-induced soluble AChE-R splice variants, likely to modulate different transmission circuits (2). Reciprocally modified striatal expression levels of the vesicular monoamine transporters VMAT1 and VMAT2 were observed in these two transgenic lines, suggesting prominent and distinct changes in striatal monoaminergic neurons. Correspondingly, MPTP-exposed TgS and TgR mice presented distinct changes in transcripts which remained unchanged in FVB/N mice while otherwise showing strikingly limited changes (ca. 1% transcripts, 30% difference) in splicing-associated transcripts. The MPTP-ablated dopaminergic input to the striatum may thus induce splicing machinery modifications subject to cholinergic regulation which are causally involved in Parkinsonism.

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CO-TRANSPLANTATION OF ZUCKERKANDL'S ORGAN CELLS WITH VENTRAL MESENCEPHALIC CELLS (VMC) IN RAT MODEL OF PARKINSON'S DISEASE: ASSESSMENT OF FUNCTIONAL RESTORATION

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Our earlier studies have exhibited that glial cell line-derived neurotrophic factor (GDNF) has ability to increase the cell viability of the remaining host dopaminergic neurons, which in turn help in functional restoration for short period in rat model of Parkinson's disease (PD). Zuckerkandl's organ (ZK) is an extra adrenal paraganglionic tissue, which lies near the bifurcation of abdominal aorta and has ability to endogenously secrete GDNF and catecholamines including dopamine and norepinephrine. In the present investigation, an attempt has been made to validate the role of ZK cells co-transplantation along with fetal VMC on long-term functional restoration and viability of transplanted VMC in 6-OHDA lesioned rat model of PD. VMC were isolated from rat fetuses (ED-13) while ZK organ dissected out from adult rats. Single cell suspension of VMC and cultured ZK cells was co-transplanted in lesioned rats. Twelve weeks post transplantation, functional restoration was assessed using neurobehavioral and immunohistochemical parameters. A significant restoration ($P < 0.01$) in d-amphetamine induced rotations and spontaneous locomotor activity in rats co-transplanted with VMC & ZK cells was observed as compared to VMC alone transplanted rats. The functional viability of transplanted VMC was confirmed by tyrosine hydroxylase (TH) expression in the striatum. A significant restoration of TH-IR fibers density in co-transplanted (VMC + ZK) animals over VMC alone transplanted animals was observed. Results suggest that co-transplantation of VMC & ZK cells may be a better approach towards functional restoration in 6-OHDA lesioned rat model of PD as compared to VMC alone.

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ALPHA-SYNUCLEIN INCREASES CELL ADHESION MOLECULE, CD44 AND MT1-MMP

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Parkinson's disease (PD) is the most common neurodegenerative disorder that is characterized by movement disorder such as bradykinesia, rest tremor, rigidity and postural instability. The loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the round cytoplasmic inclusions, termed Lewy bodies (LBs) are signature hallmarks of PD. The cause of PD is not fully understood, but both genetic and environment factors are believed to be involved in the PD development. Recently, several genes have been identified in patients with familiar PD. Alpha-synuclein gene is the one found to be associated with an autosomal dominant form of familial PD. And also alpha-synuclein is an abundant component of Lewy bodies that characterize the pathological hallmark of PD. Microglial activation and increased levels of proinflammatory cytokines and decreased levels of neutrophins support an involvement of immune mechanism in pathogenesis of PD. And intracellular alpha-synuclein inclusions in glial cells in PD patients have been found. CD44 is a transmembrane glycoprotein that is involved in inflammation, adhesion, migration and invasion. Matrix metalloproteinases (MMPs) are believed to play a role in the invasion and metastasis in tumor cells by extracelluar matrix (ECM) degradation. One of the MMPs, membrane type 1 matrix metalloproteinase (MT1-MMP) has been shown to cleave CD44 ectodomain at the cell surface and promote cell migration. We therefore examined CD44 expression in the alpha-synuclein transfected murine microglial cell line, BV2. And we also examined its proteolytic enzyme MT1-MMP expression. These findings may indicate importance of alpha-synuclein expression in immune cell migration and further increment in inflammation of PD patients.

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PLEIOTROPHIN MEDIATES THE MATURATION OF DOPAMINERGIC NEURONS IN CULTURE

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Pleiotrophin (PTN) is a secreted heparin-binding growth factor that is highly expressed during early postnatal brain development and is upregulated after experimental lesions in the hippocampus of adult animals. Recently we have found that it is overexpressed in the striatum of 6-OHDA lesioned rats treated for 6 months with levodopa. To understand better the mechanism of action of PTN on DA neurons, we plated mixed primary mesencephalic cultures in plates precoated with PTN. These neurons had more elaborate neurites than control cultures. The number of TH-positive neurons also increased 20–30% in cultures plated on PTN compared with control, but this was due to better adhesion of the cells to the wells, not better survival. Furthermore, by adding a neutralizing anti-PTN antibody to the culture medium, we showed that endogenous PTN is implicated in the initial, and cAMP-dependent enhancement of, differentiation of the dopaminergic neurons. By in situ hybridization, we showed that ptn is mainly expressed by astrocytes in the cultures, whereas double fluorescence immunocytochemistry revealed that the PTN protein and its receptors, syndecan 3 and RTPT-z/b, are found on all cell types, including dopaminergic neurons, supporting the hypothesis that PTN is a trophic factor for these cells. PTN itself, or together with other neurotropic factors, might therefore increase the benefit of cell-based therapies for patients with Parkinson's Disease in which dopaminergic neurons degenerate.

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Abstract has been withdrawn.

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A PIVOTAL ROLE OF MIXED LINEAGE KINASES (MLK) IN MICROGLIAL AND ASTROCYTE INFLAMMATION

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CEP-1347 is a potent inhibitor of the mixed lineage kinases (MLKs), a distinct family of mitogen activated protein kinase kinase kinases (MAPKKK). It blocks the activation of the c-Jun/JNK apoptotic pathway in neurons exposed to various stressors and prevents neurodegeneration in animal models of Parkinson's disease (PD). Glial activation may involve kinase pathways controlled by MLKs, and might contribute to the pathology of neurodegenerative diseases. Therefore, the possibility that CEP-1347 modulates the inflammatory response was explored. Indeed, the MLK inhibitor reduced cytokine production in primary cultures of human and murine microglia, in primary astrocytes, and in monocyte/macrophage-derived cell lines, stimulated with various endotoxins, a cytokine mix or the plaque forming peptide Ab1-40. Moreover, CEP-1347 inhibited brain TNF production induced by intracerebroventricular injection of lipopolysaccharide in mice. As expected from a MLK inhibitor, CEP-1347 acted upstream of p38 and c-Jun activation in glia. The dampening of the activity of both pathways resulted in a complex pattern of inflammatory modulation as revealed by chip analysis. These data imply MLKs as important, yet unrecognized, modulators of brain inflammation, and demonstrate a novel anti-inflammatory potential of CEP-1347.

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CELL-PENETRATION AND MEMBRANE LEAKAGE BY PEPTIDES DERIVED FROM THE N-TERMINI OF UNPROCESSED PRION PROTEINS

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Using confocal fluorescence microscopy, we show that a peptide with sequence derived from the N-terminus (residues 1–30) of the unprocessed bovine prion protein (bPrPp) translocates the plasma membrane of live HeLa cells and therefore functions similar to a cell-penetrating peptide (CPP). The peptide includes the hydrophobic signal sequence (residues 1–24) and a short basic domain (KKRPKP, residues 25–30). The bPrPp peptide and its counterpart in the mouse prion protein (mPrP, residues 1–28) were found to cause hemolysis in human red blood cells and leakage in neuroblastoma N2A cells. These membrane effects were compared with those of two extreme cases, the typical CPP penetratin, and the pore-forming toxic peptide melittin. Intermediate effects between the two extremes were found for the two prion-derived peptides, with mPrP being more potent than bPrP. The CPP-like property and membrane leakage induced by the prion-derived peptides suggests that PrP with an uncleaved signal peptide has properties in its N-terminal domain which may be important for transfer between cellular membranes (infectivity) and destruction of membrane integrity (cytotoxicity).

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ROTENONE-INDUCED AGGREGATION OF ALPHA-SYNUCLEIN IS REVERSED BY RAPAMYCIN

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The intracellular aggregation of proteins may play a role in the pathogenesis of several neurological disorders. Intracellular aggregates of alpha-synuclein are found in several disorders, including Parkinson's Disease. Rapamycin is known to induce autophagy, a process of bulk degeneration of cellular inclusions and organelles. It has been suggested that rapamycin might clear pathological protein aggregates by stimulating autophagy, and that rapamycin might therefore be useful for treatment of such diseases (JBC v.278, p.25009, 2003). In order to test this hypothesis, we employed a rotenone model of alpha-synuclein aggregation (JBC v.277, p.5411, 2002). CHO K1 cells transiently expressing human alpha-synuclein were treated with rotenone (0.1–2 μM) in order to induce intracellular aggregation of alpha-synuclein. This treatment resulted in the appearance of multiple high molecular weight alpha-synuclein immunoreactive bands on Western blots using monoclonal Syn-211 antibody. High molecular weight alpha-synuclein aggregates were not observed when cells were co-treated with rapamycin (200 μg/ml). Our results are consistent with the clearance of alpha-synuclein protein aggregates by rapamycin-induced autophagy.

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ALPHA-SYNUCLEIN MUTATIONS INCREASE SENSITIVITY OF HUMAN NEUROBLASTOMA CELLS TO CYTOTOXICITY

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Parkinson's disease (PD) is the most common progressive neurodegenerative movement disorder. It is clinically characterised by rigidity, slowness of movement, resting tremor and postural instability. Approximately 1% of the population over 65 is affected, and this increases to 5% of the population by age 85. The presynaptic protein alpha-synuclein was the first gene linked to PD. Three missense mutations have been identified in this gene, and duplication and triplication of the gene has also been shown to lead to PD. We have treated a dopaminergic human neuroblastoma cell line BE(2)M17 transfected with different alpha-synuclein mutants with different agents to induce cytotoxicity and to study changes in PD-associated proteins in this model system. We have found that mutant alpha-synuclein A30P and E46K potentiated the toxicity of dopamine and 6-hydroxydopamine in these cells. Alpha-synuclein positive cytoplasmic inclusions were also observed after dopamine or 6-hydroxydopamine treatment. We have also measured DJ-1 levels in treated cells and found a change in expression of this protein after 6-hydroxydopamine treatment.

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DIFFERENTIAL NEUROPROTECTIVE EFFECTS OF SALICYLATE IN MPTP AND NIGRAL 6-OHDA MODELS OF PARKINSON'S DISEASE

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It is well established that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-OHDA (6-hydroxydopamine) can produce dopaminergic damage in mice and rats which is similar to that observed in Parkinson's disease (PD). While, both toxins have some similar actions there are also many potential differences in the mechanisms by which neurotoxicty is produced. For example: central versus peripheral administration, direct uptake into neurones versus metabolism to active toxin (MPP+), rats versus mice. We have established and characterized a sub-chronic mouse MPTP (8 days × 30 mg/kg i.p.) and an acute rat 6-OHDA (4 mg infused directly into the substantia nigra) model of PD. We then evaluated the effects of sodium salicylate (75 mg/kg i.p.) in both models. Data indicated that sodium salicylate provided robust protection ($P < 0.001$) of both striatal dopaminergic terminals and substantia nigra neurones in the MPTP model but was inactive in the 6-OHDA model. In contrast, AMPA receptor potentiators have a greater effect in the rat 6-OHDA model were we observed their neurotrophic actions. These results indicate that the mechanisms contributing to cell death and repair are different in these models. The results also suggest that putative neuroprotective and neurorepair agents for PD should be studied in more than one model or that a particular model may be more appropriate for certain types of pharmacological intervention.

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ALTERATION OF MICROGLIAL p38MAPK IN THE RAT BRAIN FOLLOWING INFUSION OF 6-OHDA INTO THE SUBSTANTIA NIGRA OR THE STRIATUM

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Parkinson's disease (PD) is characterized by a progressive degeneration of nigrostriatal dopamine neurons. Oxidative stress and neuroinflammation have been implicated in the pathogenesis of PD. Although recent studies have linked phosphorylated (P-p38MAPK) to both neuroinflammation and neurodegeneration, the role of p38MAPK in PD is still unclear. We set out to investigate the changes in P-p38MAPK expression and microglial activation in rat brain. 6-OHDA was infused into the substantia nigra and brains perfusion-fixed at 6 h, 1, 2 and 3 days after lesioning or in a second study 6-OHDA was infused into the striatum and the brains perfusion-fixed 5, 7, 11 and 17 days after lesioning. Brains were processed for immunofluorescence. The nigral 6-OHDA lesion produced a progressive loss (over 3 days) of tyrosine hydroxylase (TH) positive dopaminergic neurons and terminals, while in the striatal model there was a slow partial loss of nigral neurons at days 11 and 17. The loss of TH staining was associated with progressive microglial activation characterized by a thickening of microglial pseudopodia and an increased expression of the microglial marker OX-42. Double immunofluorescence labeling showed an increase in P-p38MAPK immunostaining which co-localized with activated microglia ipsilateral to the 6-OHDA lesion. These observations suggest that increased p38MAPK expression in microglia may activate downstream pro-inflammatory processes that could contribute, at least in part, to dopaminergic neuronal death in PD.

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POSSIBLE ROLE OF CYCLOOXYGENASE-2 IN RESERPINE-INDUCED OROFACIAL DYSKINESIA AND COGNITIVE DYSFUNCTION

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Tardive dyskinesia is one of the major side effects of long-term neuroleptic treatment. Recent evidences suggest that cyclooxygenase (COX) may be an important mediator of neuronal injury and implicated in neurodegenerative processes associated with acute and chronic neurological disorders. Reserpine-induced orofacial dyskinesias in rats are widely accepted as an experimental model for tardive dyskinesia in humans. Orofacial dyskinesia in rats was induced by repeated treatment with reserpine (1 mg/kg, sc) on each other day for a period of 5 days. Rats with established dyskinesia were chronically (28 days) treated with various COX2 inhibitors. Using elevated plus maze and passive avoidance paradigms cognitive dysfunction was assessed. Reserpine treatment significantly induced orofacial dyskinesia in rats. Chronic treatment with selective COX2 inhibitors nimesulide (5, 10 mg/kg) or rofecoxib (2, 5 mg/kg) for a period of 28 days significantly reversed the reserpine-induced orofacial dyskinesia. Reserpine treated rats showed poor performance on elevated plus maze and passive avoidance paradigms indicating memory impairment. Chronic treatment with COX2 inhibitors significantly reversed reserpine-induced memory impairment. Biochemical analysis revealed that reserpine treatment significantly induced lipid peroxidation, elevated nitrite levels and decreased the glutathione levels which were reversed by COX2 inhibitors. The results of the presents suggest the potential role of cyclooxygenase in the pathophysiology of reserpine-induced orofacial dyskinesia and COX inhibitors could be useful neuroprotective agents for the treatment of tardive dyskinesia in humans.

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DUAL ROLE OF TESTOSTERONE IN MOTONEURONAL SURVIVAL IN SBMA

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Spinal and Bulbar Muscular Atrophy (SBMA) is a motor neuron disorder due to elongations of a polyglutamine (polyQ) tract present in the androgen receptor (AR). We have recently shown that SBMA AR aggregate only after testosterone activation in the cytoplasm and in neurites. Cytoplasmic aggregates do not affect cell survival, while neuropil aggregates alter mitochondria transport along neurites and kinesin distribution, thus affecting anterograde/retrograde transport along neuronal processes. While, testosterone may induce motor neuronal death, the same hormonal steroid may contribute to enhance neuronal survival through a different mechanism. In fact, we have analysed the androgen effect on motor neurons and found that neuritin, a protein controlling neurite outgrowth, is induced by testosterone and by its more active derivative dihydrotestosterone (DHT). Interestingly, the 5alpha-Reductase 2 enzyme, responsible for this convertion, has been found to be highly expressed in motor neurons. The data support the notion that androgens may have a dual role in SBMA: 1) they increase cell trophism and survival of motor neurons even when they contain the mutant SBMA AR, 2) they may induce AR aggregation in several cell compartments; the cellular localization of the aggregates may then be responsible for their toxicity.

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NEUROPROTECTIVE POTENTIAL OF DOPAMINE AGONISTS ON PRIMARY DOPAMINERGIC CULTURE RELEVANT TO PARKINSON'S DISEASE

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Great attention has been paid to DA agonists as first choice in de novo and young parkinsonian patients not only to delay the onset of L-dopa therapy but also to protect DAergic neurons from the ongoing degenerative process. To investigate the neuroprotective role of DA agonists, primary DAergic cultures were prepared from mouse mesencephala at gestation day 14 according to Radad *et al*. (2004). Different concentrations (0.001 to 10 μM) of α-dihydroergocryptine (DHEC) and rotigotine were added on the 6th DIV for 8 days. On the 10th DIV, MPP+ (10 μM) and glutamate (500 μM) were added for 48 h and 15 min, respectively. On the 14th DIV the culture medium was used for lactate dehydrogenase (LDH) determination and the cultured cells were stained immunocytochemically for tyrosine hydroxylase (TH). The total number of TH+ neurons as well as their neurite numbers were counted. It was found that each DA agonist neither increased TH ir neurons nor the neurite numbers when added alone to the culture. MPP+ and glutamate significantly decreased the total number of TH+ cells, disrupted their morphological characteristics and increased LDH release. α-DHEC did not produce significant protection against MPP+ or glutamate. On the other hand, rotigotine exerted marked protection against MPP+ or glutamate. It attenuated the decrease of TH+ neurons produced by MPP+ and glutamate up to 25 and 34% respectively. Such protection appeared to be receptor-dependent as it was blocked by DA antagonist sulpiride. The results show that DA agonists protect various forms of oxidative stress with different potencies.

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CO-TRANSPLANTATION OF CAROTID BODY (CB) AND VENTRAL MESENCEPHALIC CELLS (VMC) AS AN ALTERNATIVE APPROACH TOWARDS FUNCTIONAL RESTORATION IN RAT MODEL OF PARKINSON'S DISEASE

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Exogenous administration of various neurotrophic factors has been shown to protect neurons in animal model of Parkinson's disease (PD). Several attempts are being made to search a tissue source simultaneously expressing many of these neurotrophic factors. Carotid body (CB) contains oxygen-sensitive glomus cells rich in dopamine (DA) and expresses glial cell line-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). We have attempted to study the functional restoration following co-transplantation of CB cells and ventral mesencephalic cells (VMC) in 6-hydroxydopamine (6-OHDA)-lesioned rat model of PD. A significant recovery ($p < 0.001$) in d-amphetamine induced circling behavior (80%) and spontaneous locomotor activity (85%) was observed in co-transplanted animals 12 weeks post transplantation as compared to lesioned animals. Similarly a significant ($p < 0.001$) restoration was observed in DA-D2 receptor binding (77%), striatal DA (87%)/ DOPAC (85%) level and nigral DA (75%) and DOPAC level 74%. Functional recovery was further confirmed by a significantly high expression of tyrosine hydroxylase (a rate limiting enzyme of DA biosynthesis pathway) immunoreactive (TH-IR) fiber density in stria-

tum as well as TH-IR neurons in SNpc in co-transplanted animals over VMC transplanted animals. The results suggest the supportive role of CB cells co-transplantation approach along with VMC in long-term (12 weeks) functional restoration in rat model of PD possibly by supporting the survival of newly grafted dopaminergic cells from fetal brain as well as remaining host DA neurons.

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PROGRESSION OF BRAIN OXIDATIVE DAMAGE INDUCED BY 6-HYDROXYDOPAMINE IN A MODEL OF DOPAMINERGIC NEURODEGENERATION IN RATS

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6-Hydroxydopamine (6-OHDA) is a neurotoxin widely used to create experimental models of Parkinson's disease. It is generally accepted that the dopaminergic neurodegeneration induced by 6-OHDA is related with the oxidative stress generated. Our aim was to quantify the time-course of the oxidative damage induced by the stereotaxic administration of 6-OHDA (20 μg) into the right side of the rat striatum (St). The oxidative damage was assessed by determination of both TBARS as an index of lipid peroxidation and protein carbonyl content (PCC) as an index of protein oxidation. The substantia nigra (SN) and St corresponding to both the right and left sides of the brain were removed at different times after 6-OHDA injection and analyzed as whole tissue. Our data showed a progressive increase for both parameters to find a peak value after 48 h since 6-OHDA injection. Peak values in the right side represent an increase of 54 and 83% for TBARS and 38 and 42% for PCC in St and SN, respectively. Although the increase affected the right side in particular, some significant changes were also observed in the left side. The SN usually exhibited more pronounced changes than those found in St. Seven days after 6-OHDA administration, both parameters returned to values close to those found in controls. We conclude that the oxidative stress caused in the first days is responsible for the histological dopaminergic neurodegeneration observed after one week.

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ROLE OF ALPHA-SYNUCLEIN IN SYNAPTIC (DIS)FUNCTION

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Open questions concern the physiological role of alpha-synuclein (α-syn) and whether mutations induce a loss of function of the protein. The analysis of the effects of α-syn in neuroblastoma and astrocytoma cells was carried out in order to investigate its role in the various steps of transmitter release. We analyzed in details the role α-syn plays in the targeting of dense core granules occurring via the rearrangement of the cytoskeleton. The steps of granule docking and fusion have been studied in vitro, and the role of α-syn as a positive or negative regulator of secretion, was investigated, with particular attention to the calcium sensitivity of the process. We observed that, in neuroblastoma cells, α-syn increases the release of POMC-beta-Gal at steady state, while, in cell homogenate, a decrease of the release of the granule marker was observed. Studies on the regulation of the release have shown that, in the cells overexpressing α-syn, granules dock and fuse already at basal levels of cyosolic Ca^{2+}. Two mechanisms have been found to contribute to this finding: i) an alteration of the exocytic process itself and ii) a cytoskeletal rearrangement, with ensuing more easy targeting of granules to the plasma membrane. We found a stimulatory effect of α-syn, and especially of the A53T mutant, on actin polymerization, in vitro, due to increased rate of filament elongation and decreased critical concentration. At the morphological level, α-syn-transfected cells showed changes in the actin cytoskeleton: actin filaments were no longer organized in stress fibers and peripheral bundles, but instead appeared fragmented and accumulated as aggregates in the perinuclear region. This result appears to agree with those reported on secretion.

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DYSREGULATION OF STRIATAL NEUROTRANSMITTER RELEASE IN THE ROTENONE MODEL OF PARKINSON'S DISEASE

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It is believed that both mitochondrial dysfunction and oxidative stress play important role in the pathogenesis of Parkinson's disease (PD). We studied the effect of chronic systemic exposure to the mitochondrial inhibitor rotenone on the uptake, content and release of striatal neurotransmitters upon neuronal activity and oxidative stress, the latter simulated by H_2O_2 perfusion. The dopamine content in the rat striatum is decreased simultaneously with the progressive loss of tyrosine hydroxylase (TH)-immunoreactivity in response to chronic intravenous rotenone infusion. However, surviving dopaminergic neurons, take up and release only a slightly lower amount of dopamine (DA) in response to electrical stimulation. Striatal dopaminergic neurons showed increased susceptibility to oxidative stress by H_2O_2, responding with enhanced release of DA and with formation of an unidentified metabolite, which is most likely the toxic dopamine quinone (DAQ). In contrast, the uptake of [^{3}H]choline and the electrically induced release of acetylcholine increased, in coincidence with a decline in its D2 receptor mediated dopaminergic control. Thus, oxidative stress-induced dysregulation of DA release/uptake based on a mitochondrial deficit might underlie the selective vulnerability of dopaminergic transmission in PD, causing a self-amplifying production of reactive oxygen species (ROS), and thereby contributing to the progressive degeneration of dopaminergic neurons.

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ROLE OF MICROGLIAL ACTIVATION FOR THE NEURODEGENERATION IN A TRANSGENIC α-SYNUCLEIN MOUSE MODEL OF MULTIPLE SYSTEM ATROPHY

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Multiple system atrophy (MSA) is the most common atypical Parkinsonian syndrome. Pathologically MSA is characterized by neuronal cell loss, reactive astrogliosis and microglial activation in striatonigral and olivopontocerebellar systems. α-Synuclein (αSYN) positive oligodendroglial inclusions are the pathological marker for the definite diagnosis of the disease. We have recently developed a novel model of MSA which reproduced the main neuropathology of the disease. In the present work we aimed to study the role of microglial activation for the neuronal cell loss in this model. We used (PLP)-αSYN transgenic

(tg) mice that have targeted expression of human αSYN in oligodendrocytes. Five groups of animals were used including 2, 4, 6, 8 and 12 months old mice. Immunohistochemistry for CD11b, CD3, and 15G7 was applied to evaluate the time course of microglial activation and its correlation to the neuronal cell loss and αSYN expression. We analysed microglial activation in the striatum, substantia nigra, cerebellar cortex, pons and inferior olives. Further, the temporal changes of the neuronal numbers in these areas were determined and correlated with the degree of microglial activation. Next, microglial activation in the white matter tracts was determined in correlation with αSYN expression in the oligodendrocytes. Finally, the possible participation of T-cells in the pathology of the (PLP)-αSYN tg mice was determined. Our results demonstrate that microglial activation in the (PLP)-αSYN tg mice increases with age and neuroinflammatory response may play a significant role for the neurodegenerative process.

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POSSIBLE INVOLVEMENT OF NOVEL PROTEIN KINASE C ISOFORMS IN PARAQUAT-INDUCED NEURONAL CELL DEATH

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Reactive oxygen species (ROS) is thought to be a major cause of neurodegenerative diseases including Parkinson's disease (PD), which is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra. DA neurons are highly susceptible to damage from toxins, in part due to additional ROS generated through DA metabolism. Paraquat, a herbicide, has been shown to generate ROS intracellularly. It is chemically similar to the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) which is the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Oxidative stress has been shown to activate and cause translocation of certain protein kinase C (PKC) isoforms from cytoplasm to the mitochondria and nucleus. In this study, human neuroblastoma (SH-SY5Y) cells were used to test activation and translocation of PKC isoforms induced by paraquat. These cells express the Da transporter through which paraquat is generally believed to get into the DA cells. We have shown that paraquat caused a dose-dependent decrease in the viability of cells as measured by MTT and LDH assays. Immunoblot analysis indicated presence of the novel PKC isoforms delta and epsilon in SH-SY5Y cells. Paraquat caused translocation of PKC delta and epsilon to the membrane. Immunoprecipitation with anti-phosphotyrosine antibody and immunoblot also indicated the ability of paraquat to stimulate tyrosine phosphorylation of PKC delta and to a smaller extent PKC epsilon. Identifying the signaling pathways involved in paraquat toxicity will help to develop better therapies for reducing Parkinson-like symptoms induced by environmental toxins.

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IMMUNODETECTION OF PLEIOTROPHIN IN STRIATAL INTERNEURONS AND NIGRAL DOPAMINERGIC NEURONS

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Parkinson's disease is characterized by a selective damage of the dopaminergic nigrostriatal pathway. Surviving neurons undergo both spontaneous and levodopa induced plastic changes. We have recently reported an increased expression of Pleiotrophin (PTN) in the striatum of rats with a unilateral 6-OHDA lesion of the nigrostriatal tract chronically treated with levodopa. Interestingly, recent reports have shown that PTN stimulates the differentiation of dopaminergic mesencephalic neurons in vitro. The aim of this study was to identify the cellular types that contain PTN in the striatum and substantia nigra (SN) and to detect the presence of the PTN receptor N-syndecan in the SN. Using double-immunofluorescence we found PTN to colocalize with NeuN but not with GFAP in the striatum. Immunolabeling of PTN with specific markers for interneuronal subpopulations at this level ruled out calretinin (+) and parvalbumin (+) GABAergic neurons. Conversily around 40% of PTN immunolabeled neurons corresponded to GABAergic interneurons that contain nitric oxide synthase (NOS) and somatostatin (SST), while around 60% of them were cholinergic interneurons. We also studied the distribution of the N-syndecan in the SN and found it to colocalize with TH. Our results indicate that PTN is selectively localized in cholinergic interneurons and GABAergic interneurons that express SST/NOS in the striatum. At the SN both PTN and N-syndecan are present in a small number of dopaminergic neurons. In conjunction, these results suggest that PTN would possibly mediate some of the compensatory mechanisms that surviving dopaminergic nigrostriatal neurons undergo in the Parkinsonian brain.

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IMPACT OF L-dopa ON VITAMINS C AND E IN PC-12 CELLS

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L-dopa (3,4-dihydroxy-L-phenylalanine) is used as the standard therapy for Parkinson's disease (PD), which is caused by abnormal dopaminergic transmission. Interestingly, both dopamine and L-dopa have been shown to cause oxidative stress. The latter can be reduced by the antioxidant activities of vitamins C (ascorbate) and E (tocopherols). However, little is known about the interactions between L-dopa and tocopherols and ascorbate. In this study, PC-12 cells (as models of neurons) were cultured in matrigel-coated plastic culture flasks under standard conditions. When the cells had reached near confluence they were incubated for 22 hours with ascorbate (400 μM) and/or alpha tocopherol (25 μM) and in the presence or absence of L-dopa (250 μM). After incubation, the cells were harvested and analyzed for the various biochemical components by HPLC. As expected, the addition of L-dopa to the medium resulted in almost a three-fold increase in cellular dopamine content. The PC12 cells contained small concentrations of alpha tocopherol. When alpha tocopherol was added to the flasks cellular alpha tocopherol levels went up markedly. Similarly, the addition of ascorbate resulted in substantial increases in its concentration in cells. These increases in concentrations were strongly attenuated by the presence of L-dopa in the medium. The data indicate that: a) the uptake systems for ascorbate and tocopherols in these model neuronal cells are inhibited by L-dopa and/or b) L-dopa treatment causes an increase in the rate of utilization of the two nutrients. Thus L-dopa (and possibly dopamine) modulate the cellular dynamics of ascorbate and tocopherols.

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Novel techniques in neurochemistry

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IN VITRO 1H MAGNETIC RESONANCE SPECTROSCOPY DIFFERENCES BETWEEN HUMAN BRAIN TUMOURS

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In vivo MRS shows only small mobile molecules or moving groups of larger ones, while in vitro MRS shows all types of low molecular weight molecules. Aim of our work was to study differences in the presence of metabolites among different types of human brain tumours (meningioma, astrocytoma and glioblastoma) by in vitro measuring of perchloric acid extracts. In meningioma, we observed the highest content of alanine (36 ± 4). Significant differences in the alanine content were between meningioma and glioblastoma ($P < 0.001$), and between meningioma and astrocytoma ($P < 0.001$). Levels of glutamate/glutamine were different only between meningioma and astrocytoma ($P < 0.01$). No significant differences were in content of choline compounds. Intensity of inositol/glycine signals was different in meningioma and astrocytoma ($P < 0.001$). We observed also differences in presences or absence of individual detectable peaks in 1H MR spectra of tumours. We did not find signal of NAA and creatine in meningioma and taurine in astrocytoma. We conclude that in vitro 1H MRS provides information about biochemical differences in different type of human brain tumours and corresponds to *in vivo* measurements.

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RAPID, NON-RADIOACTIVE ASSAY FOR *EX VIVO* DETERMINATION OF FAAH ACTIVITY FROM RAT BRAIN AND LIVER

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Fatty Acid Amide Hydrolase (FAAH) is an anandamide (AEA) hydrolysing enzyme which abundantly occurs in the brain and liver. Animal studies suggest that FAAH inhibitors can be useful for the treatment of anxiety and pain. Two methods have been described for the determination of FAAH activity. One is using hydrolysis of tritiated AEA(1). Another one is a simple colorimetric assay based on the hydrolysis of (4-nitro-phenyl)-nonanamide (NpNA). This latter method has been described for enzyme activity determination from FAAH-transfected cells (2). Here we demonstrate that the non-radioactive colorimetric assay is appropriate for determination of ex vivo FAAH activity from rat brain and liver. The known irreversible FAAH inhibitor URB597 was given to rats and FAAH activity was determined from brain and liver microsomal membrane preparations using [3H]-AEA and NpNA. The NpNA hydrolysis (i.e. change in the absorbance at 414 nm) was determined by using 96-well plates. The known irreversible FAAH inhibitor, URB597 showed dose-dependent and almost similar reduction of FAAH activity at doses of 0.03, 0.1, 1 mg/kg in both assays. In the brain, FAAH activity (in the percentage of control activity) was 98% (0.03 mg/kg), 52% (0.1 mg/kg), 26% (1 mg/kg) in radioactive assay and 90% (0.03 mg/kg), 22% (0.1 mg/kg), 0% (1 mg/kg) in non-radioactive assay, respectively. In the liver FAAH activity was 68% (0.03 mg/kg), 36% (0.1 mg/kg), 19% (1 mg/kg) in radioactive assay and 47% (0.03 mg/kg), 1.5% (0.1 mg/kg), 0% (1 mg/kg) in non-radioactive assay, respectively. It is concluded that the assay using artificial substrate NpNA is a simple, rapid and useful method for ex vivo FAAH activity determination from tissues.

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VISUALIZING NONSENSE MEDIATED DECAY IN LIVE CELLS

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Intracellular RNA trafficking in eucaryotic cells includes: processing, nuclear export, transport, localization, translation and degradation. RNAs containing premature termination codons (PTC) are processed in the nucleus but then diverge from the normal RNA trafficking pathway and are targeted for degradation by the Nonsense Mediated Decay (NMD) pathway. NMD is a surveillance mechanism that causes PTC-containing RNA to be degraded, thereby preventing accumulation of truncated proteins. All RNA molecules are believed to undergo a pioneer round of translation soon after export from the nucleus, during which PTC-containing RNAs are recognized and targeted for NMD. A2RE is an RNA trafficking sequence that targets specific RNAs to dendritic spines in neural cells. Translation of A2RE-containing RNA is suppressed until the RNA is localized. Here we determine when, and where, in the RNA trafficking pathway PTC-containing RNA is distinguished from normal RNA, and whether RNA molecules containing both PTCs and A2REs, are resistant to NMD. Differentially fluorescently labeled normal and PTC-containing A2RE RNAs were microinjected into the nuclei of neural cells and intracellular trafficking of the injected RNAs was analyzed using time lapse confocal microscopy. Both normal RNA and PTC-containing RNA are exported from the nucleus and localized in the cytoplasm, but PTC-containing RNA is degraded after localization. This suggests that A2RE RNA is not resistant to NMD. The pioneer round of translation that targets the RNA for NMD does not occur immediately after export from the nucleus but is delayed until after RNA localization.

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INDUCIBLE GENE EXPRESSION IN RAT NEURONS *IN VITRO* AND *IN VIVO*

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Novel methods, enabling tight control of gene expression in neurons are desirable. We have applied tetracycline inducible system to neonatal cortical neurons in vitro as well as to transgenic rats *in vivo*. We have transfected cortical neurons with combination of Tet regulatory proteins: tTR (tetracycline TransRepressor) and rtTA (reverse tetracycline TransActivator) (plasmid vector CMV-RIA) and compared this with application of rtTA alone (plasmid vector pUHG17.1). Expression of EGFP driven by tetracycline responsive promoter (PhCMV*-1) was quantitatively measured with the aid of laser scanning cytometry. Cul-

tures were stimulated with 1 μg/ml of doxycycline. Transfection with rtTA alone resulted in a high basal (without doxycycline) level of EGFP expression (1110 a.u. ± 67), addition of doxycycline produced an increase of the fluorescence level to 8770 a.u. ± 909, thus allowing to achieve ca. 8-fold induction. In contrast, the use of CMV-RIA construct resulted in a very low basal fluorescence (29 a.u. ± 1) that was enhanced by doxycycline ca. 90-fold (to 526 a.u. ± 76). We have next developed two kinds of transgenic rats: first that carries EGFP transgene under control of PhCMV*-1 promoter (TR-GFP) and second that carries rtTA transgene under control of EF1α promoter (EF1-rtTA). Then we cross-bred these two kinds of animal lines to obtain double transgenic rats. Animals were given a doxycycline (2 mg/ml) in the drinking water for 72 hours. We could observe dox-induced EGFP expression in several organs. To conclude, application of the tetracycline inducible system described herein appears to have a great potential for future studies of gene function into rat CNS.

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AUTOMATED DETECTION OF FLUORESCENT MOLECULES AND THEIR SUBCELLULAR DISTRIBUTION: PKC-GFP TRANSLOCATION USING CONFOCAL MICROSCOPY

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The protein kinase C (PKC) family encompasses 12 different isoforms that have been shown to transduce signals mediated by phospholipid hydrolysis. PKC activation in neurons is associated with the control of ion channel function, desensitization of receptors and enhancement of neurotransmitter release. Recently, Green Fluorescent Protein-tagged PKC (PKC-GFP) has been demonstrated to transiently translocate in a calcium-dependent manner from a cytoplasmic to a plasma membrane localization in response to receptor stimulation. Here we present a segmentation and tracking method for quantitative analysis of PKC-GFP translocation from confocal microscopy data. The method is based on parametric contours and includes several adaptations that address important difficulties of cellular imaging, such as: the presence of low-contrast boundary and the occurrence of multiple contacts between cells. 1) We use an edge detection algorithm based on the average intensity of relative background homogeneity to detect both membrane and interfaces between adjacent cells. 2) We introduce a residual algorithm based on the difference between the whole cell detection mask and the edge detection mask. 3) A change in fluorescent distribution between cell compartments merely indicates that one or more processes have been altered. Our tracking technique was validated on a realistic data set by comparison with a manually drawn regions corresponding to the areas of interest and was successfully applied to study the PKC translocation in a neurochemistry research project.

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CAN THE LOCALIZATION OF PROTEINS IN PRE- AND POSTSYNAPTIC COMPARTMENTS BE DISTINGUISHED USING FLUORESCENCE MICROSCOPY?

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The molecular heterogeneity of CNS synapses plus changes of synaptic composition induced by differentiation and specific experience necessitate that the precise expression and distribution patterns of synaptic proteins are established. Double-immunofluorescence labeling is a potent and widely used method to determine the localization of synaptic proteins compared to the position of pre- and postsynaptic markers. But can the location of proteins on the opposite sides of the synaptic cleft be distinguished by fluorescence microscopy? Here we probed the limits of resolution of double immunofluorescence labeling and we describe the relative distribution of markers of the synaptic vesicle compartment (synapsin, vGlut, vGAT), the presynaptic active zone (RIM1 and 2, piccolo, bassoon), and the postsynaptic membrane compartment of excitatory (NMDA-R, AMPA-R, PSD-95) and inhibitory ($GABA_A$-R) synapses in mouse hippocampal neurons. Many of these proteins reveal a striking difference in their staining patterns. Center-to-center measurements of fluorescence clusters allows the localization at a resolution higher than the limits of light microscopy. But heterogeneous orientations of synapses introduce variability, and therefore demand statistical analysis of the measurements. In addition, intensity correlation analysis provides independent quantitative information on the degree of colocalization of pairs of individual marker proteins. For example, the center-to-center distances of synapsin, PSD-95, and RIM indicate that the localization of the synaptic vesicle compartment can be distinguished not only from the postsynaptic density but also from the active zone.

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Pain and inflammation

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NEURONAL EXPRESSION OF THE IB4-BINDING VERSICAN

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Nociceptors are highly specialized primary afferent nerve fibers (PAFs) that transmit noxious stimuli to the dorsal horn of the spinal cord. A subpopulation of nociceptors, the nonpeptidergic C-fibers express a cell-surface glycoconjugate that can be labeled by the plant isolectin B4 (IB4) from Griffonia simplicifolia. We have previously demonstrated that a V2-related variant of the glycosaminoglycan versican is one IB4-binding protein in porcine spinal cord tissue (O. Bogen, M. Dreger, C. Gillen, W. Schröder and F. Hucho: Identification of versican as an isolectin B4-binding glycoconjugate from mammalian spinal cord tissue. FEBS Journal 272 (2005) 1090–1102). By Western blotting with splice variant specific antibodies (kindly provided by R.U. Margolis) and isolectin-B4-conjugated peroxidase we show that this versican variant is also an IB4-binding molecule in rat spinal cord tissue. Moreover, using in situ-hybridization and colocalization of IB4- and anti-versican immunoreactivity of rat dorsal root ganglia (DRG) cells, we proof the neuronal expression of the IB4-binding versican. Our data clearly demonstrate that versican V2 is the molecule that renders the subset of nociceptive C-fibers IB4 positive.

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COMPARISON OF OPIOID RECEPTOR BINDING PROFILES OF 6-AMINO ACID SUBSTITUTED DERIVATIVES OF 14-O-METHYLOXYMORPHONE AND OXYMORPHONE

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Currently, treatment of severe pain relies mostly on the use of centrally acting opioid analgesics such as morphine, fentanyl and oxycodone. Clinical use of these opioids is limited by a number of adverse actions (e.g. sedation, tolerance, addiction), which are mediated predominantly via the central nervous system. This has led to an active search for novel opioid compounds exhibiting more favourable pharmacological features. A series of 6-amino acid conjugates (e.g. alanine, phenylalanine) of the highly potent opioid analgesic 14-O-methyloxymorphone was developed in an effort to obtain agonists that would have potentially limited ability to cross the blood brain barrier. In addition, 6-amino substituted derivatives of oxymorphone were chemically synthesized. Binding affinities to opioid receptors were determined using displacement binding assays in rat (μ, δ) or guinea-pig (κ) brain membranes. All derivatives displayed high affinities (Ki: 0.52–3.20 nM) at the μ-opioid receptor. The 14-O-methyloxymorphone derivatives showed significantly higher binding affinity towards all three opioid receptors compared to the 14-hydroxy substituted counterparts. The newly developed ionisable derivatives could find clinical applications as potent analgesics without the adverse actions of centrally acting opioids.

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TRPV1 AND MICROTUBULE CYTOSKELETON: A BIDIRECTIONAL REGULATION

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TRPV1, a nonselective cation channel, is a polymodal pain receptor expressed in a subpopulation of nociceptors. Activation of this receptor results in high Ca^{2+} influx into the cell. Previously, we have shown that the recombinant C-terminus of TRPV1 interacts with tubulin in a Ca^{2+}-sensitive manner and provides stability to microtubules under certain experimental conditions (C. Goswami, M. Dreger, R. Jahnel, O.Bogen, C. Gillen and F. Hucho J. Neurochem. 2004, 91, 1092–1103). Using both biochemical fractionations and immunofluorescence microscopy, here we show that activation of TRPV1 results in a selective decomposition of the microtubule cytoskeleton into soluble tubulin, but the actin cytoskeleton remains unaffected. Moreover, our results also indicate that the TRPV1 has a bi-directional influence on microtubuler dynamics, activation of the TRPV1 channel destabilizes microtubules while the C-terminus of TRPV1 stabilizes them. We postulate that such stabilization-destabilization of microtubules is important for some biological functions, for example for the determination of neurite outgrowth, neurite morphology and growth cone morphology.

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EXPRESSION OF CRTH2 AND DP RECEPTORS IN MOUSE SPINAL CORD

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Prostaglandin (PG) D2 is the most abundant PG in CNS. In addition to its well known effects on sleep and temperature regulation, PGD2 seems to be involved in spinal nociceptive transmission. PGD2 binds and activates two distinct receptors, DP and CRTH2. The localization of DP receptors has been investigated previously in mouse lumbar spinal cord, suggesting occurrence mainly in neurons of the ventral horn and, to a lesser degree, in dorsal horn neurons of the substantia gelatinosa. Although CRTH2 expression was detected by northern blot in human spinal cord, its localization is not known to date. The aim of the study was, therefore, to compare expression and localization of the two PGD2 receptors in mouse spinal cord by real time RT-PCR and immunohistochemistry, respectively. For immunohistochemistry, commercially available antibodies were employed; the specificity was checked by pre-absorption of the antibody with the peptide antigen. CRTH2-like immunoreactivity was mainly detected in the grey matter. Positive cells were located throughout the dorsal horn as well as in the ventral horn, where a large number of motorneurons were immunoreactive. The distribution pattern of CRTH2 thereby closely resembled the pattern we observed with DP receptors, where immunoreactivity was primarily observed in motorneurons and cells of lamina I and II of the dorsal horn. In agreement with immunohistochemistry, there was expression of CRTH2 and DP receptor mRNA in spinal cord of mice. The results show that DP as well as CRTH2 receptors are present in mouse spinal cord. Localization seems to be restricted mainly to neu-

ronal cells located in ventral and dorsal horn, thus raising the possibility that spinal PGD2 can influence efferent as well as afferent transmission.

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PIROXICAM ALLEVIATES PAIN ASSOCIATED WITH INTRAMUSCULAR INJECTION OXYTETRACYCLINE LONG-ACTING IN DOGS

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Intramuscular injection of drugs and other substances is a common practice in man and animals. Although some injected substances are quite painful, they are administered to achieve a therapeutic goal. Oxytetracycline long-acting (TLA) is used in the treatment of canine ehrlichiosis even though it produces a severe pain, stress and pain related adverse effects. This work evaluates the efficacy and safety of piroxicam in alleviating adverse effects of intramuscular administration of oxytetracycline long-acting (TLA) in dogs using six healthy dogs. The dogs were injected with TLA alone intramuscularly and, after a two-week interval, were injected with TLA and piroxicam concurrently. All the dogs were physically examined after each injection, and blood sample was taken for complete blood count and assay of some serum enzymes. Blood was taken 24 h before (the experiment) and at 0, 0.5, 1, 3, 6, 9, 12, 24, 48, and 96 h after the treatments. Swelling was noticed at the injection site of TLA in six of the dogs and, of these, four were not able to use their injected legs temporarily. Pain was also noticed at the injection site in six of the dogs. Appetite was reduced in six dogs and they showed reluctance to move. Five dogs cried out during injection. Injecting TLA and piroxicam concurrently suppressed all the signs except muscular stiffness which was observed in two dogs for 12 h. Serum creatine kinase showed a gradual increase from 1 h, and peaked at 6 h and gradually returned to normal range by 48 h after injection. Area under curve was calculated for all the dogs from which the degree of muscle damage was calculated. These findings are discussed as they relate to effectiveness and safety of TLA and piroxicam combination in clinical practice.

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THE EFFECTIVENESS OF ADENOSINE AND KYNURENIC ACID COMBINATION ON THERMAL HYPERALGESIA

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Several receptors are involved in pain transmission including the adenosine and NMDA ones. In vitro data suggest a complex interaction between the two systems. The endogenous ligands for these receptors are adenosine and kynurenic acid (NMDA-receptor antagonist), respectively. The goal of the study was to characterize the antinociceptive interaction of these ligands at the spinal level on inflammation-induced thermal hyperalgesia in rats. A paw withdrawal test was used for nociceptive testing. The intrathecal infusion (60 min) of these drugs was administered alone or in combinations (0.01–4 μg/min kynurenic acid and 0.3–3 μg/min adenosine), which was followed by an additional 60-min observation period. The latencies were obtained before the induction of inflammation, 3 h after that and then in every 10-min interval for 120 min. Kynurenic acid alone resulted in dose-dependent antinociception, but higher doses (0.25–4 μg/min) also caused motor impairment. Adenosine had a very low potency by itself. All of the combinations caused dose-independent antihyperalgesia but dose-dependent motor-impairment. Thus the mixtures of low doses produced effective antihyperalgesia ($P < 0.05$) without side effect. During and after the infusion they also completely relieved carrageenan-induced thermal hyperalgesia. The combinations were almost ineffective on the normal side. In conclusion, these endogenous substances have low potencies by themselves; however, their combinations furnished potentiated antihyperalgesia with decreased side effects, suggesting their synergistic interaction *in vivo* in respect of influencing pain signalling.

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ELEVATION OF S-100BETA IN THE BRAIN AND BLOOD UNDER POSTOPERATIVE PAIN

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There is increasing evidence to indicate that nervous system plasticity determining some external influence depends on not only neurons work but in a great extent on glial cells also. Unfortunately, the information about astrocytes reaction under postoperative hyperalgesia is very limited. The aim of our work was investigation of S-100β concentration dynamic under postoperative pain and pharmacocorection. Wistar rats were used for experimental postoperative pain model according T.J. Brennan (1996). Animals were divided into six groups: I – control, II – incision, III – control + glutamate (intraperitoneal injection, 1 mg/kg), IV – incision+glutamate (5 min before incision), V – control+MK-801 (0.5 mg/kg), VI – incision+MK-801. The S-100β concentration was measured in soluble fractions of different brain regions: thalamus/hypothalamus, hindbrain, and in the blood serum by ELISA. Postoperative hyperalgesia (2 h after incision) leads to elevation of S-100β level in the hindbrain and in the blood (by 2 times). Hyperalgesia decrease during 6 days after incision correlates to decrease of S-100β content in the blood. Injection of NMDA receptors agonist glutamate was accompanied by much more increasing level of studied protein. The noncompetitive antagonist of NMDA receptors prevents the changes of S-100β level connecting with postoperative pain, excepting hindbrain. Our data suggest that glial Ca^{2+}-binding protein S-100β takes part actively in the central mechanism of postoperative pain and alteration of S-100β level in the blood correlates with degree of postoperative hyperalgesia.

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CONTRIBUTION OF THE PERIPHERAL 5-HT_{2A} RECEPTOR TO NERVE GROWTH FACTOR- AND NITROGLYCERIN-INDUCED HYPERALGESIA IN RAT

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5-HT_{2A} receptor antagonist has been shown to be effective peripherally acting analgesics in neuropathic pain accompanied with post herpetic neuralgia in some clinical trials. We have already reported that 5-HT_{2A} receptor antagonists attenuated hyperalgesia in chronic constriction injury model (CCI model), neuropathic pain model rat. In the present paper, we tested the possible involvement of nerve growth factor (NGF) and/or NO-cGMP system in the inhibitory effect of 5-HT_{2A} receptor antagonists. We found that local s.c. injection of NGF induced a mechanical hyperalgesia in the rat hind paw. The NGF-induced hyperalgesia was inhibited by 5-HT_{2A} receptor antagonists, such as sarpogrelate and ketanserin. Furthermore, local NO production induced by application of nitroglycerin ointment also induced a mechanical hyperalgesia in the rat hind paw. The 5-HT_{2A} receptor antagonists, however, did not affect the nitroglycerin-induced hyperalgesia. These results indicate that peripheral NGF release, but not local NO produc-

tion, might involve in the 5-HT_{2A} receptor antagonist-sensitive hyperalgesia in neuropathic pain model rat.

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ANTINOCICEPTIVE EFFECT OF TRAMADOL IN THE RAT OROFACIAL FORMALIN TEST: POSSIBLE INVOLVEMENT OF OPIOIDERGIC AND SEROTONERGIC SYSTEM

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Objective: Intractable cranial and facial pain is difficult to treat in humans. Tramadol is the most commonly used opioid analgesic in the management of acute and chronic pain, however, its effect in orofacial pain is not known. Thus the present study was performed to investigate the effects of tramadol and possible neurotransmissions involved in its antinociceptive effect in the rat orofacial formalin test.

Methodology: Orofacial pain was induced by subcutaneous injection of a 50 µl of 5% formalin into the vibrissa pad of rat and the time spent grooming, rubbing, and/or scratching the facial region proximal to the injection site were recorded for fifteen successive 3 min intervals. We measured the effects of tramadol administered before the injection of formalin in rat, in the presence and absence of drugs that act on the opioidergic and serotonergic systems.

Results: The orofacial formalin responses exhibited two distinct phases with early (0–9 min) and continuous prolonged (10–45 min) responses. Tramadol significantly and dose dependently reduced the duration of late phase nociceptive behaviors of the orofacial formalin test. This effect was partially reversed by the 5-HT2 receptor antagonist ketanserin and by the opioid antagonist naloxone. Naloxone or ketanserin alone did not affect the duration of nociceptive behavior in animals.

Conclusion: These data demonstrate that both the opioidergic and serotonergic mechanisms are responsible for the antinociceptive effect of tramadol in the orofacial formalin test and suggest that tramadol may produce analgesic effect in the orofacial pain state in a clinical situation.

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BIOLOGICAL AND PHARMACOLOGICAL CHARACTERIZATION OF HIGHLY POTENT 14-ARYLALKYLOXY SUBSTITUTED *N*-METHYLMORPHINAN-6-ONES

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A significant drawback of opioid analgesics is a variety of side effects, such as sedation, constipation, nausea, confusion, respiratory depression, and dependence. In an attempt to develop opioid analgesics with fewer adverse effects we synthesized a series of 14-arylalkyloxy substituted *N*-methylmorphinan-6-ones. Their binding affinities to µ, δ, and κ opioid receptors were determined using receptor binding assay in rat (µ, δ) or guinea-pig (κ) brain membranes. The new compounds displayed high binding affinities to the µ opioid receptor, which were comparable to that of the parent compound 14-*O*-methyloxymorphone. In the guinea pig-ileum and mouse vas deferens preparations, these compounds behaved as potent agonists. Antinoceptive potencies of most of the new compounds in the hot-plate test after s.c. administration in mice were considerably higher than the potency of 14-*O*-methyloxymorphone and morphine. In the colonic propulsion test, the most potent analgesic, the 14-benzyloxy substituted derivative, showed negligible constipating activity at the analgesic dose after s.c. administration. The nature of the substituent at position 14 has a major impact on the abilities of morphinans to interact with opioid receptors leading to qualitative and quantitative differences in biological and pharmacological activities.

Purines

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CROSSTALK BETWEEN INHIBITORY A1 AND P2Y1 PURINOCEPTORS TO CONTROL EVOKED [3H]-ACH RELEASE FROM MYENTERIC MOTONEURONS

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ATP breakdown products are responsible for modulating gastrointestinal function. Myenteric ATP actions are rapidly terminated by intense ATP diphosphohydrolase activity, generating AMP and adenosine. Alternative formation of ADP by ecto-ATPase might be physiologically relevant to restrain acetylcholine (ACh) release from myenteric motoneurons via P2Y1 purinoceptors. The co-existence of inhibitory P2Y1 and adenosine A1 receptors in the rat myenteric plexus lead us to investigate their role to control [3H]-ACh release from stimulated (5 Hz, 200 pulses) cholinergic motoneurons. Blockade of A1- and P2Y1-receptors respectively with 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX, 10 nM, 43 ± 12%, $n = 3$) and MRS2179 (300 nM, 27 ± 3%, $n = 4$) increased [3H]-ACh release. Exogenous activation of adenosine A1 receptors, with R-N6-phenylisopropyl adenosine (R-PIA, 300 nM), and of P2Y1 purinoceptors, with ADPbetaS (30 μM), significantly reduced [3H]-ACh release by 36 ± 4% ($n = 4$) and by 27 ± 3% ($n = 4$), respectively. MRS2179 (300 nM) blocked only the inhibitory action of ADPbetaS (30 μM), without affecting the effect of R-PIA (300 nM). In contrast, DPCPX (10 nM) attenuated the inhibition by R-PIA (300 nM), while significantly enhancing (to 42 ± 5%, $n = 4$) ADPbetaS (30 μM)-induced depression. While ADPbetaS (30 μM) failed to modify the action of R-PIA (300 nM), activation of A1 receptors occluded the inhibitory effect of ADPbetaS (30 μM). Data suggest that the magnitude of ACh release depression may be regulated by adenosine build-up at the synapse. Therefore, over stimulation of inhibitory P2Y1 purinoceptors may be cut-short by sequential activation of inhibitory A1 receptors.

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EXTRACELLULAR METABOLISM AND CONTRACTILE POTENCY OF ADENINE NUCLEOTIDES IN HUMAN DETRUSOR SMOOTH MUSCLE TAKEN FROM PATIENTS WITH OBSTRUCTED URINARY BLADDER

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Nerve mediated contraction of the stable human bladder is exclusively under cholinergic motor control. However, detrusor from bladders with a number of pathological conditions (e.g. interstitial cystitis, neuropathic and obstructed bladders) exhibit non-cholinergic contractile responses. The atropine-resistant contractions are abolished by high concentrations of alfa,beta-methylene ATP, a nonhydrolyzable ATP analogue that is able to desensitize P2X-purinoceptors. In most tissues, extracellular ATP activity is rapidly terminated by enzymatic breakdown, via the ecto-nucleotidase pathway. Thus, we investigated the extracellular ATP catabolism (by HPLC analysis of the metabolites) in order to probe its role on contractile responses of human detrusor smooth muscle from patients with obstructed urinary bladder. In the human detrusor, the rate of extracellular hydrolysis of ATP (30 μM, 15 min incubation period) was about half of that observed in the pig detrusor, and much slower than ATP metabolism in rodents. ATP (30 μM) was dephosphorylated predominantly into AMP with subsequent formation of adenosine, which has a relaxant activity in the detrusor. ATP (0.010–3 mM) was significantly more potent in the pig detrusor than in detrusor from human obstructed bladders, when contractions were normalized by the responses to 10 μM ACh. Data indicate that increased activity of ATP in human detrusor from obstructed bladders is probably not due to a higher sensitivity of P2-purinoceptors, but to a reduced extracellular hydrolysis of the nucleotide increasing its access to smooth muscle cells.

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DUAL MODULATION OF [3H]NORADRENALINE RELEASE FROM THE SPINAL CORD BY P2 PURINE RECEPTORS

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Under normal conditions, pain is associated with electrical activity in small-diameter dorsal root ganglion (DRG) of the spinal cord (Raja *et al*., 1999). Numerous studies have shown that the descending noradrenergic pathway from the locus coeruleus plays a crucial role in pain transmission in the spinal cord and this effect might be modulated by the purinergic system. ATP is stored and released together with noradrenaline (Burnstock, 1990) from noradrenergic nerve terminals, which are equipped with a wide variety of presynaptic auto- and heteroceptors. In this study we have stimulated the spinal cord slices electrically and examined the effect of different purinergic agonists and antagonists on [3H]noradrenaline release. The nucleotides ADP and ATP decreased electrically evoked tritium overflow in high concentration. These inhibitory effects can be counteracted by reactive blue 2 (RB2 30 μM) and by the P2Y12/13 receptor antagonist 2-methylthioadenosine 5′-monophosphate (2-MeSAMP, 10 μM), but not by the P2Y1 receptor antagonist MRS 2179 (10 μM), or by suramin (300 μM) and PPADS (30 μM). RB2 (30 μM) and 2MeSAMP (10 μM), per se, had no significant effect. On the other hand the P2Y agonist 2-methylthioadenosine-5′-triphosphate (2-MeSATP), and ADP increased electrically evoked tritium overflow in low concentration. The facilitatory effect of ADP was antagonized by PPADS, which by itself decreased evoked tritium overflow. It seems likely that nucleotides exert dual and opposite modulation of noradrenaline release in the spinal cord of the rat. The inhibitory effect was probably mediated by P2Y12 and/or P2Y13 receptors, whereas the identity of stimulatory receptor awaits further investigation.

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FACILITATORY MUSCARINIC M1 AND ADENOSINE A2A RECEPTORS OPERATE A COMMON PATHWAY INVOLVING PROTEIN KINASE A AND L-TYPE CALCIUM CHANNELS AT THE RAT MOTOR ENDPLATE

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At rat motor nerve terminals, activation of facilitatory muscarinic M1 and adenosine A2A receptors are mutually occlusive. During brief trains, M1-facilitation of [3H]-ACh release prevails over the A2A-receptor-mediated tonus. Upon increasing endogenous adenosine accumulation (e.g. during high-frequency bursts) there is a neuromodulatory shift and A2A facilitation becomes predominant. Here we evaluated the mechanism responsible for the crosstalk between M1 and A2A receptors. The A2A receptor agonist, CGS21680C (2 nM), attenuated M1 facilitation of [3H]-ACh release by McN-A-343 (3 μM). Crosstalk between A2A and M1 receptors involves intracellular cyclic AMP accumulation, as it was exaggerated by the phosphodiesterase inhibitor, rolipram (300 μM). Stimulating the cyclic AMP/protein kinase A (PKA) system with forskolin (3 μM), rolipram (300 μM) or 8-bromo-cyclic AMP (1 mM), also attenuated McN-A-343 (3 μM)-induced facilitation. Inhibition of PKA with Rp-cAMPS, applied in a concentration (30 μM) that abolished the effect of CGS21680C (2 nM), reduced (>50%) M1 facilitation. Moreover, facilitation of [3H]-ACh release by both M1 and A2A receptors was blocked by the L-type channel blocker, nifedipine (1 μM). Recruitment of Ca^{2+} through nifedipine-sensitive channels was revealed upon activating M1 and A2A receptors respectively with McN-A-343 (3 μM) and CGS21680C (2 nM), and their effects were not additive. The results indicate that crosstalk between facilitatory muscarinic M1 and adenosine A2A receptors on motor nerve terminals is operated by a common signalling pathway involving PKA activation and recruitment of Ca^{2+} via L-type channels.

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