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[A1]

Long-term locomotor exercise regulates differentially neuronal plasticity-related BDNF and TrkB and NCAM/PSA-NCAM molecules in the lumbar spinal cord in the intact and spinalized rat: implications for spinal cord repair

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Our research project focuses on a better understanding of the mechanisms underlying the recovery processes following injuries to the spinal cord. An involvement of proteins crucial for the recovery processes, i.e., neurotrophins, their receptors and cell adhesion molecules, which may cause synaptic remodelling of the spinal neuronal network, is our main target. Our preliminary data suggested that the synthesis of both groups of proteins is enhanced in the spinal cord due to physical exercise (Skup, 2002; Macias, 2002). Since locomotor exercise improves stepping ability in animals after spinal cord transection, this prompted us to formulate the hypothesis that an up-regulation of endogenous pools of BDNF and NT-4, their $TrkB^{FL}$ receptor as well as cell adhesion molecules N-CAM and L1 due to exercise may underlie this effect.

A beneficial effect of locomotor training in animals after complete spinal cord transection has often been attributed to the modulation of neurotransmission. Clear links of BDNF and neurotransmitter release prompted us to address also the problem how locomotor exercise influences the segmental distribution of neurotransmitter pools in the spinal cord.

Based on these presumptions, we divided our study into two parts:

(1) firstly, we aimed to characterize in detail the impact of moderate, long-term locomotor exercise (28 days) on various populations of neurons and non-neuronal cells in the intact lumbar spinal cord of the rat by evaluating gene and protein expression of these molecules.

(2) secondly, we characterized the impact of complete spinal cord transection on these molecules and the effect of training on them.

Immunohistochemical (IR) as well as *in situ* hybridisation techniques were employed, supported by the Image ProPlus Analysis System to quantify these results. ELISA (BDNF), Western (TrkB and NCAMs) were used to quantify tissue levels of the proteins. For the segmental distribution of neurotransmitters HPLC whole tissue homogenate analysis was performed.

Our study revealed that lumbar segments of the spinal cord in the intact animals contain high levels of BDNF (100 ng/g wwt, S1 fraction) which is present in a mature and predominating, proBDNF form. BDNF levels were increased by training. The use of two exercise regimens showed that both short-lasting and long-lasting exercise is effective (125% *vs* 116% of control, respectively) (Ziemlinska, 2006). An investigation of the cellular distribution of BDNF mRNA signal disclosed that the stimulatory effect of training at the transcriptional level was the strongest in the ventral horn and affected predominantly large neurons of lamina IX, most probably α motoneurones (silver grain cluster area >1800 μm^2). This effect corresponded to that on BDNF protein distribution as revealed with IR, which showed, in addition, an exercise-related increase of BDNF staining in dendrites, with deposits apposed to motoneurones, suggesting that exercise may affect not only the synthesis but also the

translocation of BDNF protein to the synaptic compartment (Macias, 2005).

The training effect on TrkB mRNA signal distribution only partly matched that on TrkB protein. In the dorsal horn a population of small cells (silver grain cluster area <40 μm^2) responded with a higher hybridization signal. This was in line with an increased number of small TrkB-immunoreactive cells with the soma profile ranging from 20-80 μm^2 found in the spinal grey. On the contrary, in the ventral horn there was no increase in signal intensity, but a number of cells with mRNA grain cluster area between 500 and 800 μm^2 was increased. This indicates that new cells are recruited to TrkB expressing pools due to training. A comparison of BDNF and TrkB expression patterns showed that long-term locomotor activity targets both molecules expressed in partly overlapping cell populations.

With respect to the NCAM and L1 molecules, we have shown that exercise doubles the level of their mRNA in the grey matter of the lumbar enlargement (Macias, 2002). An evaluation whether that effect is translated into proteins revealed that out of three forms of the NCAM protein: 200-220 kD band representing polysialylated form of NCAM (PSA NCAM), a weak band of 145-160 kD, and a strong 103-111 kD band, exercise caused a significant increase of PSA NCAM (200% of control) and nearly tripled the values for 103-111 kD band (Macias, 2006). An HPLC study revealed that the described changes in proteins are accompanied by a neurochemical remodeling of the rostro-caudal gradient of monoamines and amino acids (AA) in the spinal cord, leading to an increase of concentration of all tested compounds in the rostral and their decrease in the caudal lumbar segments (Skup, 2006).

In the second part of the study rats with a complete section of the spinal cord at T9/10 segments were tested on the treadmill. There were generally no movements of the hindlimbs for the first 8-10 d after spinalization. By the third week, the rats began to place the plantar surface of their paws on the treadmill and showed a certain weight support during the stance phase when the tail was stimulated. Regular sequences of steps with weight support began to be observed from the fourth week after spinalization on. In the course of the training, the number of steps performed on the plantar aspect of the paw increased gradually. Pressure stimulation of the tail was a prerequisite of this improvement. Following testing, spinal cords were isolated from these animals and subjected to an immunohistochemical analysis of BDNF/TrkB and L1/NCAMs. Additional groups underwent a biochemical study. An HPLC analysis of the segmental distribution of neurotransmitters after spinal cord transection showed that the rostro-caudal gradient of amino acids (AA) was modified in the segments caudal to the lesion, whereas the concentration of monoamines dropped down and was close to the level of detection. Locomotor training in spinal animals reversed the effect of lesion on AA, leading to an increase of AA concentration in L1/L2 and to its decrease in L3-L5 and S segments comparing to spinal, non-exercising animals. First results show that spinalization also affects the expression of investigated molecules. Training partly normalized the effect of lesion. Potential changes in the synaptic terminals distribution after injury and the effect of locomotor training on them were tested with a presynaptic marker, synaptophysin. There was a substantial increase in synaptophysin IR on the surface of large neurons in the ventral horn of the lumbar enlargement in animals trained after spinalization comparing to lesioned non-trained rats. This observation was not followed by a similar increase in synaptic zinc staining. Overall, the data indicate that moderate locomotor training is a promising non-invasive approach to modulating plasticity-related molecules and disclosing its selectivity in addressing only some populations of spinal neurons.

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[A2]

Antiepileptic drugs and neuronal death in the developing brain: pathogenetic mechanisms and behavioral consequences

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Antiepileptic drugs (AEDs) may cause a widespread apoptotic neurodegeneration in the developing rodent brain. In this project we explored molecular pathomechanisms of AEDs-induced neurodegeneration as well as resulting changes in neuronal excitability and behavior.

AEDs-induced neuroapoptosis is associated with a reduced expression of neurotrophins and an impairment of intracellular signaling pathways activated by neurotrophins in the infant rat brain. Erythropoietin did not exert a protective effect against AEDs-induced neurodegeneration but protected from apoptosis induced by the NMDA antagonist MK801. A long-term treatment with erythropoietin significantly increased the duration of miniature Inhibitory Postsynaptic Currents (mIPSCs) of hippocampal neurons. 17β-estradiol ameliorated neurotoxicity of AEDs in the infant rat brain. This neuroprotective effect was reversed by tamoxifen and could not be reproduced by 17α-estradiol. 17β-estradiol did not affect $GABA_A$- or NMDA-currents in hippocampal neuronal cultures, indicating that direct modulation of neurotransmitter receptor/channel properties by this compound cannot explain the neuroprotective effect. However, a long-term treatment with 17β-estradiol resulted in an increase in the amplitude and time duration of mIPSCs. 17β-estradiol increased levels of phosphorylated ERK1/2 and AKT, suggesting that an activation of these prosurvival proteins may represent one mechanism for its neuroprotective action.

Benzodiazepines (BZDs) are believed to enhance the affinity of $GABA_A$R binding sites to their agonist. We evaluated whether BDZs affect the conformational transitions (opening, desensitization) of neuronal and recombinant ($\alpha1\beta2\gamma2$) $GABA_A$Rs. Flurazepam strongly increased the amplitude of currents elicited by low concentration of GABA, consistent with an enhancement of the binding site's affinity. However, flurazepam also decreased the amplitude and enhanced a desensitization of currents elicited by saturating [GABA]. Altogether, we found that flurazepam not only increases the affinity but also interferes with the conformational transitions of $GABA_A$Rs.

In behavioral studies it was found that neonatal exposure to valproate (VAL) and phenobarbital (PHE) significantly enhanced the horizontal activity and stereotypy-like behavior at different stages of development. The vertical activity remained unaffected. Exposure to phenytoin (DPH) did not affect motor activity. VPA and PHE administration resulted in worse performance in the Morris water maze test, whilst DPH treated groups performed similarly well to the controls. Our results clearly show that neonatal exposure to the AEDs VPA and PHE may result in an enhanced motor activity and an impairment of learning and memory skills in rats of various age. The results further suggest that 17β-estradiol and related compounds may be neuroprotective agents suitable for use in critically ill infants and toddlers. Estrogen supplementation may particularly help improve neurocognitive outcome in preterm infants who are prematurely deprived of maternal estrogen and are often treated with AEDs.

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[A3]

Microglia in brain tumor pathology and as a potential target for anti-tumor drugs

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Tumor cells seem to recruit stromal and inflammatory cells to the tumor site and transform into tumor-supportive cells. An invasion of glioma cells into the brain tissue contributes to the failure of current therapeutic treatments. Activated microglial cells are abundant in brain tumors and may support tumor invasiveness. We have previously demonstrated that Cyclosporine A (CsA) can affect the growth of glioma cells *in vitro* by inhibiting signaling pathways which are essential for tumor proliferation and invasiveness. In this work, we demonstrate that the migration of EGFP-transfected glioblastoma cells in organotypic brain slices was significantly inhibited by a treatment with 1, 10 and 30 μM CsA. In the average 77% of untreated cells migrated beyond 500 μm, while only 28-33% cells migrated as far in the brain slices treated with CsA ($p<0.001$). This inhibitory effect on glioblastoma invasion was lost when glioblastoma

cells were injected into microglia-depleted brain slices. In *in vitro* studies we demonstrated that microglia-derived factors increase glioma invasiveness in Matrigel assays, which is associated with an activation of the PI-3K/Akt signaling pathway. The invasion promoting effect of microglia was abolished in the presence of CsA. Cell co-cultures were employed to study mutual microglia-glioma cells interactions. Microglial cultures exposed to glioma-derived factors increase the production of pro-tumorigenic cytokine – TGFβ1, which increases tumor invasiveness and migration. Furthermore, glioma-derived soluble factors induce the morphological transformation of microglia and activate MAPK signaling, although no production of pro-inflammatory factors was observed. Our finding that CsA may impair the invasive growth of glioma cells at clinically relevant concentrations provides a novel therapeutic strategy.

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[A4]

The transduction of neuronal Ca^{2+}-signals via the EF-hand calcium-binding proteins caldendrin and calmyrin in Alzheimer's disease and psychotic disorders

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In a joint effort of four German and Polish research teams (2 from each country) we are investigating how the transduction of Ca^{2+}-signals contributes to the pathological processes in brains of Alzheimer's and psychiatric patients. More specifically, we investigate Ca^{2+}-activated processes by analyzing the expression, signal transduction and possible pathophysiological role of two Calcium-binding proteins (Calmyrin and Caldendrin) in these disease states. The research program is based on i) the observation that Calmyrin interacts with presenilin 2, a protein associated with Familiar Alzheimer's Disease (FAD) and ii) a recently established link between the recruitment of Caldendrin to excitatory synapses, glutamate-receptor activation and the NMDA-receptor hypothesis of schizophrenia. Our work on Calmyrin's included studies on the neuronal localization of CaMy1 as well as its analysis in brains of AD patients. Although we could show an altered distribution of CaMy1 in AD patients, the involvement of CaMy1 in the pathogenesis of AD is questionable. Thus, we found that CaMy1 interacted specifically with the AD associated protein presenilin2 (PS2) in vivo. However, the CaMy1/PS2 appeared to be Ca^{2+}-independent and not regulated by a Ca^{2+}-dependent translocation of CaMy1. Moreover, only a limited co-localization of CaMy1 with PS2 was found at the cellular level in human and rat forebrain (major area affected by AD). Accordingly, subfractionation of rat brain forebrain yielded only a limited overlap of both proteins in subcellular membranes. In summary, our results cast some doubts on the original hypothesis that a dysregulation of Ca^{2+}-homeostasis is triggered via the CaMy1/PS2 interaction in the AD brain. Beside these important findings, we conducted a variety of biophysical studies with CaMy1 and realized in the course of these studies that it is a member of a protein family including at least one further member tentatively called CaMy2. We compared the neuronal localization, structure, Ca^{2+}-binding, N-terminal myristoylation and target interactions of CaMy1 and its homologue CaMy2. This work provided some insights into the structure-function relationships of a new EF-hand calcium binding protein family sharing some structural similarities with Neuronal Calcium Sensors and Calcineurin. Finally, while on the search for a cellular function of CaMy1 we identified SCG10, a novel interaction partner in the human brain. SCG10 is a member of the stathmin family of proteins which is involved in the regulation of microtubule polymerization in neuronal growth cones. CaMy1 seems to couple Ca^{2+}-signaling with microtubular polymerisation.

To analyze synaptic Ca^{2+}-signaling in psychotic disorders we focused on Caldendrin, a neuron-specific cytoskeletal Ca^{2+}-binding protein that is highly enriched in synapses. Its synapse-association

is activity-dependent and significantly altered in animal models of psychosis as well as in the brain of chronic schizophrenic patients. Caldendrin binds to a membrane-associated guanylate kinases (MAGUKs) termed SAP97 that is dramatically reduced in the forebrain of schizophrenic patients. The binding is Ca^{2+}- and Zn^{2+} dependent and leads to a conformational change in SAP97, which is associated with a differential binding of other interaction partners and their association with the synapse. Our data suggest that the Caldendrin/SAP97 seems to be an important mechanism for the dynamic organization of the synapse and might contribute to the formation of defect synapses in schizophrenia. Additionally, we could establish a morphogenetic pathway involving Caldendrin and a novel protein that we termed Jacob and which links the activity of NMDA receptors to nuclear signaling events that will model synapto-dendritic cytoarchitecture. Upon NMDA receptor activation, Jacob is recruited to the nucleus, resulting in the stripping of synaptic contacts and a drastically altered morphology of the dendritic tree. Caldendrin controls Jacob's extranuclear localization and thereby renders it transcriptionally inactive. We assume that Jacob is either a transcription factor by itself or part of a macromolecular protein complex that regulates plasticity-and disease related gene expression. Finally, we utlilized proteomics to analyze the protein composition of synapses in normal and psychotic brain. Using this approach, we can show that the 'schizophrenic' synapse has a clear signature and that a protein termed prohibitin in particular might have a profound role in the alterations of spine synapses found in chronic schizophrenia.

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[A5]

Behavioral and cellular correlates of depression: putative roles of FGF-2

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Depression is, according to the WHO, a common mental disorder affecting about 121 million people worldwide. Although some antidepressant agents seem to be beneficial in the treatment of depression, the neuroanatomical, physiological, and neurochemical mechanisms underlying depression and the action of antidepressant drugs are only fragmentarily understood. Our project focuses on the role of FGF-2 in an animal model of depression, the olfactory bulbectomy. We analyzed the effects of chronic antidepressant (amitriptyline, citalopram) treatments and intacerebroventricular (icv) infusion of FGF-2 upon the behavioral and morphological changes produced by bulbectomy in male adult wild-type and FGF-2 knockout mice. Bulbectomy was found to increase the locomotor-activity and exploratory behavior. Moreover, bulbectomized mice showed deficits in the passive avoidance test. Bulbectomy was also found to induce neuronal degeneration in the piriform cortex (Pir) and in the posterolateral cortical amygdaloid nucleus (PLCo). We found that antidepressant treatment, among other effects, reduced the hyperactivity in bulbectomized mice almost to the control level as compared to sham-operated mice. Moreover, in bulbectomized antidepressant-treated mice, neurodegeneration in the Pir and PLCo was reduces as compared to bulbectomized saline-treated mice. These data indicate that treatment with antidepressants reduces behavioral deficits as well as neurodegeneration induced by bulbectomy. With regard to the role of FGF in these processes related to depression, we also analyzed FGF-2 deficient mice. As compared to the bulbectomized wild-types, bulbectomized FGF-2 knockout mice showed nearly the same deficits in different behavioral tests. In addition, there was no major difference in the pattern of neurodegeneration between these groups. However, in the FGF-2 knockout mice amitriptyline loses its ability to counteract any behavioral effects of bulbectomy. Moreover, we found that the effects of a single icv infusion of 40 ng FGF-2 were comparable with those of a single ip dose of amitriptyline (10 mg/kg) in the tail suspension test, indicating that FGF-2 might have an antidepressant-like activity in wild-types. We also analyzed the effects of icv infusion of FGF-2 in bulbectomized wild-types. In these mice icv infusion of

FGF-2 reduced the hyperactivity produced by bulbectomy to levels observed in sham-operated mice. Moreover, four (administered every 3 days) icv infusions of FGF-2 abolished the learning deficits induced by bulbectomy, as measured in the passive avoidance step-down test, indicating that exogenous FGF-2 mimics amitriptyline effects in this animal model of depression.

The ability of FGF-2 to reverse some of the deficits caused by bulbectomy and the lack of sensitivity of FGF-2 knockout mice towards amitriptyline treatment may indicate that FGF-2 possesses an antidepressant-like activity and might be involved in mechanisms underlying the action of antidepressant drugs. These data support the notion that trophic factors may be implicated in depression and may have a therapeutic potential in the treatment of neuropsychiatric disorders.

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[A6]

Molecular genetics and experimental therapeutics of orphan neurodegenerative diseases (ALS, MSA, PSP)

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Our research project was aimed to further investigate the molecular basis of human ALS and transgenic mouse model of ALS/MND. In the second part of the project we explored the possibility of cell transplantation into transgenic mice.

As a first step, we established immortalized lymphoblast culture banks in both centers in Ulm and Warsaw, providing a long-term DNA resource for the ongoing and future collaborative studies. This was complemented by setting up clinical phenotype databases at both centers. Currently, the Warsaw bank contains 300 DNA samples of ALS, MSA and PSP patients, which makes the total number of approximately 3000 samples together with Ulm resources. This genetic material has been already used for mutation-directed studies including tau, dynactin, VEGF, and GluR2 genes. In 416 ALS patients the A0/A0 tau genotype was not associated with ALS. However, tau genotype may contribute to the multifactorial etiology of ALS [Munch et al. 2005]. We have found 5 novel mutations and 1 polymorphism in the p150 subunit of the dynactin (DCTN1) gene [Sperfeld et al. 2006]. A novel mutation in the DCTN1 p150 subunit was also detected in a family with a co-occurrence of ALS and FTD [Munch et al. 2005]. No significant association for a single marker analysis or haplotype pairs of the VEGF gene was found in 580 ALS patients and 628 controls. Similarly, no disease-causing mutations were found in the coding region of the GluR2 gene [Ludolph et al. 2006].

Detailed studies on tau alternative splicing were performed in a transgenic mouse model harboring the human $SOD1^{G93A}$ ALS-associated mutation. Considering the well established role of tau in Alzheimer's disease, tau splicing was also studied in the APP23 transgenic mouse model, overexpressing the amyloid precursor protein, as a positive control for the ALS transgenics. A significant decrease of the total tau expression was found in the cerebral cortex and hippocampus of the $SOD1^{G93A}$ animals, but not in the APP model. Interestingly, an increase of the total tau expression was found in the cerebellum of the ALS transgenics. Unexpectedly, no correlation was ultimately found between the total expression of tau RNA and disease progression in the spinal cord of $SOD1^{G93A}$ transgenic mice. The findings indicate the influence of the $SOD1^{G93A}$ mutation on alternative splicing of tau in the brain, providing an interesting link between Cu/Zn SOD associated motor neuron diseases and the so-called tauopathies [Usarek et al. 2005; 2006].

In the second part of the project, we conducted a xenogenic cell transplantation into experimental animals (G93A mouse). In initial experiments, culture techniques for the cells used (human bone marrow mesenchymal stem cells – BMSC-, BMSC-derived

neural progenitor cells – BMSC-NPC- ; human umbilical cord cells – HUBC – and neural progenitor cell-like cells – HUBC-NPC) were optimized. Neural stem / progenitor cells derived from both sources were characterized as clonogenic, multipotent cells with an ability to differentiate into three main neural phenotypes: neurons, astrocytes and oligodendrocytes [Hermann et al. 2004; Jurga et al. 2006]. An electrophysiological analysis confirmed their neuron-like functional properties acquired under differentiation promoting conditions [Sun et al. 2005]. Furthermore, our experiments revealed that a certain level of stem/ progenitor cells commitment is important for the optimal response of HUCB-derived cells on neurogenic signals provided by the host astrocytes or the brain tissue after *in vitro* transplantation. Transplantation studies in 240 animals were performed by a new intrathecal cell injection technique into the cisterna magna [Janowski et al. 2006]. This technique guarantees both minimal cell leakage and their widespread distribution within the subarachnoidal space as confirmed by the tracking of transplanted CMFDA labelled cells. In contrast to other reports, we could not show any effect of cell transplantation on G93A mice survival, but a positive and gender-dependent effect on the motor function of the animals was observed. We established and optimized transplantation techniques, and developed MRI and PET techniques to track the homing behavior of grafted cells in future studies.

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[A7]

Peripheral analgesic effects of endomorphins in neuropathic and inflammatory pain

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A selective activation of peripheral opioid receptors can lead to an efficient and safe pain relief. This can be achieved with exogenous compounds and with opioid peptides secreted from immune cells in response to experimental (cold water swim) stress and local application of the corticotropin-releasing factor (CRF) (Stein et al., 2003). Endomorphins (EMs) are endogenous selective mu-opioid receptor agonists (Przewlocki and Przewlocka, 2001). Their role in neuropathic and inflammatory pain has not been fully elucidated. Here we examine peripheral analgesic effects elicited by exogenously applied EM-1 and EM-2, and the contribution of EM-containing leukocytes to the inhibition of chronic pain. We also compare the effects of EMs with classical mu-receptor ligands such as morphine, DAMGO and beta-endorphin. We used ligation of the sciatic nerve as a neuropathic pain model, and two models of inflammatory pain i.e. carrageenan-induced knee inflammation and Freund's adjuvant-induced inflammation of the hind paw in rats. To this end we applied behavioral (pain) testing, real-time polymerase chain reaction, radioligand binding, immunohistochemisty and flow cytometry. The number of mu-opioid receptors in dorsal root ganglia (site of the peripheral opioid receptor synthesis) was reduced in neuropathic pain but unchanged in knee inflammatory pain. EMs injected into the injured hind paws or into knee joints alleviated mechanical and thermal allodynia, decreased joint stiffness and increased animal mobility. The effects of EMs were comparable to DAMGO but were stronger than these produced by morphine. Analgesic effects of all agonists were reversed by naloxone methiodide (a peripherally restricted opioid receptor antagonist) indicating an involvement of peripheral opioid receptors. Peripheral EM-induced analgesia in Freund's adjuvant inflammatory pain model was blocked by locally applied mu- but not kappa-receptor selective antagonists. Delta-receptor antagonists did not influence EM-1-, but dose-dependently decreased EM-2-induced analgesia. Antibodies against beta-endorphin, Methionine-enkephalin or Leucine-enkephalin did not significantly change EM-2-induced analgesia. Both EMs bound to mu-receptors in dorsal root ganglia. Numerous beta-endorphin- and fewer EM-1- and EM-2-containing leukocytes were detected in the subcutaneous tissue of the inflamed paws. Leukocyte-depleting serum decreased the number of immigrating opioid-containing immune cells and

attenuated swim stress- and CRF-induced analgesia in the inflamed paws. Both forms of analgesia were strongly attenuated by antibodies against beta-endorphin and to a lesser degree by antibodies against EM-1 and EM-2 injected into the inflamed paws.

Together, exogenously applied and immune cell-derived EMs alleviate prolonged inflammatory pain through the selective activation of peripheral opioid receptors. Exogenous EM-2 in addition to mu- also activates peripheral delta-receptors, which does not involve actions via other opioid peptides. A higher peripheral analgesic efficacy of EMs, as compared to morphine, offers EMs as an alternative treatment for intractable neuropathic pain without producing side effects of centrally acting opioids and of non-opioid analgesics such as nonsteroidal anti-inflammatory drugs, antidepressants and anticonvulsants.

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[A8]

Investigation of pathomechanisms of Parkinson's disease and search for neuroprotective therapies: clinical, in vitro, and in vivo studies

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The aim of the study was to investigate the role of endo- and exogenous toxins in models of Parkinson's disease (PD) and to search for neuroprotective agents, as well as for early markers of the disease. To this end β-carbolines (BCs), tetrahydroisoquinolines (TIQs) and pesticides [rotenone, paraquat (PQ)] were studied in vivo and in vitro. Moreover, the expression of proteins characteristic for Lewy bodies was examined in the brains and muscles of humans without clinical symptoms of PD.

In vitro studies: Permanent cell lines, either wild type or transfected with human/ mouse dopamine transporter (DAT) or organic cation transporter (OCT) 1, 2, and 3 were applied to investigate the transport by which BCs are taken up by neurons. In addition, these models and the primary culture of embryonic mesencephalic cells from mice were used to study the neurotoxic and neuroprotective properties of BCs and PQ.

Main findings: Among the 27 BCs examined, several were neurotoxic, some only at high concentrations, and a single BC was clearly neuroprotective. The neurotoxic potency of a specific BC occurring in human s. nigra was in the same range as MPP^+ in several tests. Detailed investigations revealed that apoptosis is the main mechanism of neurotoxicity. The uptake of [^{3}H]DA and [^{3}H]MPP^+ by DAT was inhibited up to 70% by non-ionic BCs but not by ionic BC^+s. Most of the BCs inhibited the OCT up to 100%. Similar effects were observed in primary cells. An incubation of mesencephalic cells with the neuroprotective BC caused an apparent 20% increase in the number of dopaminergic neurons characterized by TH-positve immunostaining, RT-PCR and DA content. Moreover, a decrease of LDH release and an increase of the ATP content were determined.

In vivo studies: Neurotoxins were administered either systemically, or directly into rats' brains. PQ administered acutely (10 mg/kg ip) released dopamine in the striatum. PQ injected subchronically induced a loss of ca. 22% of dopaminergic neurons of the s. nigra and decreased the striatal dopaminergic transmission. The long-term (6 months) PQ administration induced a slowly progressing degeneration of 40% dopaminergic neurons in the s. nigra and ventral tegmental area, a shrinkage of both structures, delayed deficits of striatal dopaminergic transmission, as well as an increase in α-synuclein(α-Syn)-immunostaining of the s nigra. No cytoplasmic inclusions (α-Syn"+", synphilin-1"+", ubiquitin"+") or signs of apoptosis were found. Direct intrastructural injections of PQ (0.3-3 µg/1 µl), 2,9-dimethyl-carbolinium ion (2,9-DiMe-BC^+, 10-40 nmol/2 µl) or rotenone (2 µg/5 µl) also produced lesions in the s. nigra and a concomitant reduction of DA and its metabolites in the striatum.

Neuroprotection: Among the substances tested in the above-mentioned models of neurodegeneration in vivo, 1,2,3,4-tetrahydroisoquinoline (TIQ) seemed to exhibit some neuroprotective properties.

Clinical studies: α-Syn was shown in the skeletal muscles of older patients with neuro-muscular

disorders, as well as in patients with inclusion body myopathy. The s. nigra of neurologically healthy elderly subjects showed the presence of intranuclear ubiquitin"+" and α-Syn"-" inclusions, as well as a decreased levels of TH, increased of levels ubiquitine and synphilin-1, and unchanged levels of α-Syn.

Conclusions: The study indicates that both BCs and pesticides may contribute to the degeneration of dopaminergic neurons, while some TIQs and BCs may exert neuroprotection. The effects of BCs seem to depend on their intraneuronal uptake by DAT, OCT, and/or diffusion, as well as on their storing within dopaminergic neurons. The neurotoxic BCs trigger apoptosis by inhibiting the respiratory chain and glycolysis. An examination of protein expression in skeletal muscle fibers may constitute an approach to an early diagnosis of neurogenerative disorders.

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[A9]

The successful use of adeno-associated virus as a vehicle for adhesion molecule L1 expression in mouse spinal cord regeneration

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We applied an adeno-associated virus (AAV) construct such that it encodes for the full-length of the L1 molecule. The advantage of the AAV vector is that it infects both postmitotic and replicating cells in vivo and in vitro, shows a stable and efficient integration into the host genome, has a cloning capacity up to 4.5 to 5 kb (full-length L1 encodes with 4 kb), has a long-term gene expression profile and little evidence of toxicity and immune responses. We did not succeed to make a construct that codes for both L1 and green fluorescent protein, since this appeared too large for insertion into the small virus. Thus AAV construct expressing green fluorescent protein was used to see whether this virus would infect cells successfully in the lesioned spinal cord of adult mice. A stable expression of L1 was observed with the AAV5, but not AAV2 construct and expression of L1 was observed in neurons and glia when L1 and green fluorescent protein were driven by the CMV promoter. An expression of green fluorescent protein was seen up to an amazing 1 cm rostral and caudal to the injection site in unlesioned and spinal cord lesioned mice. A less successful, but neuron-specific expression was found when AAV 5 was driving L1 and green fluorescent protein expression under the synapsin promoter. We found that L1 and green fluorescent protein expression was already detectable one week after the injection of the virus into the intact and injured spinal cord and that it increased its expression level with time over three to four weeks, to remain stably expressed up to four months after the injection, the longest time period tested. We tested for reaction of the immune system after the injection of the virus and could not find an increase in the numbers of macrophages, CD4 positive or CD8 positive lymphocytes. We monitored the functional recovery of the motor behavior over five weeks and found that in the systems that measure plantar stepping responses, namely the BMS score and the foot-beam ankle recovery index, a novel quantitative evaluation paradigm that we had developed, that the L1 carrying AAV improved the locomotor functional recovery in a group of 15 animals, but not in AAV carrying green fluorescent protein. First results show that there was an enhanced serotonergic axonal regeneration and prevention of cortico-spinal tract axon retraction (a dying back of axonal stumps that had been anterogradely labelled by the fluorescent dye fluoro-ruby). Interestingly, the expression of the inhibitory extracellular matrix glycoprotein, the chondroitin sulphate proteoglycan NG2 was drastically decreased in the spinal cord that had been infected with L1 carrying AAV, along with the suppression of immunoreactive GFAP positive astrocytic processes, a marker for astrogliosis following a spinal cord injury. Thus, these preliminary experiments suggest that L1, when administered to both neurons and glial cells in an area covering about 1 cm rostral and caudal to the injection site was able to enhance serotonergic axonal regeneration, prevent dying back of axons of the corticospinal tract and enhance the functional locomotor recovery.

Furthermore, the ability of AVV expressing L1 to repress an inhibitory component of neurite outgrowth, namely NG2 and inhibiting astrogliosis was an unexpected, but possibly functionally very important observation that we are pursuing to dissect mechanistically.

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[A10]

The role of CREB and CREM in neuronal survival, synaptic plasticity and drug addiction – a combined molecular and neurobiological approach

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The coupling of transcription to neuronal activity is a crucial molecular mechanism that confers functional plasticity to neurons. Among the several stimulus-dependent transcription factors expressed in the brain, the cAMP response element binding protein, CREB and its related protein CREM, have been studied in the Heidelberg group by a molecular genetic approach. A simultaneous disruption of CREB and CREM leads to a progressive neurodegeneration. Using these mutants, we analyzed at the genome-wide level the contribution of these stimulus-induced transcription factors in the activity-regulated gene expression. We have found that only a subset of immediate early genes (IEGs) depends on CREB signaling, since most IEGs are still induced in the absence of CREB and CREM. A deletion of the serum response factor (SRF) in neuronal populations of adult mice also results in a deficient expression in the activity-dependent genes (in collaboration with D. Ginty, Johns Hopkins University). Whereas a loss of CREB and CREM leads to a degeneration of neurons, mice deficient for SRF in adult neurons show no impaired survival. Interestingly, SRF which is crucial for the induction of many IEGs in the forebrain is not required for the regulation of the identified set of CREB-dependent IEG genes. These results reveal an unexpected specificity of activity-dependent gene transcription in dependence of these two major stimulus-dependent pathways in the brain.

The CREB transcription factors are important in the onset of the manifestation of opioid withdrawal, since the major signs of morphine abstinence are strongly attenuated in CREB mutant mice. The Locus coeruleus, the major nucleus of noradrenergic signaling in the CNS and the primary source of noradrenaline in the cortex, is thought, but not proven, to be responsible for many symptoms observed after drug withdrawal by the upregulation of the cAMP signaling pathway. To characterize CREB function in noradrenergic neurons genetically, we have generated mouse mutants in which the CREB gene is specifically inactivated in noradrenergic neurons using the Cre/loxP recombination system. We tested the opioid withdrawal behavior in these mutants. Surprisingly, we did not see any impairment in the manifestation of the morphine withdrawal response consistent with other evidence. The role of CREB and SRF in addiction is being studied by a specific inactivation of these genes in dopaminergic and dopaminoceptive neurons. These mutations are based on the expression of the Cre recombinase after BAC transgenesis using the dopamine transporter gene for inactivation in dopaminergic neurons or the dopamine receptor 1 gene for inactivation in the Nucleus accumbens. The Warsaw group is interested in biological and pathological roles of ICER, Inducible cAMP Early Repressor, which antagonizes all members of the CREB family, since elevated CREM/ICER mRNA expression was observed *in vivo* in response to treatments evoking neurodegeneration. An expression of ICER leads to neuronal apoptosis in vitro and is able to induce neuronal cell death in organotypic hippocampal cultures. A field-specific pattern of evoked cell death was observed with dendate gyrus neurons displaying cell death already at day3, CA3 neurons at day7, and CA1 neurons only after 14 days. These observations suggest that the endogenous antagonist of the CREB protein family may have specific functions in the control of cellular survival in the brain.

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[A11]

Impaired maturation and altered regulatory function of plasmacytoid dendritic cells in multiple sclerosis

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Plasmacytoid dendritic cells (pDCs) represent a DC-subtype which exerts divergent functions in innate and adoptive immunity including the immediate reaction to microbial factors and the induction of immunoregulatory responses. It is thought that different DC-subtypes may be critically involved in the pathogenesis of multiple sclerosis (MS). In our study we assessed the phenotype, maturation and functional properties of peripheral blood pDCs from 35 clinically stable, untreated MS patients, 30 healthy controls and 9 patients with pneumonia used as non-specific inflammatory conditions (NIC). *Ex vivo* expression of CD86 and 4-1BBL was significantly lower on pDCs from MS patients than from controls and patients with NIC (22% vs. 47% vs. 41 % and 12% vs. 35% *vs.* 32% respectively). When stimulated with IL-3 and CD40L, pDCs of MS patients showed inefficient maturation as demonstrated by a significantly lower or delayed up-regulation of CD86, 4-1BBL, CD40 and CD83. Additionally, in MS stimulation of pDCs by unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN) resulted in a significantly lower IFN-alpha secretion than in controls. In MS but not in controls, pDCs failed to up-regulate proliferative responses and IFN-gamma secretion of autologous PBMC in a co-culture system. Moreover, the depletion of pDCs in MS patients, but not in controls had no effect on the generation of $CD4^+Foxp3^+$ regulatory T cells. We also provide data showing that glatiramer acetate treatment partially restores the phenotype and function of pDCs in MS patients. These findings suggest functional abnormalities of pDCs in MS patients, which might be of importance in the understanding of the development of immune dysregulation in this disease.

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[A12]

Temporal-information processing in aphasic patients: new horizons for aphasia therapy

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It has been shown that the perception of temporal order (TO) of two events (corresponding to a proposed time frame of around 20 to 40ms) is associated with the ability to identify individual phonemes (especially consonants), whereas the perception of TO of more than two events (corresponding to a proposed time frame of approximately 200 to 300ms) has been linked to speech perception on the syllable level. Children with language-learning disabilities and patients with aphasia, for example, show elevated temporal-order thresholds (TOT) and also have difficulties in discriminating stop consonants. However, whereas over the last years several tools have been developed to assess and train temporal-processing mechanisms in children with language-learning impairments, standard diagnostic procedures and training devices for assessing and training temporal-processing abilities of adult patients with aphasia have not been developed so far. Therefore, the present study was designed (1) to find adequate methods for the diagnosis of impairment in the perception of TO, (2) to gain normative data with these methods in healthy adults, (3) to train the perception of TO in aphasic patients in order to improve their speech-perception abilities. The study was performed in both German and Polish subjects to test whether the association between temporal-processing abilities and speech perception is dependent on the respective language, i.e. the ratio between consonants and vowels.

For normative data collection 82 German and 87 Polish subjects between 20 and 70 years were investigated using five different TO tasks. The perception of TO of two 1 ms click stimuli appeared to be strongly influenced by age and gender. The deterioration of performance in elderly subjects may reflect the general slowing of information processing. These influences were independent of the subjects' nationality, which suggests a strategy-independent common mechanism responsible for the perception of TO. TOTs for two tones were lower than for clicks, suggesting a stimulus dependency of performance. Regarding the perception of TO of four auditory stimuli (complex tones, simple tones, syllables), results show significant effects of age and nationality: higher TOTs were found in older adults and in Polish participants. Thresholds were also dependent on the stimulus set, with the four complex tones yielding the largest values. The lowest thresholds were found for the four simple tones. These results stress the importance of establishing task-, gender- and age-specific norms when evaluating aphasic patients' performance on these tasks.

With respect to training aphasic patients' TO perception, data acquisition is still ongoing. The first results of approximately ten German and Polish participants indicate that a training of TO perception can improve temporal-processing, as well as language abilities. Further training will show whether this effect is task-dependent and whether improvements in speech perception can also be seen in complex language tasks.

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[A13]

Potassium channels in mitochondria: novel neuroprotective targets?

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In the inner mitochondrial membrane, several potassium selective ion channels have been identified. They have been suggested to be involved in the cytoprotective action of various cell types. In our recent work we describe the identification of a novel potassium channel in brain mitochondria. In isolated mitochondria, $Ca2^{+}$ was found to depolarize the mitochondrial inner membrane and to stimulate respiration in a strict potassium-dependent manner. These potassium-specific effects of $Ca2^{+}$ were completely abolished by charybdotoxin or iberiotoxin - well known inhibitors of large conductance, calcium activated potassium channel (BK_{Ca}-channel). Furthermore, NS1619 - a BK_{Ca}-channel opener - mimicked the potassium-specific effects of calcium on the respiration and mitochondrial membrane potential. A light microscopy and immunological studies identified the BK_{Ca} channel preferentially in the mitochondrial inner membrane of rat brain. The channel activity also was measured after the reconstitution of the purified inner mitochondrial membrane into a planar lipid bilayer. The activity of potassium channel was recorded. The mean conductance of the channel was 250 pS in 50/450 mM KCl gradient. A single-channel activity of this reconstituted protein showed properties of the big-conductance potassium (BK) channel: it was activated by Ca^{2+} and blocked by charybdotoxin. Additionally, a stimulation of the channel activity was observed upon the application of BK channel openers, benzimidazolone derivatives, NS1619 and NS004. Additionally, the effect of BK channel openers on isolated brain mitochondria and neuronal HT-22 cells were studied. We propose that an activation of the mitochondrial K^{+} transport by BK_{Ca}-channel opening might be important for neuroprotection, similar as reported previously by us for the antiepileptic drug topiramate. This drug was found to affect the calcium-dependent depolarization of the mitochondrial inner membrane causing *in vivo* neuroprotection of hippocampal CA1 and CA3 pyramidal cells and antiepileptogenic properties in the pilocarpine-treated chronic epileptic rat.

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[A14]

Lesion-induced brain plasticity: experimental and clinical evaluation of strategies to improve functional recovery from stroke

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In the project, the adaptive plasticity of functional representations in the cerebral cortex was investigated. The work packages addressed plastic changes of cortical representations in the brain after stroke, long-term molecular and cellular alterations that may facilitate or hinder plasticity, a possibility of brain repair by neural stem cells transplantation and, in a clinical trial, the effect of enhancement of dopaminergic function upon neurorehabilitation after stroke. In animal experiments, focal cortical stroke was achieved by photothrombosis with Bengal Rose.

(1) A metabolic mapping of the functional brain activity with [14C]2-deoxyglucose (2DG) revealed that following lesion centred on the cortical representation of vibrissae, the barrel cortex, the metabolism of the injured hemisphere rapidly decreased, while in the non-injured hemisphere the activation-driven 2DG uptake was enhanced. This pattern evolved during two months post lesion and resulted in the emergence of a new pattern of activation of the cortex by a stimulation of vibrissae – a new representation appeared in the peri-stroke cortex. (2) The influence of stroke upon cortical plasticity was investigated in the same sensory pathway. We found that plastic changes of cortical vibrissal representations induced by a sensory deprivation of vibrissae and by associative learning involving a stimulation of vibrissae were impaired by a cortical lesion in the vicinity of the barrel field. (3) Brain plasticity and the effects of stroke are modulated by age. Experimentally, we found a reduction of functional inhibition in the brains of aged healthy animals. Following stroke, there was a facilitation of brain activity in the surround of ischemic cortical lesions in young animals, and a suppression in the brains of aged animals. In aged animals, the receptor subunit composition of GABA receptors regressed to a "younger" pattern in the surround of a stroke. (4) Neuromodulators, such as dopamine and noradrenaline, were shown to be involved in post-stroke neurorehabilitation. We investigated - using in vitro quantitative autoradiography - the distribution and levels of dopamine1 and beta1 noradrenergic receptors at different times after phototrombotic stroke. Both receptor sites showed a decreased ligand binding exclusively in the core of infarct and all other investigated areas remained unchanged. After 4 weeks post stroke, binding levels returned to control values probably due to a shrinkage of the infarct volume. Simultaneously, we examined the spatiotemporal activation of astroglia and microglia. We found that activated astroglia are present not only in the vicinity of the infarct but also in very remote cortical and subcortical areas. This astroglia activation was present even 4 weeks after phototrombosis. The activation of microglia was limited to periinfarct areas at short survival times (up to 1 week) and was not visible 4 weeks after the stroke. (5) Stroke induces spreading depressions in the surrounding brain. Using microarray technology and subsequent quantitative RT PCR, we could show that such waves of spreading depression induce long-lasting changes of gene expression up to 4 weeks. Selective proteins regulated by these genes were analyzed with immunohistochemistry. (6) As spreading depressions (SD) occur in association with ischemic brain injury, they may constitute an important mediator of stroke-induced neurogenesis. To analyse their effects on neurogenesis, we induced repetitive SD by a cortical application of 3 M KCl for ?2 hours and labelled the newborn cells with bromodeoxyuridine (BrdU). Compared to sham operated controls, SD rats had significantly more BrdU+ cells in the ipsilateral dentate gyrus (DG) at each time point. This effect was abolished by the NMDA receptor antagonist MK-801. Using confocal laser microscopy, we identified the vast majority of the newborn cells as neuronal precursors (BrdU+/DCX+) after 3 or 5 days and neurons (BrdU+/NeuN+) after 6 weeks. Rats were further tested in a spatial version of the Morris Water maze beginning 5 weeks after SD. During the acquisition phase spatial learning was improved by SD (% time in the target quadrant) but during the probe trials no difference was observed

relative to controls. Our results indicate that cortical SD significantly enhances neurogenesis in the adult rat DG, that the newly generated cells survive for at least 42 days and that SD (possibly by the increased neurogenesis) slightly influence hippocampus-dependent learning. (7) Umbilical cord-derived neural stem cells were labelled by viral transfection with Green Fluorescent Protein and injected into the subcortical white matter medially to the stroke site. After one week the labelled cells were visible around the injection site and appeared to migrate towards the lesion. (8) The clinical trial tested the effects of L-DOPA (Madopar 125) on the outcome of standard aphasia therapy. The patients received 100 mg of the drug 30 min before rehabilitation session, 5 times a week. The trial lasted 3 weeks and was double blind and placebo controlled. Statistically significant improvement was found in tests of answering questions, naming body parts word repetition and verbal fluency in patients with strokes localized in the frontal lobe, but not in the parietal and temporal lobe.

In summary, several processes causing perilesional dysfunction following stroke were identified. These processes do influence perilesional plasticity. The response of the brain to an ischemic lesion is age-dependent. Endogeneous neurogenesis is increased by spreading depressions as they are induced following stroke. Umbilical cord-derived neural stem cells migrate towards the lesion. Finally, clinical strategies for rehabilitation employing drugs which influence brain plasticity are able to support recovery from stroke.

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[A15]

Nitric oxide – specific role in neuronal death. A unifying feature in dementive disorders? Age and β-amyloid related alteration of gene expression for different isoforms of NOS in different parts of the brain. Role of NO in synapse degeneration

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The role of nitric oxide (NO), the molecular switch in cell life and death, was analysed in amyloid beta toxicity and in Alzheimer Disease. The study was carried out using different brain parts from Wistar rats, synaptosomal and synaptoneurosomal and subcellular fractions. Moreover, the amyloid beta precursor (APP), transfected cells (PC12, HEK cells) with wild human APP (APPwt) and with double Swedish mutation (APPsw) and also APP transgenic animals, then serum and leucocytes from Alzheimer patients and corresponding healthy aged controls were used for the study. A broad range of biochemical, radiochemical, immunochemical, electron microscopic and molecular biology methods were applied.

Our study indicated several important new original findings:

1. A lower endothelial NO synthase (eNOS) activity in all investigated aged brain parts and higher cGMP phosphodiesterase (PhDs) activity. The specific inhibitor of PhDs2 enhances cNOS activity through cGMP/PKG pathway and the memory function which indicated that these inhibitors may be very useful in the therapy of dementia and also may enhance the memory function in adults. The alteration of covalent phosphorylation processes is responsible for eNOS modification. An analysis of eNOS gene expression on the level of mRNA and protein presented an alteration of translation processes in aged brain for eNOS isoform.
2. High neuronal NOS activity in aged brain participates in the enhancement of macromolecules oxidation. However, the prooxidative ability of aged brain parts to