Restorative Neurology and Neuroscience 23 (2005) 159–164
IOS Press


# THEME FIVE PLENARY

# New Developments in Neurotrauma

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## APPLYING PROTEOMICS IN BRAIN INJURY STUDIES

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Traumatic brain injury (TBI) represents a major CNS disorder without any clinically proven therapy. Biomarkers that strongly correlate with disease severity, time course of disease progression and drug response can facilitate therapy development. We have shown that alphaII-spectrin breakdown products (SBDPs) are prototypic protein markers for TBI since they are an index of structural injury and provide Information on injury mechanisms. With the recent technological advances in proteomics and the availability of the human proteome database, disease marker research will be a uniquely productive application of proteomic technologies. Proteomic methods can be readily used to identify unique markers specific to a disease process such as TBI. Once novel markers are identified, sensitive detection tools (such as ELISA assays) can be developed for their detection in affected brain tissue, CSF and blood samples. We are employing an integrated, proteomics-based approach to discover novel biomarkers for TBI. We will be presenting data on: (1) 2D-gel electrophoresis-MALDI-TOF, (2) 1D and 2D-liquid chromatography, (3) (LC)-gel electrophoresis-MS/MS and (4) high throughput immunoblotting analyses.

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## BONE MARROW STROMAL CELLS - A PROMISING TOOL FOR THERAPY OF BRAIN AND SPINAL CORD INJURIES

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Magnetic resonance (MR) imaging provides a noninvasive method for studying the fate of transplanted cells *in vivo*. The fate of implanted rat bone marrow stromal cells (MSCs) and mouse embryonic stem cells (ESCs) labeled with superparamagnetic iron-oxide nanoparticles was studied in rats with a cortical photochemical lesion or with a balloon-induced spinal cord compression lesion. MSCs were co-labeled with bromodeoxyuridine (BrdU) while ESCs were transfected with pEGFP-C1 (eGFP ESCs). Cells were either grafted intracerebrally into the contralateral hemisphere of the adult rat brain or injected intravenously. *In vivo* MR imaging was used to track their fate; prussian blue staining and electron microscopy confirmed the presence of iron-oxide nanoparticles inside the cells. During the first week post-implantation, grafted cells migrated to the lesion site and populated the border zone of the lesion. Less than 3% of MSCs differentiated into neurons and even fewer into astrocytes; 5% of eGFP ESCs differentiated into neurons, while 70% of eGFP ESCs became astrocytes. The implanted cells were visible on MR images as a hypointense area at the lesion site, and the signal persisted for more than fifty days. The presence of GFP-positive or BrdU-positive and nanoparticle-labeled cells was confirmed by histological staining. These studies demonstrate that both grafted MSCs and eGFP ESCs labeled with iron-oxide nanoparticles migrate into the injured CNS. Iron-oxide nanoparticles can therefore be used as a marker for the long-term noninvasive MR tracking of implanted stem cells. There may be various ways in which MSCs may interact with the host CNS tissue.

**SESSION 5.1***:* Imaging

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## REGIONAL PET ANALYSIS OF GRAY VS. WHITE MATTER METABOLISM: IMPLICATIONS OF FOCAL PHYSIOLOGICAL MONITORING ACUTELY FOLLOWING TBI

*M Bergsneider* (*Los Angeles, USA*)

*Introduction*: A variety of highly focal physiological monitors, such as tissue oxymetry, are used at to guide head-injury management. The data typically are interpreted with regards to presumed ischemia. Theoretically, there may be differences between white matter (WM), gray matter (GM), and pericontusional edematous regions. The latter is generally assumed to exhibit ischemia.

*Methods*: 32 TBI patients were studied using PET imaging (mean 2.5 days post-injury). Regional maps of CMRO2, OEF, CBF, and CMRglc were generated. The images were co-registered with concurrent CT/MRI. Based on MRI, GM and WM masks were generated of cerebral cortex remote from contusions. A separate analysis was made of the pericontusional edematous region (identified by CT and MRI FLAIR imaging). 16 normal healthy volunteers were studied in a similar manner.

*Results*: Normal appearing remote cortex demonstrated differential WM vs. GM metabolic changes. There was a significant reduction in the subcortical WM oxygen-glucose ratio (OGR) following TBI compared to normals, whereas the mean cortical GM and whole brain values remained unchanged. WM metabolic changes, which were diffuse throughout the hemispheres, were characterized by a reduction in CMRO2 without a concomitant drop in CMRglc. The pericontusional edematous region showed the most severe metabolic abnormalities immediately adjacent to the hemorrhagic core, but high OEF values, suggestive of ischemia, were rarely encountered.

*Conclusion*: Knowledge of the location of focal monitor sensors may be important in the interpretation of values. With current ICU management, ischemia may be less common than assumed based on focal monitoring.

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## MAGNETIC RESONANCE SPECTROSCOPIC IMAGING AT 3 TESLA AND COGNITIVE FUNCTION IN TRAUMATIC BRAIN INJURY

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Traumatic brain injury (TBI) unleashes a series of events including cell death and metabolic dysfunction with few proven tools for predicting patient-specific sequelae. Magnetic resonance spectroscopy (MRS) can quantify specific brain markers of neuronal injury (N-acetylaspartate; NAA) and inflammation (choline; Cho) non-invasively. Moreover, these markers have been shown to predict cognitive recovery. However, most previous MRS studies measured small volumes of radiologically normal-appearing tissue remote from the impact. Our aim was to determine whether a large sample of brain tissue was also associated with cognitive performance in patients in the post-acute phase of recovery from TBI ($\sim$ 6 weeks). Eight patients with moderate to severe TBI and eight uninjured controls were studied. Attention, language, memory, executive, and motor skills were tested and a mean z-score calculated. Three Tesla MR spectroscopic imaging (MRSI) sampled a 16 by 16 grid of spectra corresponding to approximately 100mls of supraventricular gray and white matter (PRESS, TR $=$ 1500 ms, TE $=$ 30 ms). T-tests revealed that patients had lower mean cognitive z-scores ($-1.33$ vs. 0.08; $p < 0.01$), lower NAA (12.7 vs. 14.8 mM; $p < 0.05$) and higher Cho (2.98 vs 2.65 mM; $p < 0.05$) than controls. Cognitive z-scores were correlated with NAA ($r = 0.87$; $p = 0.007$) and Cho ($r = -0.93$; $p = 0.001$). MRSI data also revealed an inhomogeneous distribution of NAA and Cho indicating that the cellular sequelae to TBI vary by location presumably dependent upon individual injury. However, the cognitive deficits associated with particular patterns of injury remain to be determined. MRSI might provide a better understanding of the cognitive sequelae, clinical management, and interventional strategies following TBI.

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## EVIDENCE FOR NEURO-PLASTICITY AFTER TRAUMATIC BRAIN INJURY: A PROSPECTIVE 2-YEAR MAGNETIC RESONANCE STUDY

*P Goetz, A Blamire, B Rajagopalan, C Mackay, T Cadoux-Hudson* (*Oxford, UK*)

Patients show clinical improvement after diffuse traumatic brain injury (TBI). We have previously showed that MR abnormalities correlated with severity and outcome. We hypothesized that longitudinal changes and functional MRI could reveal mechanisms underlying clinical recovery. 18 patients (mean age 36, range 17–63) diagnosed as head injury (mean admission GCS 7.5, 9 mild, 6 moderate and 3 severe) were studied. Measurement of micro-structural (diffusion-weighted imaging), biochemical (proton spectroscopy) and vascular (cerebral blood volume) changes in normal appearing white matter (NAWM) were made acutely (average 7.5 days after injury, range 1–40). The former two were repeated at 6.8 months (range 6–10.5) and 21 months (16.5–26). Determination of functional reorganisation (functional MRI, fMRI) and outcome assessment (GOS) were performed at 2 years. Results were compared with a control group ($n$ = 18, av. age 35, 18–59). In normal appearing white matter (NAWM) N-acetyl aspartate (NAA) is reduced and apparent diffusion coefficient (ADC) is increased; these changes correlate with both initial injury severity (ANOVA $p = 0.03$ and $p = 0.04$) and 2-year GOS (ANOVA $p = 0.002$ and $p = 0.047$). There were no significant ADC or NAA changes over time. Areas of low CBV had high ADC suggesting no ongoing ischaemia. fMRI using a word generation paradigm showed significantly increased areas of activation in the left frontal lobe in the patient group. Initial structural and biochemical MR indices correlate with severity and outcome but appear to be irreversible. Thus, the likely mechanism for clinical improvement is functional reorganisation suggested by fMRI. Rehabilitation strategies aimed at promoting plasticity may be beneficial.

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## EARLY CHANGES OF EXPERIMENTAL SPINAL CORD INJURY IN MICE BY MRI DIFFUSION TENSOR IMAGING AND MULTI-EXPONENTIAL T2 ANALYSIS

*M Gaviria, J Bonny, H Haton, M Teigell, J Renou, A Privat* (*Montpellier; Champanelle, France*)

Therapeutic windows for neuroprotection in SCI depend on time/space pathophysiological changes. Our objective was to characterise early SCI changes in mice using *ex vivo* MRI during the acute phase (24 h) by means of two complementary quantitative approaches: diffusion tensor imaging (DTI) and multi-exponential T2 analysis (MET2).

For this purpose, SCI was performed at T8 level, spinal blocks (5 vertebrae) were excised and analysed using an Avance DRX400 micro-imaging system. The delays post-injury were $+2$ h, $+4$ h, $+8$ h, $+16$ h, $+24$ h. Images of five slices perpendicular to the spinal cord were collected, each one covering a single thoracic metamer. The values of the different parameters were measured within 11 regions of interest in the spinal cord in both white (WM) and grey matter (GM). In WM, a rapid decrease of the largest diffusivity parallel to the WM tracts was observed until 8h after SCI, whereas the radial diffusivity increased slightly. The pathological process led to an acute decrease of the amplitude of the short-T2 component at 8 h. In GM, all the diffusivities reached a minimum at 8 h. DTI and MET2 represent quantitative markers, both sensitive to the pathological events in the early period following SCI. The changes in diffusivity are consistent with those reported in previous studies. The alteration of the amplitude of the short-T2 component in WM indicates a demyelination, especially at 8 h. This non-invasive characterisation of spatial and temporal evolution of SCI helps to define optimal parameters for innovative therapeutic strategies.

**SESSION 5.2***:* Proteomics and Genomics

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## CALPAIN AND CASPASE - 3 ACTIVATION IN PATIENTS WITH SEVERE TRAUMATIC BRAIN INJURY

*SB Lewis, KK Wang, MC Liu, K Barami, JJ Tepas III, RL Hayes, JA Pineda* (*Gainesville, USA*)

*Introduction*: Previous work in our laboratory demonstrated the role of two important proteases (caspase-3 and calpain) in protein breakdown with formation of distinct breakdown products after traumatic brain injury (TBI). Lines of evidence in animal models suggest that calpain plays a major role in the pathophysiology of TBI. Moreover the role of caspase-3 protease as key executioner in mammalian apoptosis is well established. Non-erythroid alpha-II-spectrin is a cytoskeletal protein that is a substrate of both calpain and caspase-3 cystein proteases. Cleavage of important cytoskeletal proteins such as alpha-II-spectrin (240 kDa) by calpain and caspase-3 results in accumulation of protease-specific breakdown products that can be used to monitor the magnitude and temporal duration of protease activation.

*Methods*: We performed a longitudinal protein analysis in patients diagnosed with severe TBI (GCS 8 or less) to immunolabel specific proteolytic fragments of alpha-II-spectrin produced by both caspase-3 and calpains (150 kDa), calpains (145 kDa) and caspase-3 (120 kDa) proteases. Similar analysis was conducted for specific breakdown products of additional proteins of distinct sub-cellular localization. Calpain products were present immediately after injury in all patients, gradually decreased, but not disappeared over several days after injury. Caspase-3 activation was also evident after injury in all patients.

*Conclusions*: Calpain and caspase-3 specific protein breakdown products are persistently elevated after traumatic brain injury, suggesting an important role for cystein proteases in the pathophysiology of TBI in humans. Using a functional degradomics approach we identified potential diagnostic and therapeutic targets in patients with severe TBI. Similar studies of subarachnoid hemorrhage are ongoing.

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## GENE EXPRESSION PROFILE OF RAT OLFACTORY ENSHEATHING CELLS

*AJ Vincent, JM Taylor, JC Vickers, AK West, MI Chuah* (*Hobart, Australia; Oxford, UK*)

In recent years olfactory ensheathing cells (OECs) transplanted into the injured spinal cord has led to variable degrees of anatomical and functional recovery. Although the therapeutic potential of OECs is commonly inferred by their *in vitro* expression of several growth-promoting factors (e.g. NGF, BDNF, NT4/5) and extracellular matrix molecules (e.g. N-CAM, L1), we hypothesised that microarray analysis will uncover additional molecules which are likely to be involved in interactions with the injured CNS. We have compared the gene expression profile of OECs with Schwann cells (SCs) and astrocytes (ACs). Cells were harvested from neonatal Hooded Wistar rats and purified in culture. Two biological replicates of RNA from OECs, SCs and ACs were hybridised to long oligo rat 5 K arrays (Clive & Vera Ramaciotti Centre for Gene Function Analysis, Australia) against a common reference RNA pool (50% fibroblast, 50% brain). The data were filtered for noise (low and saturated signal) and 1841 datapoints were accepted for further analysis. The results demonstrated that OECs are a unique cell type and are more closely related to SCs than ACs. Venn diagram combined with statistical analysis (ANOVA) revealed a genelist of 18 transcripts that were $> 1.5$-fold enriched in OECs relative to the SCs and ACs ($p < 0.05$). From this list, real-time RT-PCR and immunohistochemistry have been used to confirm the expression patterns for lysozyme, TIMP2, CTGF, Gro (CXCL1), MCP1 (CCL2) and C/EBPb. The expression of chemokines such as Gro, MCP1 and C/EBPb suggests that OECs could be involved in regulating inflammation in the injured CNS.

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## ACTIVATION AND INTERACTION OF P53 AND NFKB DURING CELL DEATH - ROLE FOR SECONDARY BRAIN DAMAGE AFTER CONTROLLED CORTICAL IMPACT IN MICE

*LV Baumgarten, C Culmsee, A Baethmann, N Plesnila* (*Munich, Germany*)

Programmed cell death (PCD) is a well-known feature of secondary brain damage following TBI. The objective of the current study was to characterise the role

and the interaction of the transcription factors p53 and NFkB for PCD. C57/Bl6 mice were subjected to CCI and sacrificed 15 min, 3, 6, 12, or 24 h thereafter for quantification of p53 expression by Western blotting or immunohistochemistry ($n = 6$ per group). In a second series p53 was selectively inhibited with pifithrin (4 or 8 mg/kg) either given before or 15 min, 1, 3, or 6 h after trauma. NFkB activity and its interaction with p53 after TBI were investigated in NFkB-luciferase reporter mice. 24 h after TBI p53 was maximally upregulated in and around the contused cortex ($+230\%$; $p < 0.01$). p53 inhibition prevented secondary lesion expansion by more than 40% ($n = 10$ per group; $p < 0.01$) even when given up to 6 h after trauma ($n = 7$ per group; $p < 0.05$). NFkB transcriptional activity in the contused cortex was reduced by 50% (at 3 and 24 h; $n = 3$; $p < 0.05$). Inhibition of p53 increased NFkB activity in naive brain and prevented the decline of NFkB activity in contused tissue. Our results demonstrate that p53 is upregulated after TBI and its inhibition up to 6h after the insult reduces contusion volume. In addition to the activation of apoptotic signalling, p53 seems also to inhibit intrinsic neuroprotective signalling by NFkB. This dual way of action may make p53 an interesting drug target for the treatment of TBI.

**SESSION 5.3***:* Cell Therapy/Regeneration - Young Investigators

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**ADULT MARROW STROMAL CELLS ENHANCE OLFACTORY ENSHEATHING CELL ATTACHMENT AND PROCESS ELONGATION**

*M Abrams, I Lee, D Prockop, L Olson,* (*Stockholm, Sweden*)

Olfactory Ensheathing Cells (OEC) are a specialized population of glial cells that have been reported to promote axonal regeneration following spinal cord injury. Marrow Stromal Cells (MSC) have been reported to facilitate the growth and attachment of neurosphere cells *in vitro*; therefore, the purpose of this research was to investigate the potential of MSC to influence OEC. MSC and OEC obtained from adult rats were cocultured in either a monolayered feeder culture or a mixed cell culture. MSC monolayered feeder cultures were characterized by increased OEC attachment and extensive process elongation. In mixed cell cultures, OEC attachment and process elongation was unchanged by the presence of MSC. Our results suggest that MSC monolayered feeder cultures promote OEC attachment and process elongation, thereby suggesting possible benefits in spinal cord injury models of MSC transplantation followed by the transplantation of OEC.

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**DIFFERENCES IN THE NEURONAL STEM CELLS SURVIVAL, NEURONAL DIFFERENTIATION AND NEUROLOGICAL IMPROVEMENT AFTER TRANSPLANTATION OF NEURAL STEM CELLS BETWEEN MILD AND SEVERE EXPERIMENTAL TRAUMATIC BRAIN INJURY**

*T Shindo, N Kawai, S Nagao* (*Kagawa, Japan*)

*Objective*: We evaluated the differences in the neuronal stem cells survival, neuronal differentiation and memory function after transplantation of the neuronal stem cells (NSCs) between mild (2 atm) and severe (7 atm) experimental traumatic brain injury.

*Methods*: C57BL/6 mice were subjected to mild (2 atm) or severe (7 atm) lateral controlled cortical impact brain injury (by fluid percussion). Fourteen days after the injury, animals were randomized to receive stereotactic injection of NSCs or vehicle into the cortex-hippocampus interface in the ipsilateral hemisphere. One month after the transplantation, memory function was evaluated using an eight direction maze. Animals were sacrificed to examine the NSCs survival and neuronal differentiation by immunochemical staining.

*Results*: Mildly injured-animals showed significantly better improvements of memory function compared with severely injured-animals and vehicle treated animals. Histological analysis showed that NSCs survive in hippocampus and/or cortical areas adjacent to the injury cavity in mildly injured-animals. Transplanted NSCs in mildly injured animals expressed significant neuronal (Hu) or cholinergic (ChAT) or synaptic (synaptophysin) differentiation. Severely injured-animals rarely showed such differentiations.

*Conclusion*: These data suggest that transplanted NSCs can survive in the traumatically injured brain and differentiate into neurons with the improvement of memory function in mild traumatic brain injury.

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## THE USAGE OF KI-67 IMMUNOREACTIVITY IN STUDIES OF CELL PROLIFERATION AFTER TRAUMATIC BRAIN INJURY

*S Ekmark, A Lewén, L Hillered* (*Uppsala, Sweden*)

The recent discovery of the adult cerebral neuronal stem cell pool has opened up for possibilities of recruiting stem cells to brain regions suffering cell loss after traumatic brain injury (TBI). The methods used to study regulation of neuronal stem cells usually include 5-Bromo-2'-deoxyuridine (BrdU), which needs to be administered before visualisation, a caveat that limits its potential in studies of TBI. The usage of endogenous markers of cell division needs therefore to be explored. In this preliminary study we used BrdU in combination with the immunogen Ki-67 (or MIB-1) as markers of newly divided cells after a controlled cortical impact injury in mice. Immunohistochemistry was performed in five-micron thick coronal sections in formalin fixed paraffin embedded brain tissue. Animals were taken 24 h after severe injury. The expression of Ki-67 was also studied in animals taken 4h or 21 days after injury. Both four hours and 21 days after injury numerous Ki-67 positive cells appeared in the ipsilateral and contralateral thalamus. Few Ki-67 positive cells appeared in the perimeter of the cortical lesion at 4 h. BrdU and Ki-67 double labelling studies revealed that likely all BrdU labelled cells were Ki-67 positive at 24 h. A few Ki-67 positive cells were not BrdU positive, a discrepancy that may be explained by different appearances in the cell cycle. In summery, Ki-67 immunoreactivity may be useful in studies of regulation of cell division after TBI. Future studies need to address the identity of Ki-67 expressing cells.

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## NEURAL STEM CELL'S TRANSPLANTATION PROMOTES GAP-43 mRNA EXPRESSION, MOTOR AXONAL REGENERATION AND FUNCTIONAL RECOVERY FOLLOWING SPINAL CORD INJURY IN RATS

*L Li, W Hu, Z Fu, P Gu, Y Wu* (*Jiangsu, China*)

*Objective*. To investigate the effects of neural stem cell's transplantation on axonal regeneration and functional recovery after spinal cord injury in rats.

*Methods*. Forty-four rats were randomly divided into two groups: Group A transplanted with neural stem cells (NSC), Group B grafted with cell culture medium(CCM) following spinal cord contusion injury. Contusion injury to adult rat spinal cord was produced at T10 vertebra level. Neural stem cells were cultured from rat's embro (E-17), differentiated into neural progenitors and grafted into the injury site of spinal cord 7 days after contusion injury. The axonal regeneration and functional recovery was observed by using RT-PCR, immunohistochemical method and open field locomotion.

*Results*. An increase of 48.3 $\pm$ 2.3% in the expression of GAP-43 (growth associated protein) gene, an endogenous indicator of axonal regeneration, was demonstrated in the group A 24 h post injury. Ten weeks later, there were significant amount of ChAT positive motor axonal regeneration demonstrated in the group A compared to group B ($P < 0.05$). Furthermore, transplantation with NSC in the group A resulted in stimulation of hindlimb activity as well as improvement in the rate of functional recovery in open field locomotion ($P < 0.05$).

*Conclusions*. Transplantation of neural stem cells could improve functional recovery through mechanisms that include increasing GAP-43 mRNA expression and enhancing motor axonal regeneration at the injury site. Neural stem cells' transplantation may be an effective approach for treatment of spinal cord injury.