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GENOMIC CLUES TO SECONDARY INJURY MECHANISMS IN BRAIN TRAUMA

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“Something´s here I´m not quite getting
Though I try, I keep forgetting
Like a memory long since past
Here in an instant gone in a flash
What does it mean?
What does it mean?

In these little bric-a-brac a secret´s waiting to be cracked
These dolls and toys confuse me so
Confound it all, I love it though

Simple objects, nothing more
But something´s hidden through a door
Though I do not have the key
Something´s there I cannot see
What does it mean?
Hm…
…
It´s simple really, very clear
Like music drifting in the air
Invisible, but everywhere
Just because I cannot see it
Doesn´t mean I can´t believe it”
Jack’s Obsession’ / D. Elfman

ABSTRACT

Traumatic brain injury (TBI) is a heterogenous disease that can lead to persistent disability or death. The immediate mechanical disruption of brain tissue is followed by a phase of secondary brain injury. During this phase TBI challenges clinical interventions due to its complexity. Increased knowledge is needed about the operating molecules and their time-windows. We used cerebral cortical contusions by weight drop and experimental depolarisation, in order to analyse molecular and genetic responses. Alterations in gene expression were studied by microarrays, RT-PCR, *in situ* hybridisation and immunohistochemistry.

Nestin is an intermediate filament, expressed in CNS progenitor cells during embryogenesis, but restricted to residing stemcells in adults. Depolarisation induced nestin expression in astrocytes along the ipsilateral cortex. NMDA blockade by the MK-801 reduced nestin expression. The nestin expression in reactive astrocytes is interesting since they form the glial scar.

Matrix metalloproteinases (MMPs) and their inhibitors TIMPs, determine extracellular matrix turnover and cause overgrowth or disruption when disturbed. We found a time-dependent increase of neuronal TIMP-1 and MMP-9 expression in the ipsilateral cortex after depolarisation and contusion. The early expression of TIMP-1 could be a MMP-9 independent protective response to damage. TIMP-1 and MMP-9 are likely to participate in the tissue reorganisation or neuroprotection.

After experimental contusion early and delayed genomic responses were significantly different for genes involved in transcription, cell communication, cell proliferation, cell-death and metabolism. In the early phase genes involved in transcription and cell-death were prominent, while immune response and proteolysis dominated the delayed phase. Osteopontin and the CD44 receptor are involved in inflammation, and were locally upregulated at the impact site. Genes of the calcium signaling pathway i.a. hippocalcin and VILIP-1, were suppressed while genes of the complement and coagulation pathways were upregulated. Depolarisation and experimental contusion largely shared genomic responses. NMDA receptor blockade after depolarisation abolished the regulated genes to a larger extent than after contusion.

Genes for nestin, MMP-9, TIMP-1, osteopontin and CD44 were similarly regulated in both models. Almost all depolarisation induced effects were detected in traumatically injured animals, while some post-traumatic changes appeared to be independent of depolarisation. The findings corroborated that depolarisation was a mechanism in experimental trauma. Microarrays could be used to produce a comprehensive image of regulated genes at a specific time or to allow an unbiased search for relevant events.

Keywords: traumatic brain injury, cerebral cortical contusion, weight-drop injury, depolarisation, gene expression, nestin, MMP-9, TIMP-1, osteopontin, CD44, hippocalcin, NMDA receptor, microarray, rat

LIST OF PUBLICATIONS

I. Holmin S., **von Gertten C.**, Sandberg-Nordqvist A-C., Lendahl U., Mathiesen T.
”Induction of astrocytic nestin expression by depolarization.”
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II. **von Gertten C.**, Holmin S., Mathiesen T., Sandberg-Nordqvist A-C.
”Increases in matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 mRNA after cerebral contusion and depolarisation.”
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III. **von Gertten C.**, Flores Morales A., Holmin S., Mathiesen T., Sandberg-Nordqvist A-C.
“Genomic responses in rat cerebral cortex after traumatic brain injury.”
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IV. **von Gertten C.,** Lundberg J. Mathiesen T., Flores Morales A., Sandberg Nordqvist A-C.
”Depolarisation and traumatic brain injury in rat share genomic responses”
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LIST OF ABBREVIATIONS

AMPA	2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate
AP-1	activating protein -1
Apo E	Apolipoprotein E
ATP	adenosine triphosphate
BBB	blood brain barrier
Bcl	B-cell lymphoma
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CaM	Ca^{2+} / calmodulin / kinase
cAMP	adenosine 3-,5-cyclic monophosphate
CCC	cerebral cortical contusion
CCI	cortical contusion impact
CCK	cholecystokinin
cDNA	complementary DNA
CNS	central nervous system
CREB	cAMP response element binding
CSF	cerebrospinal fluid
Cy	cyanine
DAI	diffuse axonal injury
DNA	deoxyribonucleic acid
dpi	days post injury
EAE	experimental autoimmune encephalomyelitis
ECM	extra cellular matrix
EGF	epidermal growth factor
ER	endoplasmatic reticulum
ER	estrogen receptor
ERK	extra cellular regulated kinase
FDR	false discovery rate
GDNF	glial derived nerve growth factor
GFAP	glial fibrillary acidic protein
HIV	human immunodeficiency virus
ieg	immediate early gene
IF	intermediate filament
IGF	insulin like growth factor
IL	Interleukin
im	intra muscular
ip	intra peritoneal
ISH	*In situ* hybridisation
LFP	lateral fluid percussion
LPS	lipopolysaccharide
LRP	lipoprotein receptor-related protein
LTP	long term potentiation
MAPK	mitogen activating protein kinase
MFP	midline fluid percussion
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mRNA	messenger RNA
NeuN	neuronal nuclei
NFs	neurofilaments
NGF	nerve growth factor
NMDA/ R	N-methyl- D-aspartate/ receptor
NO	nitric oxide
NOS	nitric oxide synthase
NVP	neural visinin-like protein
Opn	osteopontin
PARS	poly ADP ribose
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
pi	post injury
PI3K	phospho-inositol-3 kinase
PID	peri-infarct depolarisation
PSD	post synaptic density
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
SD	spreading depression
TBI	traumatic brain injury
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumour necrosis factor
Trk/R	tyrosine kinase/receptor
VEGF	vascular endothelial growth factor
VILIP	visnin-like protein

1 INTRODUCTION

The brain controls our conscious and unconscious activities, like walking, talking, breathing and heart rate. It is also the centre for our thoughts, emotions and comprehension. Traumatic brain injury (TBI) can disrupt some or all of these functions and can result in lifelong disability or death. Therefore, in individuals surviving moderate or severe brain trauma neurological deficits, impaired memory and behavioural changes, such as personality changes, aggression and depression are common and consisting a large problem (Schalen, Hansson et al. 1994). Patients with mild traumatic brain injury, which in Sweden is the most common group (Tagliaferri, Compagnone et al. 2006), experience e.g. headache, fatigue and concentration difficulties (Ryan and Warden 2003; Kurca, Sivak et al. 2006; Stulemeijer, van der Werf et al. 2006). All of the post injury symptoms can contribute to a diminished quality of life. In Sweden 17524 cases were hospitalised in 2005 [The Swedish national board of health and welfare, personal communication]. The incidence of TBI requiring hospital care in Scandinavia is about 200 per 100 000 / year (Romner, Ingebrigtsen et al. 2000), and the numbers are approximately the same for the United States and Europe (Narayan, Michel et al. 2002; Tagliaferri, Compagnone et al. 2006). In well-developed countries TBI is the leading cause of death and disability in young adults (Maas 2001). The most common cause/s of brain injury vary between ages and countries, and include falls, traffic accidents, violence, and sports (Bruns and Hauser 2003). Violence is a more common cause in the United States, while car accidents are more frequent in Southern Europe. Falls are more common among elderly, and young men are more often involved in traffic accidents. Brain injury affects all ages, though is most frequent in men and young adults between 15 –24 years (Kraus 1993; Bruns and Hauser 2003). TBI results in a large individual suffering, similarly, for the surrounding family and friends, but it is also a great cost to society. Because trauma is common in young individuals, the TBI related loss of working years precedes cancer and cardiovascular diseases (Pontén U. 1995).

Fig 1. *Events occurring in brain trauma*

1.1 TRAUMATIC BRAIN INJURY - TBI

Brain injury is a complex event starting with an energy transfer and brain disruption, and followed by secondary processes and a potential risk of secondary complications (Reilly 2001). The heterogeneity of the disease can depend on several parameters such as genetics, age, sex, and health status of the individual as well as energy transfer, severity and impact site of the injury *per se*. Brain trauma consists of many events including depolarisation, haemorrhage, hypoxia, ischaemia and inflammation, which all

contribute to pathogenesis. The injury results in direct, irreversible cell death, due to the impact, and impending further tissue destruction, because of secondary events. The secondary phase consists of secondary damaging mechanisms and insults. Due to this complexity, trauma intervention to improve final outcome is challenging and today, trauma prevention is the best measure to fight brain injury.

Among the cells in the brain, neurons are well described as sensitive to ischaemic periods. Extended ischaemia will induce cellular changes in glia and blood vessels, which can lead to infarction where the whole tissue area is affected. Microglial cells are recognised as the prime components of the intrinsic brain immune systems. Astrocytes play an important role in homeostatic maintenance of extracellular environment and pH, clearance and release of extracellular glutamte, provision of metabolic substrates for neurons, coupling of blood flow to neuronal activity and possibly sculpting of the synapses (Sofroniew 2005). After CNS insults astrocytes become reactive with characteristics of cellular hypertrophy, changes in gene expression, and sometimes, astrocyte proliferation. Like many other processes and molecules the actions of the reactive astrocytes probably are both good and bad. They take part in wound healing though their particular role has not been defined.

Cells are surrounded by an extracellular matrix (ECM), which provides attachment, trophic factors, and information from other cells. In the brain, the matrix is mainly composed of hylauronan, proteoglycans, tenascin-C and thrombospondin (Bellail, Hunter et al. 2004). Furthermore, integrins and selectins are the anchors between filaments and the cell membrane. These factors are dynamically regulated during development and after CNS injury.

1.1.1 TBI primary injury

The primary injury can be of various forms originating from forces e.g. rotational acceleration, compression and distension resulting from acceleration or deceleration, and chafing towards the skull, all together affecting neurons, glial cells and blood vessels (Graham 1995). Traumatic brain injuries are parenchymal and/or vascular. Parenchymal injuries are contusions, lacerations or diffuse axonal injuries, whereas vascular injuries are epidural-, subdural-, subarachnoidal- or intracerebral haematomas (Graham 1995). The pathology, pathophysiology and clinical course are depending on the primary injury, which can be diffuse or focal (Marik, Varon et al. 2002). Diffuse injuries include cerebral concussion, traumatic subarachnoid haemorrhage and diffuse axonal injury (DAI), while contusions and traumatic haematomas are predominantly focal. Additionally, ischaemia-reperfusion is common and ischaemia may be focal or global, which also contributes the injury scenario.

1.1.2 Gender

There is a disagreement whether or not gender has an impact on brain trauma. Potential contributing factors are the sex hormones estrogen and progesterone (Stein 2001). Among severely head injured patients women experience significantly more intracranial hypertension and brain swelling, especially pre-menopausal women,

compared to males (Farin, Deutsch et al. 2003), while men are reported to suffer earlier lipid peroxidation than women (Bayir, Marion et al. 2004). Additionally in severe cases of TBI, there is a gender difference in glutamate and lactate/pyruvate production as reflected in the cerebrospinal fluid (CSF) (Wagner, Fabio et al. 2005). Furthermore, there is a controversy whether female patients recover better than men (Groswasser, Cohen et al. 1998; Davis, Douglas et al. 2006), or not (Coimbra, Hoyt et al. 2003; Davis, Douglas et al. 2006). More men than women are affected by TBI, hence it can be difficult to compose the appropriate groups for statistical testing. Another parameter seldom taken into account is that if hormones are important, the timing of brain injury with respect to the hormonal cycling for women should be noted (Stein 2001).

In experimental studies, female rats develop less oedema than male after an intracerebral haemorrhage and have an exacerbated oedema after treatment with an estrogen receptor (ER) antagonist, while administration of 17β-estradiol, an ER ligand, to male rats results in reduced brain oedema and neurological deficits, thereby indicating a gender dependent role for an ER mediated effect (Nakamura, Hua et al. 2005; Nakamura, Xi et al. 2006). Protection by estradiol in ischaemia is mediated by the estrogen-α receptor (Dubal, Rau et al. 2006), which expression is induced in glial cells after TBI in male rats (Garcia-Ovejero, Veiga et al. 2002). The beneficial effect could also be due to estradiol and the trophic factor insulin like growth factor (IGF)-I, and the interaction of their respective receptors, ER and IGF receptor, which could produce neuroprotection by activation of phospho-inositol-3 kinase (PI3K) and mitogen activating protein kinase (MAPK) signalling cascades (Azcoitia, Doncarlos et al. 2002). Furthermore, brain aromatases, which catalyse the biosynthesis of estrogens, are increased after brain injury and proposed to be protective (Azcoitia, Sierra et al. 2001). Other experimental findings are an earlier degradation of cytoskeletal proteins in male mice compared to female after TBI (Kupina, Detloff et al. 2003) and cell death differences between male and female organotypic hippocampal slice cultures subjected to injury (Li, Pin et al. 2005).

The possible impact of genes expressed in a more or less male-/female-dominant way could be worth consideration in future trauma research. This aspect has hardly been studied at all in trauma.

1.1.3 Genetics

Each individual has a unique set of genes. This can contribute to the way we respond to brain trauma or other sickness and disease. One gene implicated as a risk factor in TBI is the apolipoprotein (apo) E, which is a component of several plasma lipoproteins, with a role in transport and metabolism of cholesterol, triacylglycerols and phospholipids (Mahley and Rall 2000). A gene can come in alternative forms, called alleles. Carriers of the apoE ε4 allele are associated with poor outcome after brain trauma (Friedman, Froom et al. 1999). Positron emission tomography studies suggest that apoE4 individuals have an impaired cerebral glucose metabolism (Mielke, Zerres et al. 1998). ApoE4 is also a risk factor in Alzheimer's disease (Parker, Cathcart et al. 2005), which is interesting as brain trauma is a risk factor for neurodegenerative diseases (Lye and Shores 2000).

In experimental settings, the apoE4 and apoE3 proteins are related to among other things neurite outgrowth (Narita, Bu et al. 1997), facilitation of transcription of cAMP response element binding (CREB)-dependent genes, and cytoskeletal assembly (Huang, Liu et al. 2001). The neurotrophic and neuroprotective effects of apoE3 (Hashimoto, Jiang et al. 2000) and the neurotoxic effects of apoE4 (Tolar, Keller et al. 1999) are associated with the function of the apoE receptor, the low density lipoprotein receptor-related protein (LRP) (Qiu, Crutcher et al. 2003). The LRP directs apoE and another important protein α2-macroglobulin, which is a proteinase inhibitor, to degradation. Furthermore, LRP regulates the permeability of the blood brain barrier (BBB) (Wang, Lee et al. 2003; Yepes, Sandkvist et al. 2003). Chronic apoE4 treatment of neurons increases intracellular Ca^{2+} levels via the glutamate N-methyl-D-aspartate receptor (NMDAR) (Qiu, Crutcher et al. 2003). Except for the receptor mediated effect, toxicity could be due to an effect on lipid metabolism or structural changes in the receptor. The effects of apoE4 can be prevented by activated α2-macroglobulin, which can down-regulate the NMDAR (Qiu, Strickland et al. 2002; Qiu, Crutcher et al. 2003).

Most experimental reports on TBI use animals, of a single sex, and with little variation in their genetic composition to reduce the number of possible confounding parameters (Faden 2002). However, inflammation after experimental TBI performed with different animal strains shows a heterogeneous response, where increased inflammation is seen in the strain susceptible of experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis model, compared to the Dark Agouti and Piebald Virol Glaxo strains (Bellander 2004).

1.1.4 Treatment

Although many neuroprotective treatments have shown efficacy in animal models of brain injury, this has unfortunately not held true for clinical human trauma, where a well documented pharmaceutical therapy is still lacking (Fawcett 2001; Morales, Marklund et al. 2005). At present, treatment after acute insults to the central nervous system (CNS) is still largely symptomatic therapy and avoidance of secondary insults or surgery for space occupying lesions, followed by physiotherapy and rehabilitation. However, mortality from brain injury has steadily declined during the period 1984 through 1996 (Lu, Marmarou et al. 2005). Improved acute management, stabilisation of vital parameters, as well as improved in-hospital care have increased survival rates after TBI (Elf, Nilsson et al. 2002). Neurointensive care nowadays employs extensive monitoring of blood pressure, oxygenation, intracerebral pressure, microdialysis, jugular bulb oxymetry and EEG, in order to avoid secondary insults (Marmarou, Fatouros et al. 1990; Elf, Nilsson et al. 2002; Rudehill, Bellander et al. 2002). Other factors that have contributed to decreased mortality/ and disability after TBI are the preventive measures such as improved safety in cars and helmet usage.

Presently there are phase III clinical trials ongoing for free radical scavenging by NXY-079 and drugs affecting the brain erythropoietin system (Hasselblatt, Ehrenreich et al. 2006). A potential future therapeutic has emerged with stem cells (Altman and Das 1965), where research now focuses to understand and regulate the cells in hope for a

future treatment alternative (Picard-Riera, Nait-Oumesmar et al. 2004; Westerlund, Svensson et al. 2005; Wennersten, Holmin et al. 2006).

But how come clinical trails have failed? Most reviewers of these studies agree that evaluation of the trials was difficult or even impossible due to faulty trial design (Faden 2002; Narayan, Michel et al. 2002). There are several explanations: injury in humans may be variable and affect different parts of the brain and is therefore quite unpredictable at admission, resulting in patients with highly variable outcomes/disabilities. Therefore, trials have often included diverse injuries with different injury mechanisms and expected outcomes. In order for a drug to show efficacy it would need to have a major or broad effect in all different kinds of trauma. This can be compared to experimental models, where the settings are more easily controlled. Furthermore, appropriate control groups can be difficult to select. Disregarding the explanation of non-optimal set-ups, other contributing factors are the differences between animals and humans, drug concentration and timing of administration (Povlishock 1995; Fawcett 2001). First, dissimilarities between the rodent and human brain lie in the size, white-to-grey matter ratio, and lissencephalic, without convoluted cortex, versus gyrencephalic, with a convoluted cortex. One focus of neuroprotection in focal injuries is to save neurons in the peri-lesional zone where cells in the vicinity of the damage are affected but not immediately killed. The peri-lesional zone may be of the same size in both rodents and humans. This would mean that cell rescue would have a larger effect in rodents than in humans. Secondly, in humans it can be necessary to decrease dosage to avoid problems with side effects e.g. psyschomimetic and cardiovascular. Third, the drug is often delivered before or immediately after injury in the experimental setting, but possibly too late in humans. Finally, drug delivery can also pose a problem; it has to pass the BBB if not administered directly into the brain.

1.2 SECONDARY INJURY MECHANISMS

The primary insult initiates complex biochemical and physiological processes that last over a time period from hours to days. These processes are known as the secondary injury mechanisms and they also make the brain more vulnerable to secondary insults, which in their turn can enhance existing, as well as initiate new, secondary processes. The mechanisms include excitotoxicity, high intracellular calcium levels, free radical generation and lipid peroxidation, and inflammation. The severity of the primary injury as well as its location will affect the severity of the secondary injury mechanisms (Bramlett and Dietrich 2004), possibly mirrored by more induced genes after a severe experimental brain trauma than a moderate injury (Li, Lee et al. 2004).

Among the patients with mild traumatic head injury lacking clinical signs and symptoms initially, some will later on develop serious intracranial complications, which require neurosurgical interventions (Reilly, Graham et al. 1975). These “talk and die” or “talk and deteriorate” patients, who were first described by Reilly in 1975, illustrate some of the operating secondary injury mechanisms. The existence of such progressing injurious events is corroborated by detection of cell death, as well as grey and white matter disturbances up to one year after experimental brain trauma (Smith,

Chen et al. 1997; Pierce, Smith et al. 1998; Bramlett and Dietrich 2002). Clinically, damage progression was observed after TBI by magnetic resonance imaging (Anderson and Bigler 1995). This suggests that there is a time-window after brain trauma, which potentially can be used for therapeutic interventions.

It should, however, be underlined that mechanisms of repair and regeneration also operate during the time period referred to as "secondary brain damage". Several growth factors (like BDNF, NGF, IGFs, bFGF, and GDNF) are upregulated both on expression and protein level. They support cell growth and can be neuroprotective; likewise their tyrosine kinase (Trk) receptors are increased (Oyesiku, Evans et al. 1999). Neurogenesis following CNS injury has been reported in several studies (Rice, Khaldi et al. 2003; Itoh, Satou et al. 2005) and grafted stem / progenitor cells can survive after brain injury (Ikeda, Kurokawa et al. 2005; Wennersten, Holmin et al. 2006). Furthermore, inflammatory factors display dual roles in damaging and beneficial actions after the injury (Lenzlinger, Morganti-Kossmann et al. 2001). The latter will probably also hold true for some of the factors already described as damaging, depending on the time frame and environment evaluated.

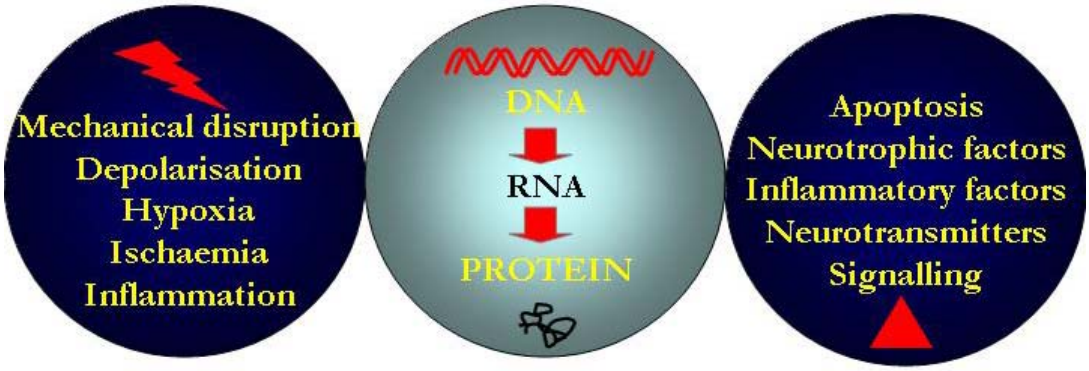

Fig 2. *Primary injury induces gene expression alterations. These will trigger regulation of several factors.*

1.2.1 Excitotoxicity

CNS injury results in neuronal depolarisation and a massive release of excitatory amino acids. Glutamate is the principal excitatory neurotransmittor in the CNS, but also a potent neurotoxin (Gibbons, Brorson et al. 1993). Increased glutamate levels after injury were found in rodents (Benveniste, Drejer et al. 1984), and in humans (Baker, Moulton et al. 1993). In trauma, the excessive amounts of glutamate can originate from damaged leaking cells or a disrupted BBB. The interstitial glutamate concentration is normally lower than in blood or inside the cells. Though glutamate signalling is vital (Hetman and Kharebava 2006), overactivation of glutamate receptors initiates cell demise, which is termed excitotoxicity. Glutamate as a neurotoxin was first described in the late 1950´s (Lucas and Newhouse 1957) and excitotoxic cell death was thereafter found to occur in almost all neurons with glutamate receptors (Olney 1969). Glutamate

signalling is carried out by transporters and two kinds of receptors; the fast acting ionotropic and the slower metabotropic receptors. The main responsible receptor for excitotoxicity is the NMDAR. At overactivation, massive influx of Ca^{2+} occurs together with internal Ca^{2+} release. When energy stores are depleted, membrane integrity is lost, leading to ion and water influx, which results in cell swelling and cytotoxic oedema. This will lead to further release of glutamate. In addition to injuring nerve cells, glutamate is also toxic to glial cells, including astrocytes (Haas and Erdo 1991) and oligodendroglia (Yoshioka, Hardy et al. 1995; Matute, Sanchez-Gomez et al. 1997). Astrocytes are better suited to cope with glutamate since they possess a certain buffering capacity and are involved in clearance of glutamate from the extracellular space (Chen and Swanson 2003). However, at energy depletion during ischaemia, the glutamate uptake system can be impaired or even reversed (Chen and Swanson 2003). Besides the NMDARs glutamate activates 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) and kainate receptors, which also contribute to the scenario.

In summary, the toxic effects of glutamate are energy depletion, cell-swelling and increased intracellular Ca^{2+}, which in turn will lead to further damage.

1.2.1.1 NMDA receptors

Targeting the NMDARs with antagonists thus seemed appropriate; however, the clinical trials failed (Narayan, Michel et al. 2002). Despite beneficial effect of NMDAR blockers in experimental systems (Faden, Demediuk et al. 1989; Sun and Faden 1995), others contradicted the efficacy (Ikonomidou, Stefovska et al. 2000; Biegon, Fry et al. 2004). The reason for the discrepancy can be the lack of a full understanding of the NMDARs despite extensive studies.

The receptor has binding sites for the ligand, magnesium (Mg^{2+}), and H^+, and needs a membrane depolarisation to free the $Mg^{2+}$and activate the receptor in addition to ligand binding (Fawcett 2001). Several subunits compose the iontropic NMDARs including NR1, NR2A-D and NR3A-C (Cull-Candy, Brickley et al. 2001). The NR1 subunit is important for pore formation, Ca^{2+} permeability, voltage dependent Mg^{2+} block, control of gating properties effector site of polyamines and affinity to glutamate and glycine. The NR2 contributes to sensitivity to Mg^{2+} block, affinity for glycine and glutamate and single channel conductance. Furthermore, both the NR2 and the NR1 link to the cytoskeleton, where neurofilaments (NFs) participate in axon transport by crossbridging to each other and to actin filaments as well as other molecules. At over-activation of NMDARs, a rapid cytoskeletal alteration occurs and axonal degradation follows (Chung, McCormack et al. 2005). NR1 interacts with neurofilament L as well as α-actinin, an actin binding protein, which may serve to anchor the receptor (Ehlers, Fung et al. 1998). Antagonizing the binding of α-actinin, the Ca^{2+} / calmodulin (CaM) affects the channel opening time (Rycroft and Gibb 2004), and triggers a signalling pathway to induce CREB-dependent gene transcription through CaM kinase (CaMK) II (Robison, Bartlett et al. 2005), which can regulate nitric oxide synthase (NOS) activity (Hayashi, Nishio et al. 1999). The CaM also interacts with the phosphatase calcineurin (Ye, Li et al. 2006), controlling the on and off switch by regulating protein phosphorylation and de-phosphorylation. The NR2 is suggested to link to downstream mediators by interaction with the cytoskeleton and connecting protein e.g. the

cytoskeletal post-synaptic density (PSD) proteins. PSD95 links among other things NMDARs with neuronal NOS production (Brenman, Chao et al. 1996). Interference of the PSD95–NMDAR interaction in transient focal ischaemia reduces cerebral infarction and improves neurological function (Aarts, Liu et al. 2002). While targeting the NMDA receptor in order to avoid Ca^{2+} influx did not produce the expected result, but rather pointed to its diverse roles, associating proteins are now prospective treatment targets in order to interfere with downstream injurious cascades (Arundine and Tymianski 2004).

Another aspect of NMDARs is the potential of an altered subunit composition. The subunit composition may differ between normal development, plasticity and aging, as well as after CNS injury (Myers, Churchill et al. 2000; Ontl, Xing et al. 2004). The neonatal brain has a high level of NR2B, while the NR2A expression increases during development. With age, decrease of both NR2B mRNA and NR2B protein levels (Clayton and Browning 2001; Magnusson, Nelson et al. 2002). This decrease may contribute to deficits in long term potentiation (LTP) and learning/memory (Tang, Shimizu et al. 1999). This is corroborated by results in genetically altered animals. NR2B knock-out (KO) mice display impaired NMDARs (Clayton, Meschesh et al. 2002), and NR2B overexpression enhances learning and memory (Tang, Shimizu et al. 1999). Interestingly, the NR2A: NR2B ratio regulates glutamate sensitivity and pore opening. A NR1/NR2B receptor lacks the Ca^{2+}-dependent inactivation, which NR1/NR2A possesses (Yamakura and Shimoji 1999). Subsequently, a low NR2A: NR2B ratio (i.e. less NR2A than NR2B) leads to increased sensitivity to glutamate and longer pore-opening. Functional and compositional changes in NMDARs occur as a consequence of experimental TBI (Kumar, Zou et al. 2002; Osteen, Giza et al. 2004). Accordingly, transient global ischaemia decrease the NR2A/NR2B subunit levels and alters receptor function (Zhang, Hsu et al. 1997). After CCI, NR1/ NR2A/ NR2B proteins decrease without a corresponding change in mRNA levels. This is suggested to be due to calpain degradation (Kumar, Zou et al. 2002). An increased susceptibility to toxicity could be attributable to an altered subunit composition. In lateral fluid percussion (LFP), the downregulation of NR2 subunits is suggested to be involved in pathophysiological cascades, even in regions without overt cell death (Osteen, Giza et al. 2004). The decrease in NMDARs can be a way for cells to protect themselves from excitotoxic damage. The NR2A subunits are decreased to a larger extent than NR2B, which could mean that NMDARs are sensitised and open for longer periods, thereby contributing to posttraumatic accumulation of Ca^{2+}. High NR2B levels during development are a probable adaptation to allow plasticity. Following trauma a decreased NR2A:NR2B ratio could also improve neuroplasticity, although with post-traumatic conditions, contribution to damage is probable.

Another possibility is that spatial localisation, not composition, is the important factor (Ivanov, Pellegrino et al. 2006). Signalling via the extra cellular regulated kinases (ERK) pathway by NMDARs will either activate ERK or promote inactivation, depending on whether the receptors are synaptically or extrasynaptically located (Ivanov, Pellegrino et al. 2006). Nevertheless, ERK activation is still dependent on the NR2B subunit (Krapivinsky, Krapivinsky et al. 2003). NMDARs do not only create havoc as they also mediate physiologically necessary reactions. The survival signalling pathways include ERK, PI3/Akt signalling, the role of CaM dependent kinases, trophic factors and glycogen synthase kinase 3β (Hetman and Kharebava 2006).

1.2.2 Calcium

Ca^{2+} homeostasis is crucial in cells. The increased levels after TBI will initiate several processes, as Ca^{2+} is a second messenger amplifying and transducing the signal triggered at a receptor. Ca^{2+} concentration fluxes depend on the nature of the mechanical insult. In a stretch model where only uniaxial stretch is applied to neurons the intracellular Ca^{2+} levels rise immediately. Though the magnitude of Ca^{2+} increase is much higher if the stretch is applied in two directions (biaxial) (Geddes-Klein, Schiffman et al. 2006). Various channels antagonists can block the Ca^{2+} increase caused by uniaxial stretch, while the biaxially stretched neurons still have a transiently increased intracellular Ca^{2+} level. Furthermore, the uniaxially but not the biaxially stretched neurons exhibit an enhanced Ca^{2+} influx at 24 hours after stimulation with NMDA (Geddes-Klein, Schiffman et al. 2006). These data indicate that the nature of the impact is important for the tissue response.

Another vital parameter is the entranceway of Ca^{2+}. During normal conditions, and neurotoxicity, the activation of distinct biochemical signalling pathways is dependent on the route of Ca^{2+} entry and its intracellular localisation. This 'source specificity' hypothesis of Ca^{2+} neurotoxicity emerged since similar Ca^{2+} loads were toxic or non-toxic depending on whether Ca^{2+} entered through NMDARs or L-type voltage sensitive channels (Tymianski, Charlton et al. 1993). The hypothesis proposed that enzymes or substrates involved in the toxicity are directly associated to NMDARs. Additionally, increased intracellular Ca^{2+} triggers further release from internal Ca^{2+} stores such as the endoplasmatic reticulum. High Ca^{2+} levels lead to Ca^{2+} accumulation in the mitochondrial matrix. Mitochondria have the ability to sequester Ca^{2+} via an electrochemical gradient generated by the electron transport chain. Influx of Ca^{2+} will however, decrease the electrochemical gradient and thereby reduce ATP synthesis. The impaired electron chain can also lead to an excessive reactive oxygen species (ROS) production. At the same time there is an increased need of ATP to extrude Ca^{2+} through plasma membrane pumps. Altogether, Ca^{2+} accumulation, a decreased ATP production and an increased need of ATP, are suggested to contribute to cell death (Schinder, Olson et al. 1996; Robertson 2004).

Ca^{2+} can alter gene expression by activating the pathways for transcription factors, like CREB (Bito and Takemoto-Kimura 2003) and the immediate early gene c-fos. Transcription factors in their turn activate several other target genes that contain appropiate binding sites. One way of controlling gene expression is through the mediator CaM kinase IV (Redmond, Kashani et al. 2002Yano, 2005 #537).

1.2.2.1 Calcium activated enzymes

Calcium will activate enzymes including lipases, kinases, phosphatases and proteases (Kampfl, Posmantur et al. 1997). Calpains are intracellular proteases, which degrade neuronal structural proteins. Their overactivation plays a major role in the neurodegenerative cascade following experimental TBI *in vivo* (Kampfl, Posmantur et al. 1997). They are involved in neuronal cell damage and death (Ray, Karmakar et al. 2006), and impaired neurobiological functions following experimental brain injury (Povlishock, Buki et al. 1999). Calpains can have profound effects on the neuronal structure and function, as they are located throughout the neuron. They also have a role

in neuronal differentiation (Hirai, Kawasaki et al. 1991), in normal turnover of cytoskeletal proteins, and LTP.

While a certain proteolysis of cytoskeletel proteins is a housekeeping function in the CNS, increased proteolysis can result in a disturbed function and structure of the neurons. The major components in the neuronal cytoskeletons are microtubules, microfilaments and intermediate filaments (IFs). Microtubules transport intracellular proteins and organelles by an ATP-dependent mechanism, and microfilaments take part in growth cone motility, endo- and excocytosis, synaptic vesicle release and adhesion of various microfilaments lattice to plasma membrane. Neurofilaments are the primary IFs, found in dendrites and axons, in the neuronal cytoarchitecture. Loss or disturbances will lead to impaired axonal transport, and structural integrity. Cell death ensues. Calpain inhibitors reduce cytoskeletal loss and contusion volume after experimental cortical impact (Posmantur, Kampfl et al. 1997) and attenuate motor and cognitive deficits after TBI (Saatman, Murai et al. 1996). They may also prolong the timewindow for saving neurons (Buki, Farkas et al. 2003).

Ca^{2+} activated phospholipases participate in the posttraumatic scenario. Phopsholipase C increases the second messenger diacylglycerol (DAG). Finally inositol trisphosphate ($IP_{3)}$ (Dhillon, Carman et al. 1999) can trigger additional Ca^{2+} release and phospholipase A2 can activate turnover of arachidonic acid, which is a precursor for several active metabolites.

1.2.2.2 Calcium sensor proteins

Ca^{2+} binding proteins that participate in the Ca^{2+} homeostasis are divided into buffering and sensor proteins. The nervous tissue contains visinin-like proteins (VILIPs, also called neural visinin-like proteins (NVPs)). The VILIPs include the family members VILIP-1, -2, -3, hippocalcin and the neurocalcins (Braunewell, Riederer et al. 2001). The Ca^{2+} sensor proteins can change their conformation due to Ca^{2+} interaction and play a role in signal transduction inside the cell, but however, their exact functions are not yet understood. Ca^{2+} homeostasis is disturbed in aging, where decreased levels of VILIP-2 and hippocalcin are seen (Furuta, Kobayashi et al. 1999) and neurodegenerative diseases. In Alzheimer's disease decreased protein levels of VILIP -1 and -3 were found (Braunewell, Riederer et al. 2001; Schnurra, Bernstein et al. 2001). Hippocalcin, exclusively expressed in neurons, can interact with neuronal apoptosis inhibitor proteins and induce phospholipase D2 via ERK activation (Oh, Yon et al. 2006). Moreover hippocalcin is neuroprotective. Hippocalcin deficient neurons show a reduced viability and hippocalcin provides protection from neuronal death (Korhonen, Hansson et al. 2005). Hippocalcin is suggested to have a crucial role in Ca^{2+} homeostasis as hippocalcin deficient mice have a defective CREB protein activation with significantly attenuated CREB phosphorylation, associated with impairment (Kobayashi, Masaki et al. 2005). VILIP can bind to RNA, specifically the trkB neurotrophin receptor (Mathisen, Johnson et al. 1999) and VILIP-3 interact with microsomal cytochrome b5, which is an activator of cytochrome P450 (Oikawa, Kimura et al. 2004). Taken together these studies and the influential role of Ca^{2+} in TBI, make Ca^{2+} sensor proteins an attractive target for therapeutic manipulations.

1.2.3 Free radicals

The increased Ca^{2+} levels trigger activation of several enzymes involved in production of free radicals. This group of molecules possess one or more unpaired electrons, and are highly prone to react with other molecules. Under normal conditions reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in low amounts e.g. in the mitochondrial oxidative metabolism. In trauma vast amounts of free radicals are produced by the enzymes nitric oxide synthase (NOS), phospholipase, and xanthine oxidase. Ca^{2+} independent routes of activation include disruption of the mitochondrial electron transport chain and acidosis; a low pH allows iron release from transferrin and ferritin. Increased levels of NO-synthase have been detected in human contusion, and after experimental brain injury (Clark, Kochanek et al. 1996; Gahm, Holmin et al. 2000). Free radicals increase microvascular permeability as well as destabilise cell membranes via lipid peroxidation (Mathew, Bullock et al. 1996). DNA damage caused by free radicals activates poly ADP ribose, PARS, which is energy-dependent and can deplete the cellular energy reserves after prolonged activity.

1.2.4 Inflammation

It is now generally accepted that CNS has an immune surveillance and can induce a potent immune response. Traumatically induced BBB breakdown disrupts the barrier that normally separates blood from interstitial fluid and parenchyma. Water and solubles can freely enter the brain, which leads to a vasogenic oedema. A cytotoxic oedema, or cell swelling, also occurs due to an altered environment or stress to the cells. In experimental models of brain trauma, disruption of the BBB is often transient and the BBB reconstitutes after a few days. Chemotaxis, diapedesis, and BBB breakdown opens new routes into the brain. Lymphocytes infiltrate from the circulation to remove debris after injury and in concert with neurons and glial cells, they secrete pro and anti-inflammatory cytokines. After experimental and clinical TBI, the pro-inflammatory cytokines interleukin (IL)-1, -6, and TNF-α increase (Holmin, Mathiesen et al. 1995; Kossmann, Hans et al. 1996; Holmin, Schalling et al. 1997; Hans, Kossmann et al. 1999). TNF-α is believed to trigger the initial inflammatory response and elicits the production of other cytokines and adhesion molecules (Lenzlinger, Morganti-Kossmann et al. 2001). TNF-α can worsen brain injury and by changing the cytoskeleton of endothelial cells cause leakiness, but it can also exert a neuroprotective role together with IL-1β, to increase NGF expression. Furthermore, mice lacking the TNF-receptor are more vulnerable to TBI (Sullivan, Bruce-Keller et al. 1999). TNF-α deficient mice exhibit attenuated cognitive deficits and motor dysfunction in an acute phase compared to the wildtype (WT), although the latter recover with time while the mice without TNF show prolonged motor deficits (Scherbel, Raghupathi et al. 1999). This argues for important roles of TNF-α in the acute stage of inflammation, and beneficial later on in regeneration and/or repair. Similarly to TNF-α, IL-1β is involved in the acute phase and increased endothelial permeability can enhance oedema (Holmin and Mathiesen 2000). IL-1β is involved in brain ischaemia, excitotoxicity, LPS and TBI. Blocking the IL-1β receptor is neuroprotective (Loddick and Rothwell 1996).

Moreover, ‘neuroinflammation’ includes the earlier phenomenon ‘reactive gliosis’, which describes the endogenous tissue response to CNS injury (Holmin, Mathiesen et

al. 1995; Holmin, Soderlund et al. 1998; Streit, Mrak et al. 2004). Macrophages and microglial cells initiate and modulate the immune response and these "activated microglia" and re-activated astrocytes secrete chemokines and cytokines; they contribute to the inflammatory phase which includes the repair responses induced simultaneously. Activated microglia in the peri-lesional zone help to clear the injury area and express complement cascade members after trauma in both human and rat (Bellander, von Holst et al. 1996; Bellander, Singhrao et al. 2001). In the perilesional zone microglia/macrophages express C3. Except for its role in inflammation, it participates in tissue regeneration (Kimura, Madhavan et al. 2003), neurogenesis (Rahpeymai, Hietala et al. 2006) and neuroprotection (Heese, Hock et al. 1998; Mukherjee and Pasinetti 2001).

Metallothionien (MT) expression, induced by IL-6, participates in protective actions with the antioxidant properties. In IL-6 KO mice, cortical freeze injury resulted in reduced MT I and II levels together with fewer activated glial cells and macrophages and a greater loss of neurons than in WT (Penkowa, Moos et al. 1999). Animals with deficient MT I and II expression show prolonged inflammatory responses, with increased cell death and neuronal loss accompanied by reduced wound healing and tissue regeneration (Penkowa, Carrasco et al. 1999; Penkowa, Carrasco et al. 2000).

Brain trauma confers a risk of developing neurodegenerative diseases later in life. After injury, the β-amyloid precursor protein, which is involved in Alzheimer´s disease, is increased. This could potentially be connected with an immune response, which transforms from acute into chronic inflammation (Holmin and Mathiesen 1999).

1.2.5 Cell death

Disruption of brain tissue leads to acute, as well as delayed traumatically induced cell death (Conti, Raghupathi et al. 1998; Newcomb, Zhao et al. 1999), which may last for up to one year after experimental trauma (Smith, Chen et al. 1997). The acute cell death is primarily necrotic (Dietrich, Alonso et al. 1994). Cells burst due to water influx when the cell no longer can retain cell membrane integrity, and spill out their contents (Dietrich, Alonso et al. 1994). Necrotic cell death is described as energy independent and characterised by swelling of organelles and nucleus. However, depending on cellular stress from growth factor withdrawal, DNA damage and free radical production (Fawcett 2001), brain trauma also causes cell death by apoptosis (Newcomb, Zhao et al. 1999). Apoptosis is characterised by internucleosomal DNA fragmentation, nuclear shrinkage, chromatin compactation, cytoplasmic condensation and disintegration. In later stages of apoptosis, the cell surface membrane blebs and breaks down into apoptotic cell bodies, which are phagocytised (Kerr, Wyllie et al. 1972). In this way, apoptosis avoids to elicit an inflammatory response.

Apoptosis proceeds either through caspase dependent pathways; the extrinsic and intrinsic pathway, or a caspase independent pathway. In the extrinsic pathway to cell death, a signal is transmitted through death receptors, such as Fas/CD95, when the ligand, FasL, binds. The following caspase-8 activation will trigger further activation of downstream caspases. In the intrinsic pathway, cellular stress leads to a disrupted pro-

and antiapoptotic protein ratio (Raghupathi, Conti et al. 2002; Raghupathi, Strauss et al. 2003), such as Bcl-2 and Bax (Wennersten, Holmin et al. 2003; Pfister, Oyler et al. 2004). Subsequently, mitochondria release cytochrome C that will bind to the apoptotic-protease-activating-factor-(Apaf)-1 and trigger activity of caspase -9 followed by additional caspases. Both pathways lead to a proteolytic caspase cascade, which mediates apoptosis.

1.3 DEPOLARISATION AND SPREADING DEPRESSION

Spreading depression (SD), first described by Leão in 1944, is a self-propagating wave of depolarisation associated with a slowly spreading suppression of electroencephalographic activity in the cortex, lasting for some minutes (Leao 1944). There are different events of depolarisation: traumatic SD in the peri-lesional areas following brain trauma (Strong, Fabricius et al. 2002), peri-infarct depolarisations (PID) in connection with the penumbra zone of ischaemia (Nallet, MacKenzie et al. 1999), and anoxic depolarisations (AD) in focal stroke (Anderson, Jarvis et al. 2005). Moreover it can be associated with migraine, epileptic seizures, and transient global amnesia (Gorji 2001). SD has also been proposed to cause the posttraumatic headache, which is one symptom following TBI.

While no exact mechanism of initiation is known, Ca^{2+}, glutamate and above all perhaps, K^+ concentrations are suggested to be important, whereas conduction or generation of action potentials and chemical synaptic transmission are not required (Balestrino 1995; Basarsky, Duffy et al. 1998). Likewise, the latter are not required for propagation. Spreading of the wave is, however, dependent on NMDARs, and SD can be blocked with the NMDAR antagonist MK-801 (Petzold, Windmuller et al. 2005) and ketamin (Gorelova, Koroleva et al. 1987). Interestingly, the inhibition does not occur when high levels of extracelllular K^+ are present in vivo (Petzold, Windmuller et al. 2005), and similarly antagonists fail to block NMDA receptors in brain slices when metabolic stress is high (Obeidat, Jarvis et al. 2000; Jarvis, Anderson et al. 2001). It is therefore suggested that a metabolic stress gradient dictates the glutamate receptor antagonist efficacy (Church and Andrew 2005). As the NMDARs are sensitive to extracellular H^+, acidosis exerts a negative regulation of SD (Tong and Chesler 2000) and magnesium, which also regulates the NMDARs, inhibits the SD threshold when present at increased levels (van der Hel, van den Bergh et al. 1998). During the early phase of focal ischaemia a high K^+ concentration in the ischaemic core and subsequent K^+ diffusion into the surrounding tissue can trigger SD to propagate from the rim of the penumbral zone (Siesjo and Bengtsson 1989). Likewise, the peri-lesional area in brain trauma often has a graded perfusion of blood flow creating partial ischaemia.

The astrocyte involvement in SD has been debated. However, gap junctions allow slowly moving Ca^{2+} waves that take part in Ca^{2+} signalling, which are similar to the spread of depolarisation (Martins-Ferreira, Nedergaard et al. 2000). SD is associated with increased intracellular and decreased extracelllular Ca^{2+} concentration that are partly caused by the astrocytes (Basarsky, Duffy et al. 1998). In addition, gap junction blockers inhibit depolarisation (Martins-Ferreira, Nedergaard et al. 2000) and the absence of the gap junction protein connexin 43 in astrocytes causes an accelerated SD

propagation (Theis, Jauch et al. 2003). The latter seemingly contradictory finding actually corroborates that gap junctions are relevant for SD. Waves of SD contribute to glutamate release (Basarsky, Feighan et al. 1999), and recent data propose that astrocytic glutamate release, and possibly neuronal, take part in events preceding SD-like depolarisation (Larrosa, Pastor et al. 2006). SD has an effect on regional cerebral blood flow, pial vessel reactivity, release of neurotransmitters and gene expression. The phenomenon links metabolic and ionic brain disturbances with changes in local blood flow, vascular reactivity and long term alterations in function associated to gene expression (Parsons 1998). In this way the depolarisation induces a variety of genes and proteins in areas away from infarct (Kariko, Harris et al. 1998). In normal brain tissue, a depolarisation does not cause morphological or metabolic damage (Nedergaard and Hansen 1988), but the brain tissue is not normal under pathological conditions. SD-like events can exacerbate posttraumatic damage (Church and Andrew 2005; Fabricius, Fuhr et al. 2006) and peri-infarct depolarisations can promote neuronal damage in focal stroke (Dijkhuizen, Beekwilder et al. 1999; Hartings, Rolli et al. 2003). The already compromised metabolism in neurons can probably not cope with SD that repeatedly disrupt the ion balances, activates NMDARs and cause Ca^{2+} fluxes. Therefore, subsequent cell death cascades will be initiated. Even more intriguing, depolarisation is involved in protection of brain tissue (Kobayashi, Harris et al. 1995; Chazot, Godukhin et al.), if elicited before injury. This phenomenon is similar to ischemic preconditioning where a short ischaemic episode prior lethal ischaemia confers protection (Yin, Zhang et al. 2005).

Depolarisation is a proposed mechanism of posttraumatic gene-regulation, since it occurs in TBI. The following genes are regulated in both SD and TBI: the ieg c-fos, MMP-9, connexin 43, BDNF, and bFGF (Hermann, Mies et al. 1999Gursoy-Ozdemir, 2004 #333; Kawahara, Ruetzler et al. 1999; Truettner, Schmidt-Kastner et al. 1999). SD is also proposed as a mechanism of microglial activation (Kato and Walz 2000). In addition, cortical SD modifies the interleukines and proinflammatory cytokines (Jander, Schroeter et al. 2001; Thompson and Hakim 2005). Ischaemic preconditioning involves the MAP kinase pathway (Yin, Zhang et al. 2005), which was proposed to mediate preconditioning in transient activation by CSD as well (Chow, Thompson et al. 2002).

1.4 EXPERIMENTAL *IN VIVO* MODELS

The brain consists of lobes, each with control of particular functions and skills. The left hemisphere is usually dominant and responsible for verbal functions while the right hemisphere is involved in visual-spatial functions. Focal contusions frequently affect cortical areas, most commonly the frontal and temporal lobes. The location of injury largely determines functional deficits: damage to the prefrontal cortex may cause motivational deficits, posterior frontal damage leads to pareses and damage to the dominant temporal lobe language deficits and memory impairments. In experimental models, the craniotomy position is critical for the extent and location of tissue injury (Vink, Mullins et al. 2001). To measure outcome in experimental injury models, morphological and behavioural data are used.

1.4.1 Traumatic brain injuries

Several experimental models exist to mimic trauma. In order to obtain reliable data these are standardised to create identified injuries when replicated (Povlishock 1995). Models provide important information about certain pathological components or phases of a clinical trauma, though no experimental model can completely replicate the heterogeneity of a 'true' trauma. In fact, the existing multitude of models may even be seen as a reflection of the heterogeneous nature of trauma. Experimental TBI models are more easily graspable than the clinical occurrence of several uncontrollable contributing events. Since many years ago TBI research is mostly performed in rodents, due to ethical, technical and financial aspects (Cernak 2005) and has provided important information about TBI. However, it should be remembered that a rat is not a human.

Experimental models can be classified according to whether a static or an indirect/direct dynamic mechanical force is applied (Cernak 2005). An explosion would cause an indirect dynamic brain injury, while direct brain injuries are either made by impact or non-impact acceleration. The head can be constrained or freely moving. Parameters can be varied to produce mild, moderate or severe experimental trauma. In this thesis, a dynamic direct brain injury was used. The resulting brain deformation caused a cerebral cortical contusion (CCC). The weight drop model (Feeney, Boyeson et al. 1981) together with midline fluid percussion (MFP) (Dixon, Lyeth et al. 1987; McIntosh, Noble et al. 1987), controlled cortical impact (CCI) (Dixon, Clifton et al. 1991; Scheff, Baldwin et al. 1997), and focal cortical cryolesion (Sun, Tani et al. 2000), result in a focal contusion in the brain parenchyma with haemorrhagic components and vasogenic oedema. CCC allows a guided weight to fall onto the brain, CCI uses a piston compressing the tissue under controlled parameters, while in the cryolesion model, a pre-cooled cylinder is applied to the brain. Finally, MFP exerts a fluid puls to the dura (Morales, Marklund et al. 2005).

Closed head injury models mimic more diffuse injuries and include the impact acceleration model (Marmarou, Foda et al. 1994) and the recently developed diffuse injury model (Cernak, Vink et al. 2004). Diffuse axonal injuries are often found in clinical TBI. Mixed focal and diffuse injuries are illustrated by lateral fluid percussion (McIntosh, Vink et al. 1989). Finally, combined injury models with a primary damage followed by a secondary event such as e.g. ischaemia, are used for resemblance to clinical TBI with secondary insults (Morales, Marklund et al. 2005).

1.4.2 Transgenic animals

Transgenic animals are frequently used in research and provide a valuable tool to study specific molecular and cellular mechanism associated with TBI (Longhi, Saatman et al. 2001). Knock-out, KO, animals, so far mainly mice, have a deleted target gene, allow analysis of gene function at different levels. The opposite i.e. animals with the target gene overexpressed, exist as well. Additionally, target genes can be mutated at specific sites or disrupted at a chosen timepoint, a so-called conditional KO. The latter technique is a suitable option if deletion results in a native lethal phenotype. An important regard is that function can be difficult to assess in KO animals, because other

genes may compensate for the gene of interest and absence of a detectable phenotype, therefore renders the evaluation more difficult.

1.5 GENE EXPRESSION

The human genome was finally sequenced in 2001 by two competing consortia (Lander, Linton et al. 2001; Venter, Adams et al. 2001). Surprisingly, the total number of genes was not more than 32000, which are only a little more than twice the amount in the lower evolutionary species *Drosophila* (fruitfly). The brain is estimated to express about 70% of the genome. Even though some gene families are held fairly constant among species, others have been preferentially expanded. Among these are genes with neurobiological functions, particularly gene families that are vital to brain development such as NGF family members and ECM proteins, but also ion channels and genes related to myelin and function. Another discovery from the final sequencing results of the human genome was that large parts of the DNA are so called "junk" DNA, which is described, so far, to lack function.

Evolutionarily differences in gene expression patterns and levels may explain alterations in brain structures. Formation of major brain structures e.g. lamination of the forebrain, where huge differences among mammals and nonmammalian vertebrates are seen, may perhaps be regulated by "junk" DNA (Lander, Linton et al. 2001). It could be that these stretches of DNA take part in important processes that are yet undiscovered. They could comprise a resource of DNA that can contribute in reorganisation of the genome by providing new material. The challenge now is how to deal with the genomic information in order for it to make sense. The genetic codes can be compared to a phonebook. Vast information is stacked in the pages, but without a map we cannot know how the streets are situated. Still, we have tools to discover some answers. With the knowledge of gene sequences, genomics, methods were developed to study large-scale gene expression, where the whole genome can be investigated in one single experiment. With all the different '- omics' emerging, focus is shifting forwards to the proteins, their modulations and interactions with each other.

The brain reacts and copes with trauma by more or less coordinated gene expression changes. Altered gene expression affects biological processes and contributes to cell dynamics. Genomics and the tools to investigate it have since a few years opened up the possibility to explore brain trauma in a new way. Instead of studying single genes, large scale analyses offer the possibility to form hypotheses with a wider perspective than in the pre-genomic era.

1.5.1.1 Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

MMPs are zinc endopeptidases, secreted into the extracellular space or membrane bound, with fundamental roles in ECM remodelling and breakdown. The MMP family has more than 20 members, which are regulated on several levels: transcription, pro-enzyme secretion and activation, and interaction with their inhibitors, "TIMPs". The MMP-TIMP system plays important roles in processes during development and plasticity, while a disrupted balance leads to either an excessive matrix accumulation or disruption. An increased MMP: TIMP ratio is suggested to be involved in CNS

diseases and described in many neurological diseases, i.a. Alzheimer's, Parkinson, stroke, human immunodeficiency virus (HIV) associated dementia and glioma.

In experimental studies affected MMP-TIMP system was detected in e.g. haemorrhage (Rosenberg and Navratil 1997), cold induced brain injury (Morita-Fujimura, Fujimura et al. 2000), and stab injury (Jaworski 2000). Increased levels of MMP-9 and MMP-2 are involved in the biphasic destruction of the BBB and oedema formation (Rosenberg, Navratil et al. 1996; Rosenberg and Navratil 1997). MMP-9 KO mice have a reduced infarct area following ischaemia (Asahi, Wang et al. 2001) and experimental trauma (Wang, Jung et al. 2000). Patients with spontaneous haemorrhage have elevated levels of MMP-9 (Castellanos, Leira et al. 2003) and high levels of MMP-9 correlate with neurological worsening (Abilleira, Montaner et al. 2003). Therefore MMP-9 has been proposed as a prognostic marker (Castellanos, Leira et al. 2003: Castillo, 2004 #307). However, IGF-1, which stimulates neurogenesis, is dependent on MMP-9 activity to induce migration of IGF-1 (Mira, Manes et al. 1999).

All four inhibitors, TIMP1-4, are expressed in the adult brain (Crocker, Pagenstecher et al. 2004). TIMP-1 expression is usually low, and TIMP-2 is the most abundantly expressed of the inhibitors (Crocker, Pagenstecher et al. 2004). TIMPs are recognised with functions other than MMP inhibition: growth-promoting properties of TIMP-1 and -2 (Hayakawa, Yamashita et al. 1992; Hayakawa, Yamashita et al. 1994), and TIMP-1 mediated neuroprotection (Tan, Heywood et al. 2003). Finally TIMP-3 is pro-apoptotic It potentiates the death receptor FasR signalling (Wetzel, Tibbitts et al. 2004), which is interesting as TIMP-3 is one of the most highly methylated genes found in brain tumours (Esteller, Corn et al. 2001). Methylation is a mechanism that makes DNA inaccessible for the transcription machinery and thereby silences the gene. TIMP-3 is therefore suggested to be a potential tumour suppressor (Lindsey, Lusher et al. 2004). MMPs and TIMPs can be activated by growth factors, matrix proteins, and inflammatory cytokines. One of the pro-MMP-9 and MMP-9 activators is Osteopontin (Opn).

1.5.1.2 Osteopontin

This secreted phopshoprotein is involved in multiple biological functions such as cell migration, inflammation and anti-apoptotic processes. It serves as a cytokine and adhesion protein, as well as a provisional matrix component. TNF-α and IL-1β can induce Opn expression. Therefore, the high Opn expression in brain trauma and other CNS injuries agrees with the other findings. The exact role of Opn is, however, not defined. In ischaemia, Opn deficient mice injuries create larger lesions and increased signs of secondary neurodegeneration than in WT mice (Schroeter, Zickler et al. 2006). The protective effect of Opn could partly be due to NO-regulation. It can induce iNOS production and thereby affect NO levels (Schroeter, Zickler et al. 2006). Opn treatment in a stroke model has shown neuroprotection (Meller, Stevens et al. 2005). Opn deficit leads to a matrix disorganisation during wound healing, pointing to a task for Opn in the repair process. Another role of Opn is in myelination where it stimulates myelin basic protein and myelin formation (Selvaraju, Bernasconi et al. 2004). In the peripheral nervous system Opn expression is downregulated after axonal injury. The downregulation is suggested to be a pre-requisite for axonal regeneration (Jander,

Bussini et al. 2002). Additionally, it was proposed but not corroborated that Opn could be a stem cell attractant (Sailor, Dhodda et al. 2003). However, Opn exerts a chemotactic effect on macrophages and astrocytes (Wang, Louden et al. 1998). Opn binding to different integrins regulates cell adhesion and migration. The integrin receptor $\alpha_v\beta_3$ is increased simultaneously with Opn after damage (Ellison, Velier et al. 1998), and suggested to be responsible for cell migration and formation of the glial scar. Another Opn receptor is CD44.

1.5.1.3 CD44

CD44 is a transmembrane glycoprotein working as an adhesion receptor. It is involved in endothelial cell recognition, lymphocyte trafficking and regulation of cytokine gene expression in inflammatory diseases. CD44 expression is induced in stab wounds (Stylli, Kaye et al. 2000), in experimental ischaemia (Wang, Xu et al. 2002), in experimental autoimmune encephalomyelitis (EAE) (Kim, Cho et al. 2004), and after an experimental cryolesion (Shin, Ahn et al. 2005). In ischaemic mice lacking CD44, a decreased infarct area and an improved neurological function are seen, proposly related to lower levels of soluble IL-1β in the KO animals (Wang, Xu et al. 2002). Hyaluronan fragments regulate CD44 gene expression. CD44 expression is also strongly upregulated in response to the inflammatory cytokine IL-1β and TNF-α. Several growth factors such as EGF, PDGF and VEGF in tumour cells, fibroblasts and endothelial cells can increase CD44 expression. CD44 expression is localised to microglia/macrophages. Upregulation of CD44 is also found in several tumours. It is detected in 75% of gliomas, and associated to their invasiveness (Kuppner, Van Meir et al. 1992; Ylagan and Quinn 1997). CD44 helps to localise MMPs for directional degradation of the ECM (Yu and Stamenkovic 1999). The receptor is also involved in wound healing. Phosphorylation of CD44 is important for the regulation of cell migration. The role of CD44 in apoptosis is not clear; it is reported to be both pro- and anti-apoptotic (Hauptschein, Sloan et al. 2005).

1.5.1.4 Nestin

Nestin is an intermediate filament which takes part in formation of the filamentous network of cells. Under development nestin is expressed in neuroepithelial cells, radial glia, germinal matrix and vascular cells (Lendahl, Zimmerman et al. 1990; Dahlstrand, Lardelli et al. 1995). In the adult brain, nestin expression is only detected in endothelial and certain subventricular cells (Morshead, Reynolds et al. 1994). After the decrease, nestin/vimentin expression is replaced by another intermediate filament: the astrocyte specific glial fibrillary acidic protein, GFAP (Liem 1993; Dahlstrand, Lardelli et al. 1995). Currently, nestin is used as a stem and progenitor cell marker. However, after CNS injury nestin is re-expressed in reactive astrocytes (Frisen, Johansson et al. 1995; Holmin, Almqvist et al. 1997) and in mitotically active astrocytes (Ernst and Christie 2005). Interestingly, external stimuli can regulate nestin re-expression (Douen, Dong et al. 2004). Cytoskeletal network can still be formed without nestin, though astrocytes without GFAP and vimentin cannot produce a functioning network and nestin is dependent on vimentin for its proper assembly (Marvin, Dahlstrand et al. 1998). Most cycling cells express nestin, and nestin expression is long lived in astroglial cell cultures (Sergent-Tanguy, Michel et al. 2006).

2 AIMS OF THE THESIS

The purpose of this thesis was to study alterations on the gene expression level after traumatic brain injury in order to increase the understanding of molecular mechanisms involved in secondary events of both destructive and restorative nature.

More specifically, the aims were:

- To investigate whether depolarisation is a possible pathogenetic mechanism in experimental contusion

- To compare early and delayed changes in gene expression using high-throughput analysis in the posttraumatic injury phase

- To evaluate the effect of NMDA receptor intervention in the delayed phase after depolarisation and experimental contusion

- To explore in more detail some of the regulated genes after traumatic brain injury and depolarisation like nestin, MMP-9 and their inhibitors, osteopontin and CD44.

3 MATERIALS AND METHODS

3.1.1 Animals

Adult male Sprague –Dawley rats (B&K Universal, Sweden) with weights around 250-300 g were used in all studies with approvals from the regional ethical committees. Before surgery, animals were sedated and anaesthetised using 0.2 ml Hypnorm™-Dormicum (1:1:2 Hypnorm: Dormicum: dH_2O) intramuscularly followed by local anaesthesia 0.1 ml Xylocain administered subcutaneously in the sagital midline of the skull. At experimental end-point, all animals were decapitated in Hypnorm™ anaesthesia.

3.1.2 Cortical contusion (II-IV)

The weight-drop model is considered a quick, easy and convenient method with possibility to adjust the height to obtain different severity of trauma (Morales, Marklund et al. 2005). Originally described by Feeney and collaborators, the model is based on weight-drop using a contusion device composed of a free-falling weight (here 24 g), a piston and a tube with air holes along sides in order to avoid air compression (Feeney, Boyeson et al. 1981; Holmin, Mathiesen et al. 1995). The rat was placed in a stereotactic frame with the skull fixed and after craniotomy, the contusion device was positioned according to co-ordinates, here 2.5 mm posterior and 2.5 mm lateral to bregma, vertically with the piston in contact with the exposed dura (fig. 3), and lowered according to the maximum depth parameter, here 3 mm. The piston, moving freely inside the tube, was pushed back by the exposed dura, while at weight drop, here at a 9.3 cm height, pushed down compressing the brain tissue to the point of chosen stop of the contusion device.

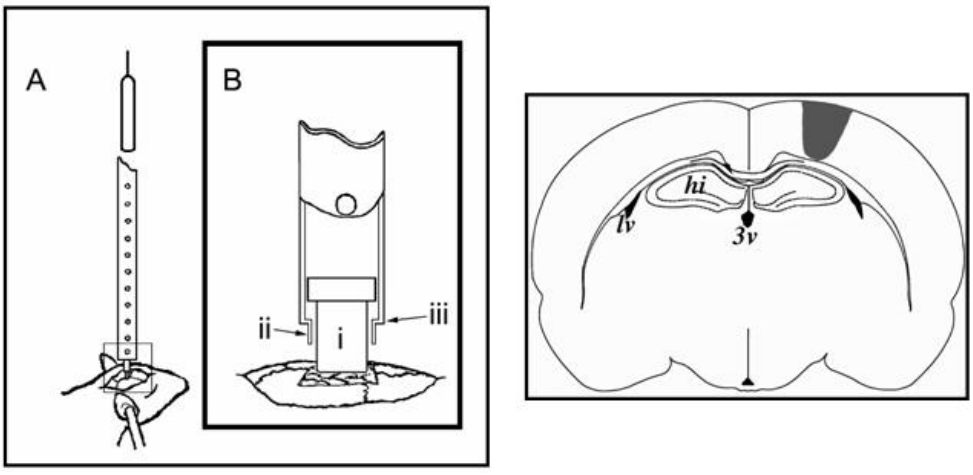

Fig. 3 *Scematic illustration of the contusion device showing the weight drop suspended in the guidance tube, positioned over the exposed skull of a rodent (A). The marked area in A is shown magnified (transparent for clarity) in B. The piston (i) is pushed up in the sleeve (ii) by the exposed dura mater and force of the falling weight is subsequently transferred to the neocortex by the piston. Finally the cuff (iii) of the sleeve stops the downward movement of the*

piston. C is showing a schematic illustration of the lesion (dark grey) in a coronal section. Hippocampus (hi), lateral ventricle (lv), 3rd ventricle (3v).

3.1.3 Depolarisation (I, II and IV)

The rat was placed in a stereotactic frame and craniotomy was made similarly to what is described for the contusion model above. The exposed dura and arachnoid were incised and retracted. A 2 mm diameter filter pad, soaked in 3 M KCl was placed onto cortex for 10 or 30 minutes. Control rats were treated with a filer pad soaked in physiological NaCl solution. After removal, the skin was sutured and the animals were allowed to recover (Bonthius and Steward 1993).

3.1.4 MK-801 (I and IV)

Dizocilpine maleate/ MK-801 is a non-competitive NMDA receptor antagonist, used in studies of glutamatergic pathways. MK-801 causes increased locomotion in rodents and at higher doses various stereotypic behaviours can be observed, while at low doses, animals display periods of immobility (Tang, Zou et al. 2006). A dose of 1 mg/kg is reported to be sufficient to block specifically NMDARs, (Wong, Kemp et al. 1986).

Here in our studies, rats were treated twice daily with intraperitoneal (i.p.) injections of 1 mg/kg. In study I, rats were pretreated with one dose of MK-801 before depolarisation, whereas in study IV, treatment started 30 minutes after depolarisation/ experimental contusion.

3.1.5 Tissue preparation

For histopathological examination, brain tissue was removed after decapitation, quickly frozen in isopentane-dry ice and sliced in coronal 14 µm cryosections through the contusion/application centre using a Leica cryostat (CM 3000, Leica Instruments GmbH, Nussloch, Germany). The sections were thaw-mounted onto Super Frost/Plus™ object glasses (Menzel-Gläser, Braunschweig, Germany) and stored at -20°C prior to use.

For RNA isolation, brain tissue from the impact/ application area and the surrounding cortex, was dissected out. RNA was isolated using TRIZOL Reagent (Life Technologies AB, Täby, Sweden) or RNeasy Qiagen kit (VWR International AB, Stockholm, Sweden) according to manufacturer's protocol.

3.2 GENE EXPRESSION

3.2.1 *In situ* hybridisation (II and III)

In situ hybridisation (ISH) allows detection of gene expression, with the advantage of providing spatial information within cells or tissue sections, something that is not obtained when analysing RNA and DNA immobilised onto membranes or in the polymerase chain reaction (PCR). Spatial gene expression is valuable when studying

development or external stimuli response in a tissue or cell. However, ISH is labour-intensive and have long processing and analysis time, which may limit the extent of use. The nucleic acid probe can be RNA or DNA, obtained by cloning, *in vitro* amplification or chemical synthesis. Labelling can be either radioactive or non-radioactive. Non-radioactive labels, biotin and digoxigen, are detected by fluorescence or enzymatically with high resolution. Sensitivity is however higher using radioactively labelled probes. If quantification of the relative gene expression is desired, this can be made in the case of radioactive probes, using a phosphoimager, which counts radioactive decomposition in relation to a standard, usually ^{14}C. Another way to perform semiquantitative measurements is to use computerised image analysis carried out by micro-densitometry.

In study II and III, oligonucleotide probes were used (table 1), which contained the reversed nucleotide code, i.e. anti-sense, to the gene of interest. ^{35}S-dATPs were 3´ end-labelled to the probe and chosen due to that ^{35}S is a good compromise between resolution and exposure time. During hybridisation, the anti-sense works as one part of two matching puzzle pieces and will attach to its corresponding piece/sequence. As a control, a sense probe with the same nucleotide sequence of the target gene was labelled, which should not be able to bind and yield a signal. In order to assure for the ISH procedure working, a control gene cholecystokinin (CCK) was included, which is well characterised and known to be expressed in brain. Radiolabelled brain sections were further processed with photo emulsion dipping.

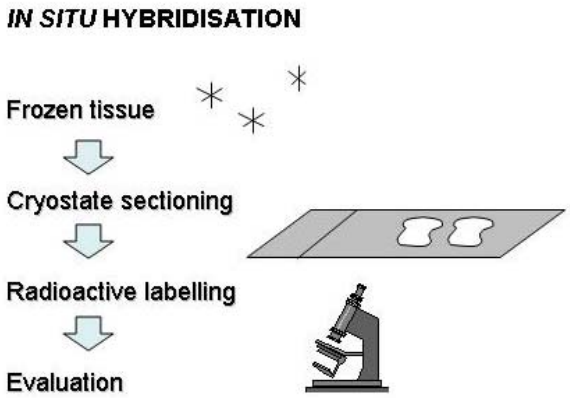

Fig. 4 *Schematic illustration of in situ hybridisation.*

Table. 1 *Oligonucleotide probes that were used for in situ hybridisation.*

Gene	Orientation	Sequence (nt no.)	GenBank no.
CCK	antisense	298-341	NM_012829
CD44	antisense	655-698	AA817820
GFAP	antisense	601-645	L27219
MMP-9	antisense	1339-1383	U36476
NF-68	antisense	780-827	AF031880
OPN	antisense	643-687	M14656
TIMP-1	antisense/sense	302-346	L31883
TIMP-2	antisense	301-345	L31884
TIMP-3	antisense	521-564	NM_012886

3.2.2 Polymerase chain reaction (II and III)

PCR is a frequently used method, which have been extensively developed with many application areas since the first appearance in 1985 when science could take a big leap forward. The technique is fundamentally simple, rapid and sensitive in vitro, which is advantageous. With the use of the Termus aquaticus, the Taq enzyme, pieces of DNA can be copied and amplified in large amounts. There are three fundamental steps in the reaction: denaturation, when the target/template gene sequence is made available by separation of the duplex strands, II) annealing, when the primers attach to the right sequence and finally III) extension, when nucleotides are added to the primer and copying the target. This procedure is repeated in several cycles, which increases the target pool. A limitation of PCR is that it does not give any information on localisation at cell level.

Messenger RNA (mRNA), expressed DNA sequences, can be converted into DNA by RNA-dependent DNA polymerases called reversed transcriptase (RT), which can create complementary DNA (cDNA). Primers are either specific for a single mRNA species or capable to anneal to all mRNA, e.g. an oligo dT primer binding to the polyA tail or a random primer. Today the real-time PCR provides quantification of such cDNAs, which represents the RNA amounts in samples of interest.

Here, PCR was performed with 2 µl cDNA, 1x buffer, 0.2 mM dNTP, 0.5 units Taq Dynazyme, 1 µM forward and respectively reverse primer (table 2), with a 4 min hot start at 94ºC and then 30 cycles of 94ºC – 45 sec, 59ºC – 45 sec, 72ºC – 1 min and finally 10 min at 72ºC.

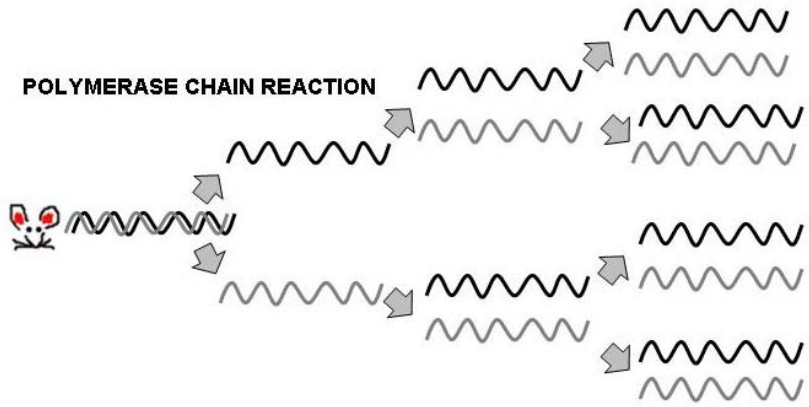

Fig 5. *The PCR reaction amplifies genetic material in an exponential way*

Table 2. *Primer pairs used in PCR and their product size.*

Gene	Primer sequence (nts)	Product (bp)	GenBank no.
PAT-1	5′- GAAGCTAGAGGCTGAGGATC -3′ (F) 5′- GTCCAGTCTCCCTCCTTCAC -3′ (R)	233	L00091
CD44	5′- CCGACCTTCCCACTTCACAG -3′ (F) 5′- TCTCCTCGCAGGACCAGAAG -3 (R)	200	AA817820
G6PD	5′- CCAGCCTCCTACAAGCACCTCAA -3’ (F) 5′- AATAGCCCCCACGACCCTCAGTA -3′ (R)	406	X07467
IGF-II	5′- GACTGAGTTGGGGCAAATAC -3′ (F) 5’- CAGGTGTTAGGAAGGTGCTC -3′ (R)	211	AA899788
MMP-9	5’ - AGGCTACAGCTTTGCTGCCCC - 3’ (F) 5’ - GCTGCTTCTGAAGCATCAGCA -3’ (R)	193	U36476
OPN	5′- CTGCCAGCACACAAGCAGAC -3′ (F) 5′- ACTCCTTGGACTGCTCCAGG -3′(R)	323	M14656
S-100	5′- GGACCTGAGAGTGCTCATGG -3′(F) 5′- GCATGCAATGATGAGCCCCG -3′(R)	222	J03627.1
TIMP-1	5’ - CGAGACCACCTTATACCAGCG -3’ (F) 5’ - CAGGAAGCTGCAGGCAGTGAT -3’ (R)	216	L31883
TIMP-2	5’ - TGCACCCGCAACAGGCGTTTT -3’ (F) 5’ - TTCCTCCAACGTCCAGCGAGA -3’ (R)	224	L31884
TIMP-3	5’ - AACTCCGACATCGTGATCCGG -3’ (F) 5’ - ATCCTCGGTACCAGCTGCAGT -3’ (R)	483	U27201
VTN	5′- ATCGACGCTGCCTTCACTCG -3’′(F) 5′- TGGCGCCATCAGAGGATCTG -3 (R)	373	U44845

3.2.3 Microarray (III and IV)

The technique has the advantage of allowing simultaneous high throughput measurements of the expression level of several thousands of genes. Microarrays can be made of cDNA or oligonucleotide probes. cDNA microarrays are made of PCR products, 100 base pairs or longer, from cDNA clones representing known genes and/ or expressed sequence tags. The advantage of cDNA microarrays is that the long probes increase sensitivity, whereas the disadvantage, also due to the size, is an increased likelihood of cross hybridisation. Furthermore, PCR products need to be produced and quality verified before spotted onto the slide, which altogether can be considered a tedious task depending on how much of the processes that are automated. Oligoarrays have the advantage of a fast and confident fabrication of oligomers, and can be produced with different probe lengths, usually 20-70 oligonucleotides. The oligomers can be synthesised directly on to the array surface or be attached after synthesis. Short probes can reduce sensitivity. However, oligomers can be used to distinguish between closely related gene family members.

In brief (fig. 6), the microarray method in this thesis is based on comparative hybridisation where the two targets RNA pools to be compared are reversed transcribed and labelled with two different fluorophores: cyanine (Cy) 3 and 5. After purification,

the samples are pooled, and let to hybridise over night to attach to target sequences immobilised on the microarray surface. Thereafter, the microarrays are scanned for detection of the two different fluorophores. Measured fluorescence intensity for each spot determines how much of the target that has bound and allows quantification of the gene expression ratio for specific genes in the original RNA pools.

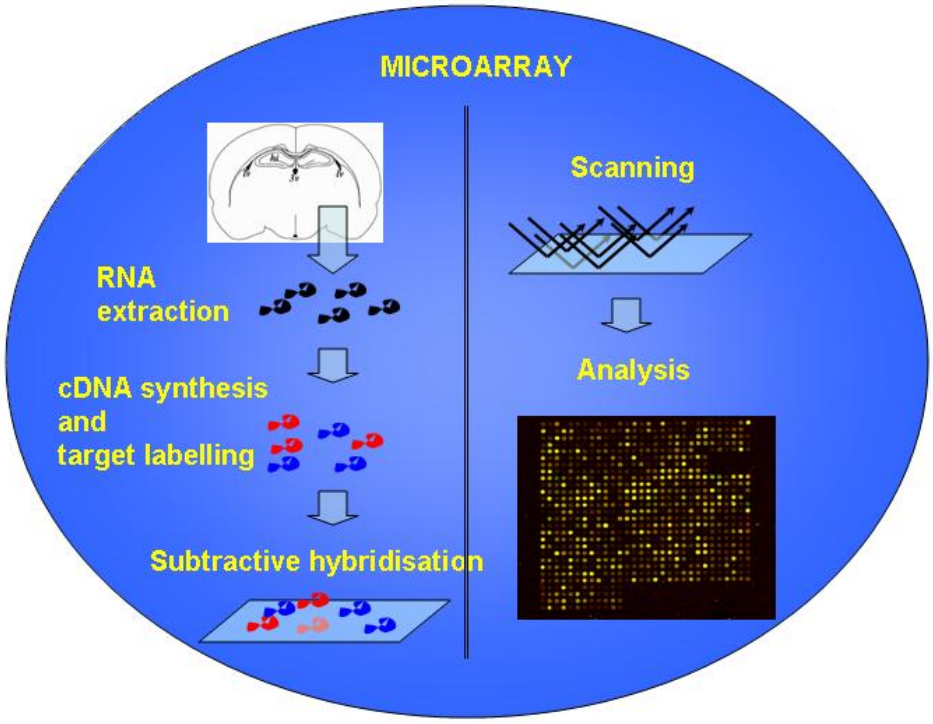

Fig 6. *Schematic illustration of the microarray method.*

3.2.3.1 Arrays

Here, we have used both cDNA and oligomicroarrays to study experimental contusion and the latter to explore gene expression in depolarisation. cDNA microarrays were made in house, Molecular Endocrinology group, Department of Molecular Medicine and Surgery, Centre for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden, and have been previously described (Flores-Morales, Stahlberg et al. 2001). The cDNA microarrays consisted of approximately 6200 probes of rat/mouse cDNA clones selected from TIGR Gene Index (www.tigr.org), Research Genetics (www.resgen.com) and in house cloned cDNAs. In study IV, oligomicoarrays were kindly provided by the Royal Institute of Technology, Stockholm, Sweden and contained 27K unique 70-mer oligos from the Operon (www.operon.com) version 3 rat genomic oligonucleotide set.

3.2.3.2 Target preparation and hybridisation

In the first microarray study (Study III), an internal control sample, the contralateral side, competed with the test sample, the injured area and surrounding cortex, for probe binding. Thirty µg of total RNA, from each side ipsilateral and contralateral, were isolated from individual animals subjected to experimental contusion. The two pools were individually labelled with fluorescent Cy3 or Cy5. Dye swap was applied due to

differences in incorporation for the Cy-labelled dinucleotides. In study IV, 5 µg total RNA of the control sample (a common pool of normal rat brain tissue labelled with Cy3), and the same amount of the test sample (contused/ depolarised or MK-801 treatment + contused/ depolarised/ normal brain tissue from rat labelled with Cy5CTP), were competitively hybridised. Labelling and hybridisation as well as pre-treatment and washing of arrays were made using the Pronto System (Promega, Madison, WI), according to manufacturers protocol. Specifically, ChipShot^{TM} Direct labelling system, for total RNA was used to generate Cy3, Cy5 labelled targets, which were later purififed using QIAquick PCR purification kit according to manufacturer's protocol (Qiagen, Hilden, Germany).

3.2.3.3 Data collection, normalisation and analysis

Arrays were scanned using a confocal laser (GenePix professional 4200A, Axon Instrument). Image analyses were performed using GenePix Pro 6.0 software (Axon instruments, CA) to quantify amount of bound cDNA from each sample to each immobilised probe. In addition to spot intensity quantification, background levels were obtained and subtracted from the measured intensity. Automatically and manually defined bad spots, including absent or weak spots, were removed from subsequent analysis. The background corrected values were used and transformed into logarithmic expression ratios with Log2, giving fold differences. Expression ratios were normalised with the locally weighted linear regression method, Lowess as implemented in the SMA (Statistics of Microarray Analysis www.stat.berkeley.edu/users/terry/Group/software.html) package (Quackenbush 2002) (Study III) or Acuity 4.0 (Axon Instruments Inc.) (Study IV). Normalising the data is done in order to remove artefacts caused by unequal quantities of input RNA and differences in labelling and detection efficiencies between the dyes. Lowess performs a local fit to the data in an intensity dependent manner (Dudoit S. 2000).

To identify differentially expressed genes statistical testing was performed with Significance of Microarray Analysis (SAM) (Tusher, Tibshirani et al. 2001). SAM is a permutation-based statistical method specific for analysis of microarrays. Each transcript is assigned a score by SAM, based on the change in gene expression for that transcript in relation to the standard deviation of replicated measurements for that gene. In addition, SAM calculates an estimated false discovery rate (FDR), which is the percentage of genes expected to be falsely identified as differentially expressed. Each gene is assigned a q-value, which is similar to the p-value, showing the lowest FDR at which that particulate gene can be called significantly differentially expressed. In study III a 2% FDR was applied for genes defined as differentially expressed, and in study IV a 1% FDR. Moreover, for genes to be called regulated, a cut off for expression ratios were set to >0.7 corresponding to > 1.6 fold regulation. Additionally in study IV, two-class unpaired SAM was used to test for differences between depolarisation versus experimental contusion, as well as depolarisation/ experimental contusion versus MK-801 treated depolarisation/ experimental contusion. Significant differences met the criteria of 1.6 fold change on a 5% FDR. Local false discovery rates were applied to genes identified as regulated in depolarisation/experimental contusion in order to evaluate a possible effect of MK-801. Local FDR can be suitable for genes with small variations (Ploner, Calza et al. 2006) and it gives a measure of how true the change in

expression is for a specific gene, e.g. a local FDR of 0.09 would mean that it has a 9% chance of being a false discovery (or a 91% chance of being a true discovery).

In study III, regulated genes were functionally grouped into gene ontology categories using the web based tool eGON (explore gene ontologies, http://nova2.idi.ntnu.no/egon/) v 1.0, developed at the Norwegian University of Science, which also was used to statistically compare differentially expressed genes 1 dpi versus 4 dpi, and 1 dpi / 4 dpi towards all genes expressed. In study IV, DAVID (database for Annotation and Visualisation and Integrated Discovery, (http://david.abcc.ncifcrf.gov/home.jsp) was used for functional annotation clustering and finding biological pathways. Additionally, PubMed, Ensembl (http://www.ensembl.org/index.html) and other gene expression reports have been used in classification or annotating transcripts.

3.3 IMMUNOHISTOCHEMISTRY

3.3.1 Immunohistochemistry (I, II and IV)

Detection of proteins and peptides by antibody-binding has provided much of the knowledge of cellular anatomy and functioning of cells. With the more frequent use of confocal microscopy, a three-dimensional view can be obtained, facilitating possible protein-protein interactions. Antibodies are visualised with either fluorescently or enzymatically, conjugated secondary antibodies (fig. 7). Though the former has the advantage of well illustrating co-localisation at stainings using two or more antibodies, it has the disadvantage of fading. The enzymatically labelling system permits the use of light microscopy and structures are more easily seen within tissue if combined with routine stainings and the risk of losing the results are low.

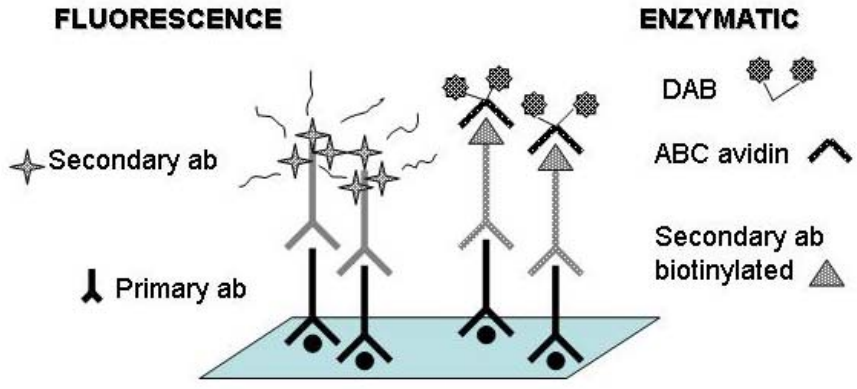

Fig. 7 *Schematic illustration of fluorescent and enzymatic immunohistochemistry.*

In our studies, antibodies were diluted in 1% or 4% bovine serum albumin (BSA) for primary, respectively secondary antibodies. In brief, sections were air-dried, rehydrated, and fixed in 4% formaldehyde. Incubation in 0.3% hydrogen peroxide was performed to quench endogenous peroxidase activity. After washing, sections were first incubated in 1% BSA to block unspecific binding and then with the primary antibody (table. 3) over night at 4ºC. In enzymatic immunohistochemistry, biotin-conjugated secondary antibodies were used, detected by the avidin-biotin-enzyme complex (ABC)

technique, and where the bound peroxidase was visualised by incubation with a diaminobenzidine kit (Vector Laboratories, Burlingame, CA). Cy3 (indocarbocyanine), and FITC (fluorescein isothiocyanate) conjugated $F(ab')_2$ fragments were used in fluorescent immunohistochemistry. Where double labelling was performed, (study III and IV) the procedure was repeated for the next antibody.

Table 3. *The primary antibodies that were used in the thesis*

Antibody	Dilution	Specificity	Source
CD44	1:100		Serotec, Oxford, UK
ED-1	1:1000	macrophages and activated microglia	Serotec, Oxford, UK
GFAP	1:500, 1:1000	astrocytes	Prof V Peter Collins, Dept of Histopathology, University of Cambridge, UK
Hippocalcin	1:1000	calcium sensor protein	Abcam, UK
MPIIIB101	1:50	Osteopontin	Developmental Studies hybridoma bank IA
Phopho-NR2B	1:200	phosphorylated NMDAR subunit 2B	Upstate, NY
Nestin	1:2000,1:500	intermediate filament	Prof. U. Lendahl, Dept of Cell and Molecular biology, KI, Sweden
NeuN	1:500	neuronal nuclei	Chemicon, Temecula, USA
2F11	1:75	neurofilament	Dakopatts a/s, Glostrup Denmark

3.3.1.1 TUNEL staining (I)

Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate-digoxigenin nick end labelling (TUNEL) labels fragmented nuclear DNA. The free 3´-OH groups of the DNA are linked to fluorescent nucleotides by the enzyme Terminal deoxynucleotidyl transferase (TdT) and thereby visualises cells with DNA breaks. The method is used to detect apoptosis, however, late stages of necrosis can also display fragmentation.

Sections were air-dried, fixed and washed as previously described and permeabilised for five minutes in a 2:1 ethanol-acetic acid solution at –20˚C. The TUNEL reaction was carried out with the "*In situ* cell death detection kit, fluorescein" (Boehringer Mannheim, Bromma, Sweden) for 30 minutes at 37˚C, followed by washing in PBS.

3.3.1.2 Fluoro-Jade staining (IV)

Fluoro-Jade is a fluorochrome, which selectively stains degenerating neurons and their processes (Schmued, Albertson et al. 1997). Although the mechanism is not yet elucidated, it has been speculated to bind to a basic molecule ubiquitously expressed by degenerating neurons as the Fluoro-Jade is strongly acidic (Schmued, Albertson et al. 1997).

Sections were air-dried, fixed and washed as previously described. After brief washing in distilled water, sections were incubated in 0.00002% Fluoro-Jade in 0,1% acetic acid under agitation for 30 minutes, RT, followed by washing in distilled water.

4 RESULTS AND DISCUSSION

4.1.1 Study I: Nestin expression and depolarisation

Depolarisation induced nestin expression in the ipsilateral cortex, after both 10 (Study I, fig.3) and 30 minutes exposure time. The nestin expression pattern resembled the pattern of basic fibroblast growth factor and GFAP, another intermediate filament, which is also increased after TBI and depolarisation (Bonthius and Steward 1993; Kawahara, Ruetzler et al. 1999). The thirty minutes exposure time resulted in a stronger and more widespread ipsilateral expression pattern, which was extended along the cortex. All nestin-positive cells co-localised with the GFAP immunoreactivity (Study I, fig. 2c-d), though not all GFAP positive astrocytes were nestin-positive. No co-localisation of nestin and neurofilaments were found. Nestin expression was markedly reduced (Study I, fig.3) by blocking the NMDA receptor with the antagonist MK-801. No cell death was detected for the 10 minutes depolarisation time and only local, minor cell death was observed at the application area after the longer depolarisation time. Evaluation of NeuN immunoreactivity showed similar numbers of NeuN positive cells in all 10 minutes exposed animals. The nestin pattern after 30 minutes exposure displayed a large variation between animals. Quantification at this time point was thus not reliable.

In accordance with our hypothesis, nestin synthesis could be induced by depolarisation of brain tissue. The nestin expression was located to astrocytes in a pattern spreading along the ipsilateral cortex, which is similar to what is found in experimental brain trauma (Holmin, Almqvist et al. 1997). The nestin upregulation did not associate with any cell death and could thus not be considered a secondary response to cell death. It was likely that the cortical post-traumatic nestin pattern was directly induced by depolarisation. The idea that trauma induced depolarisation could be responsible for genetic effects was further addressed in studies II and IV. The sporadic cases of cell death after the longer exposure time were probably caused by K^+ toxicity after prolonged exposure. As nestin expression was reduced after MK-801 treatment, we concluded, at that time, that its induction was mediated by NMDA receptors. One criticism put forward to this conclusion was that the MK-801 administration before KCl application would block the induction of depolarisation (Obeidat, Jarvis et al. 2000). This hypothesis could be further tested by post-injury drug administration. We did not find upregulation of inflammatory markers, which is at variance with Jander *et al.* where IL-1β, mRNA and protein, and TNF-α mRNA were upregulated after depolarisation at 4 hours (Jander, Schroeter et al. 2001). Furthermore, cortical spreading depression has been shown to modulate members of the inflammatory cascade (Thompson and Hakim 2005). However, different models of depolarisation have been used, and both previous studies had earlier timepoints, and a different area of investigation (Jander, Schroeter et al. 2001), which could explain the differneces between our study and the studies by Jander *et al* and Thompson *et al*.

Nestin levels are higher during prenatal stages and development than in the adult brain (Sergent-Tanguy, Michel et al. 2006). Its induction after damage may indicate a transition to a more immature state of the astrocytes, which would be in line with the

high levels of nestin in not fully differentiated astrocytes (Sergent-Tanguy, Michel et al. 2006). Similarly vimentin levels increased after TBI (Moon, Ahn et al. 2004), which also suggests a more immature or transitional cytoskeleton. Vimentin levels are also higher during development. The relationship between the three IFs GFAP, vimentin, and nestin is important, because of their contributions to the cell interior, surface proteins and the ECM. Studies of mice lacking both GFAP and vimentin show that nestin and vimentin collaboration is necessary to create a proper IF network as nestin is unable to form a network alone (Marvin, Dahlstrand et al. 1998). GFAP cannot substitute for vimentin, although it can promote nestin assembly into short filaments (Pekny, Johansson et al. 1999; Menet, Gimenez y Ribotta et al. 2001).

Of the three filaments GFAP is indicated as the major contributor to the properties of the glial scar. Absence of GFAP is associated with an increased expression of laminin and N-cadherin, which are present during CNS development and provide a permissive milieu (Menet, Gimenez y Ribotta et al. 2001). In line with the latter, GFAP deficient astrocytes are a more favourable substrate for neuronal growth than GFAP expressing cells (Menet, Gimenez et al. 2000). Recent data show that GFAP plays a role in cell swelling after SD-like events (Anderova, Kubinova et al. 2001). The characteristic hypertrophic form of reactive astrocytes could be attributable to GFAP and vimentin (Wilhelmsson, Li et al. 2004). However, the upregulated nestin levels are another feature of reactive astrocytes, in humans (Tamagno and Schiffer 2006). Intermediate filaments can regulate the motility of astrocytes (Lepekhin, Eliasson et al. 2001), and re-activation of nestin could be beneficial for neurite outgrowth. External stimuli regulate nestin re-expression, which could have a role in brain repair (Douen, Dong et al. 2004). The latter was supported by a recent report that showed an essentially protective role of reactive astrocytes. Their ablation resulted in significant tissue loss (Myer, Gurkoff et al. 2006). Reactive astrocytes form the glial scar, which seals off the inflammatory area from the surrounding tissues. However, it also obstructs re-growth into the damage zone, which is why astrocyte re-activation is of high interest for brain repair.

Presently nestin expression mainly serves as a marker for stem cells and reactive astrocytes (Ernst and Christie 2006), and the nestin promoter region is used for manipulations of genes in different constructs. Nestin is so far not ascribed any vital functional property. Studies with nestin deficient conditions after injury and stem cell transplants would be one way to closer dissect the role of nestin.

4.1.2 Study II: Gene expression of matrix metalloproteinase and tissue inhibitors of matrix metalloproteinases

After experimental contusion and depolarisation, MMP-9 and TIMP-1 increased in a time-dependent expression pattern over the timepoints 4 hours, 1, 4 and 14 dpi. The expression of TIMP-2 and -3 mRNA remained unchanged. MMP-9 mRNA peaked around 1 and 4 dpi after experimental contusion, whereas in depolarisation the MMP-9 induction was delayed and found at 4 days post application. Both models showed that MMP-9 levels returned to normal again at 14 dpi. TIMP-1 expression displayed a similar pattern in both depolarisation and experimental contusion, with increased levels

already at 4 hours and a maximum around 1 and 4 days. MMP-9 and TIMP-1 expression was localised along the ipsilateral cortex with a strong labelling to the piriform cortex in both models. Likewise, both models resulted in a neuronal MMP-9 and TIMP-1 expression.

MMPs and TIMPs were at this time reported to be involved in CNS injury (Rosenberg and Navratil 1997; Morita-Fujimura, Fujimura et al. 2000; Vecil, Larsen et al. 2000), though the main research focus were the involvement of the system in neurodegenerative diseases. The major new findings of our study were that depolarisation *per se* was enough to trigger the induction of MMP-9 and TIMP-1 mRNA. Increased TIMP-1 expression after experimental contusion was not previously described, but might have been anticipated. Today it has been shown that the depolarisation not only induces, but also activates MMP-9, which is involved in breaking down the BBB in rat brain (Gursoy-Ozdemir, Qiu et al. 2004).

In the light of new data, the early induction of TIMP-1 at 4 hours in our study was interesting as TIMP-1 is neuroprotective in excitotoxic damage to hippocampal cells in an MMP-9-independent way (Tan, Heywood et al. 2003). It is possible that TIMP-1 could be responding to the injury by reducing Ca^{2+} influx through NMDARs already at an early stage, and thereby limit further destruction. Surprisingly, TIMP-1 deficient mice have a hampered MMP activity and are resistant to seizure induced excitotoxicity (Jourquin, Tremblay et al. 2005). In addition, these mice have impaired learning and memory (Chaillan, Rivera et al. 2006). The MMP: TIMP ratio is important for regulating MMP activity, and possibly to assure for normal learning and memory processes, while impaired cognitive functions are the result of an altered proteolytic activity. Another beneficial effect of the early TIMP-1 expression could be mediated by its growth promoting properties, earlier described by Haykawa et al. but somewhat neglected in more recent reports (Hayakawa, Yamashita et al. 1992; Hayakawa, Yamashita et al. 1994).

As the time-dependent expression patterns of TIMP-1 and MMP-9 in depolarisation were similar to the experimental contusion in our report, we proposed that depolarisation could be the mechanism of upregulated TIMP-1 and MMP-9 in trauma. This hypothesis was further corroborated by other studies (Gursoy-Ozdemir, Qiu et al. 2004; Mali, Cheng et al. 2005). In addition to the suggestion that TIMP-1 may inhibit or modulate MMP-9 action, it should be added that TIMP-1 may also act independently of MMP-9. Nevertheless, it is likely that this system contributes to tissue reorganisation and neuroprotection after trauma. Several studies report increased MMP-9 levels associated with haemorrhage (Abilleira, Montaner et al. 2003; Castellanos, Leira et al. 2003; Castillo and Rodriguez 2004). Additionally, the TIMP - MMP system is involved in inflammation, NOS regulation, interaction with the plasminogen system (Wang, Lee et al. 2003), and IGF-1 migration (Mira, Manes et al. 1999). Several ongoing trials target the MMP - TIMP system but most of these are aimed at cancer (Doherty, Asotra et al. 2002). Hopefully, experimental trauma in TIMP-1 deficient mice would emerge and improve the knowledge of TIMP-1. The development of a double KO of TIMP-1 and MMP-9 could, if viable, possibly also be valuable.

4.1.3 Study III: Genomic responses in an early and delayed phase after experimental brain trauma

Genes regulated by experimental trauma at 1 and 4 dpi showed little overlap (Study III, fig. 1). One dpi demonstrated more regulated genes than 4 dpi, 211 compared to 63 respectively. We also analysed whether genes regulated at the two time points were involved in different functions. The regulated genes were assigned to functional categories according to the Gene Ontology. Significant differences between the two timepoints were found for the gene ontology groups: cell proliferation (GO:0008283), cell communication (GO:0009987), cell death (GO:0008219), transcription (GO:0006350) and metabolism (GO:0008152) (Study III, fig. 3). Upregulated genes at 1 dpi were more numerous in almost all of the functional categories compared to 4 dpi, with the exception of genes involved in transport (GO:0006810), where 1 and 4 dpi were approximately the same, and cellular defence response (GO:0006968), where there were more genes regulated at 4 dpi. Downregulated genes were found in several categories including cell communication (GO:0009987), cell cycle (GO:0000074), cell differentiation (GO:0030154), cell proliferation (GO:0008283), development (GO:0007275), regulation of cell cycle (GO:0000074), response to stimulus (GO:0050896), transport (GO:0006810) and transcription (GO:0006350). Genes involved in metabolism (GO:0008152) displayed the highest number of downregulated genes 1 dpi. Upregulated genes involved in cellular differentiation (GO:0030154), response to stimuli (GO:0050896), and defence response (GO:0006968) had a significant overrepresentation at both 1 and 4 dpi. Additionally, genes found to be regulated 1 dpi, were also involved in cell death (GO:0008219), cell growth (GO:0016049), cellular morphogenesis (GO:0000902), development (GO:0007275), regulation of cell cycle (GO:0000074) and transport (0006810). At further dissection of the defence response, a significant immune response (GO:0006955) was found 4 dpi, which was not present 1 dpi, and likewise proteolysis and peptidolysis (GO:0006508) was observed 4 dpi. Of the regulated genes, a subset including CD44, Opn, S-100, angiotensinogen, IGF-II, vitronectin, TIMP-1 and -2 were subsequently confirmed as regulated after experimental TBI using RT-PCR. Two inflammatory factors, osteopontin and one of its receptors CD44, were further studied by ISH and immmunohistochemistry. The expression of Opn and CD44 was found locally at the impact site with a corresponding immunoreactivity for the respective proteins (Study III, fig. 4). Opn was co-localised with activated microglia/macrophages.

The higher number of regulated genes at 1 dpi could either fulfil a physiological fast adaptation to the new homeostatic state, or reflect a more chaotic response after non-specific traumatic stimulation. In a study of traumatic injury in brain slices Morrison *et al.* argue for a specific response after injury due to co-ordinated expression of genes and not a generalised transcriptional impairment (Morrison, Eberwine et al. 2000). It is possible though, that a particular gene expression in response to the trauma, from being ordered, could turn into mal adaptations due to the deranged milieu, and thereby end up in chaos. It seems probable that post-traumatic genetic events would induce at least an element of non-specific and disorded reactions. The reduced number of genes in almost every category 4 dpi, compared to 1 dpi, can indicate that a new equilibrium has been reached. The total numbers of expressed genes and their variation with time after injury has not been extensively studied in trauma. There is a time series study of mice

subjected to brain trauma (Kobori, Clifton et al. 2002). The focus of the report was to identify new transcripts, and the number of regulated genes for each timepoint was not widely discussed. The amount of transcribed genes must have consequences for energy stores of the cells. In addition, the number of regulated genes depends on type of injury. Moderate and severe injuries in the hippocampus were studied at 0.5, 4 and 24 hours pi (Li, Lee et al. 2004). The number of up- and down-regulated genes in the moderate group increased over the 24 hours, while the upregulated genes decreased in numbers in the severe damage group. Downregulated genes displayed a slight increase in number of genes in the severe group. A large difference in the number of upregulated genes was seen between the moderate and severe injury, though curiously not for the downregulated genes. It would be necessary to study all three levels of TBI, i.e. mild, moderate and severe in the post traumatic period to determine the patterns, which at least differ between moderate and severe TBI in the hippocampus (Li, Lee et al. 2004).

The difference in genes involved in the cell proliferation response between 1 and 4 dpi could reflect the early proliferate response of astrocytes, which propagate after damage (Kernie, Erwin et al. 2001) to form a glial scar. The response could also reflect neurogenesis (Rice, Khaldi et al. 2003), and a microglial proliferation. As might have been anticipated, a large group of genes in cell communication were active early on. They could help in signalling to the surrounding tissues and participate in the regulation. The large number of genes involved in metabolism suggests a reply for the demand of more energy in disturbed brain tissue. The more prominent 4 dpi defence response, including an immune response, lies in line with a delayed onset of inflammation in our model (Holmin, Mathiesen et al. 1995) connected to secondary injury mechanisms. This is also the case for proteolysis, since proteases contribute to the inflammatory response by breakdown of debris. They can also play a role in tissue repair and reorganisation (Ellison, Barone et al. 1999). The finding that several functional categories displayed affected genes corroborate that TBI was a strong stimulus, which induces a complex gene expression pattern. It is necessary to apply experimental techniques that allow a broad understanding of these patterns.

We chose to study two molecules involved in inflammation in more detail. Osteopontin is a molecule attracting attention due to its highly regulated expression after injuries and its many roles in biological functions (Denhardt, Noda et al. 2001). The expression of Opn and CD44 is likely to take part in the inflammatory response, reflected in Opn IR co-localised to microglia/ macrophages in our study. We also found its receptor CD44 protein in the same area; hence it is probable that they interacted. The Opn and CD44 upregulation was probably involved in remodelling processes as Opn possess chemoattractant properties that affect astrocytes and microglia (Ellison, Barone et al. 1999), CD44 expression could contribute to the Opn chemotactic effect (Shin, Ahn et al. 2005). This could suggest that CD44 and Opn attract the reactive astrocytes to form the glial scar around the injury. Mice deficient in CD44 are protected from ischaemia (Wang, Xu et al. 2002), while Opn is ascribed a neuroprotective role in stroke (Meller, Stevens et al. 2005; Schroeter, Zickler et al. 2006). The upregulated expression of Opn endures for some time after injury and Opn may accordingly have tasks that change along the posttraumatic time axis (Ellison, Barone et al. 1999).

This study contributed to the knowledge of gene expression alterations and differences between 1 and 4 dpi. The delayed phase of TBI has not received as much attention as the initial 24 hours after injury. Immediate gene expression changes at the acute and early stage are of importance to understand TBI, but this information must be related to the delayed phase. Our results suggested that numerous genes were affected at 1 dpi but that this scenario was altered at 4 dpi. There was little overlap in genes between these two timepoints. In the early phase numerous genes were involved in transcription. These could have caused some of the gene expression changes at the later timepoint. The delayed phase displayed an immune response, which was not seen at 1 dpi, and which most likely participates in secondary injury mechanisms. Likewise genes involved in proteolysis were probably associated to an inflammatory and reparative response. It is helpful to obtain patterns of a large number of regulated genes in order to generate hypotheses of posttraumatic dynamics.

4.1.4 Study IV: Depolarisation and traumatic brain injury share genomic responses.

The regulated genes in experimental trauma and their corresponding expression in depolarisation displayed a similar pattern at 4 dpi (Study IV fig. 2). Depolarisation resulted in a total of 151 regulated genes, while following experimental contusion 436 genes were regulated. One hundred thirty three genes, of which 101 were upregulated and 32 downregulated, were common to both depolarisation and contusion. Large groups of the upregulated genes found in both models were ‘cell communication’, ‘metabolism’ ‘response to stress’ and ’immune response’, while for the downregulated genes ‘cell communication’, ‘signal transduction’, and ‘transport’ dominated. Genes with a decreased expression in both models clustered to the ‘Ca^{2+} signalling pathway’ and genes with increased expression to the ‘complement and coagulation cascade’. Solely in experimental contusion, genes significantly upregulated clustered to ‘hematopoietic cell lineage’, ‘ribosome’, and ‘cytokine-cytokine receptor interaction’ and downregulated to ‘focal adhesion’ and ‘ecm-receptor interaction’. No genes that matched our criteria for regulated genes were found in animals that received MK-801 post depolarisation or post trauma in order to antagonise the NMDARs. MK-801 treatment of normal animals resulted in 4 downreglated genes. No statistical significance was found for differences in regulated genes in animals subjected to injury/ depolarisation versus MK-801 injured/ depolarised treated animals. When applying local FDR to regulated genes in depolarisation and similarly for experimental contusion, a statistical effect of the NMDAR antagonist was found for 17 genes in depolarisation and 33 genes in experimental contusion (Study IV table. 3). Of these genes, many were involved in inflammatory-, immune- and/or defence response at manual evaluation and using functional classification program ‘immune response’, ‘signal’, ‘cell proliferation’ and ‘negative regulation of cellular physiological processes’. There were no degenerating neurons found after depolarisation and only a few following experimental contusion. Fluoro-Jade positive cells in TBI did not co-localise to the NMDAR subunit NR2B, which displayed IR in both hemispheres in injured/ depolarised animals (Study IV fig. 3). However, stronger NR2B IR was seen in normal animals compared to the other groups. The Ca^{2+} sensor protein hippocalcin

(Study IV fig. 4), localised to cell membranes of neurons at immunostaining, and was neither visibly reduced after injury/ depolarisation, nor after antagonist treatment.

Here we wanted to study the delayed phase after experimental trauma in more detail to pursue our previous study of expression profiles of 1 and 4 dpi. The rational for choosing a focus at 4 dpi, which is often neglected, was the need to understand long-term effects. The secondary mechanisms working during the delayed phase are important for prospective therapeutics. Our results corroborate our previous findings of a delayed immune response at 4 dpi (Holmin, Mathiesen et al. 1995). The inflammatory factors were affected in the experimental contusion, as well as in depolarisation, which is in line with other studies of cortical spreading depression SD (Jander, Schroeter et al. 2001; Thompson and Hakim 2005). The majority of genes found regulated by depolarisation were also regulated in the experimental contusion. Depolarisation, which makes part of a trauma, could thus be the induction mechanism of some of the regulated genes in TBI. However, the opposite was not true, which could be attributed to TBI being a stronger, more complex stimulus than depolarisation. The contribution of depolarisation in experimental contusion is of interest. Elicited before injury, depolarisation *per se* can render protection, while its occurrence after trauma may worsen tissue damage. Comparison of expression profiles in animals subjected to depolarisation with a following injury (preconditioning + injury), to our present results, in order to see how genes found in the three settings, depolarisation, experimental contusion and preconditioning + injury, would be regulated, could further explain the role of depolarisation. In our study, genes common to both models were regulated to a higher magnitude in trauma. One can speculate if pre-depolarisation would alter this regulation.

It is likely that depolarisation effects to some parts are mediated via NMDARs, which play a vital role in trauma. Somewhat surprisingly, interfering with the NMDARs at a time when they were already activated did not result in a statistical effect of the antagonist MK-801. One explanation could be that a drug effect on expression level in our models, which are considered to be mild injuries, might be too subtle to be detectable at this timepoint and would maybe be detectable in a larger material. Another contributing factor is the interrupted administration. Daily injections have created bursts of MK-801 and an endpoint closer to the last dose may have given other results. Nevertheless, additional analyses, revealed a significant subset of genes regulated by MK-801 in depolarisation and experimental contusion. Several of the genes could be grouped to inflammation or immune response.

The Ca^{2+} sensor protein members hippocalcin and visnin-like protein (VILIP)-1 were regulated after both experimental contusion and depolarisation. This was a novel finding. Several cathepsins were also regulated after experimental contusion. This had been anticipated but not extensively reported in the literature.

A microarray approach was suitable to study trauma, because the injury leads to complex gene expression. It was advantageous to create a more comprehensive molecular view, which has not really been possible before.

5 GENERAL DISCUSSION

"Secondary injury mechanisms" is a well known term in brain trauma, which leads the thoughts to excitotoxicity, calcium increases, free radicals production, inflammation and delayed cell death. Experimental attempts to interfere with the different processes have been more or less successful. The attempts mainly dealt with blocking the secondary injury mechanisms in order to salvage brain tissue. However, blocking receptors and signalling pathways may be too crude to obtain a desired result. The intervention would be like throwing a spanner in the works, instead of gently applying the brakes; shutting down a pathway or inhibiting a receptor will have consequences for all downstream events.

With a better understanding of the delayed phase, a need to modify the term "secondary injury mechanisms" emerges. Perhaps they should simply be referred to as "secondary mechanisms". The reason is that with an increasing insight, it becomes acknowledged that repair, remodelling, and even regeneration, occur as part of the "injury events". We realized the dichotomous nature of depolarisation in the discussions of papers I and II. The genetic data and analyses of TIMPs and proteases also indicated that the same signalling pathways could be used for destruction or survival depending on the context and timing. This was not least true for the inflammatory processes; several molecules are either shown or speculated to have dual roles. Because of 'bad becoming good', or vice versa, the functions of factors are in a flux. Further inductive and descriptive studies are a prerequisite to find relevant patterns and reactions in a paradigm with access to the kind of data generated in microarray studies. With an increased understanding of the delayed phase the possibility of potentiating endogenous repair mechanisms emerges in addition to the traditional attempts to obstruct the ongoing destructive processes. The intertwined beneficial and detrimental actions indicate that one simple drug, a miracle drug, would be unlikely to fulfil the need to obstruct post-traumatic destruction. Instead, cocktails of drugs may have to be designed to counteract the mechanisms that are destructive under any specific conditions. Likewise, the mechanisms that are protective under those conditions need to be supported.

The secondary mechanisms evolving after trauma are intricate. Under normal circumstances cell responses are ordered. They confer adaptation and communication with its surroundings. However, after trauma, massive changes occur. Several reports of trauma focus on upregulated genes, which seem to be more numerous than the downregulated. One can speculate how these contribute to the pathogenesis. The induction of a few relevant genes may be beneficial, but too many genes activated at the same time might not be physiological, but rather signify a reaction that will end up out of control. The transcription machinery capacity could be exceeded at a certain point and the cell energy stores be emptied; a cell death program or necrotic cell death might ensue. Certain cells will remain intact and can react normally to the incident, while others no longer possess that property. The resulting tissue response should therefore be a mixture of chaos and order. Additionally, certain molecular responses that would be correct in the normal state may instead lead to maladaptation in the pathological condition. Neurons with lost connections will suffer from lack of input signals and trophic factors. Although they are the most vulnerable, astrocytes are

gaining more attention and are no longer considered to be mere glue or scaffolding for neurons. Their contribution to damage could be relevant when their buffering capacities have been exceeded. Additionally, lost neuronal contact probably also affects the astrocytes as a communication between astrocytes and neurons does exist.

The contributions of different insults such as hypoxia, ischaemia, inflammation and depolarisation are useful to characterise, as the avoidance of them improves outcome. The timing of their occurrence during the posttraumatic axis probably determines their function. Ischaemia is a well studied and frequently occurring secondary insult following brain trauma. However, depolarisations within a traumatised area also worsen the injury. Although prophylactic treatment is not relevant in the case of TBI, protection via depolarisation is still interesting in experimental models, as some regulated genes are common to TBI and depolarisation. These will either induce protection or destruction depending on their context and should therefore be good candidates for treatment – but only if the context is well understood and defined. An important parameter in brain trauma is age, as the mature brain tissue does not possess the same plasticity as the immature. The ECM, receptors and transcription factors among others, showed post-traumatic changes that were reminiscent of their expression during development. The upregulation of several genes that are normally expressed in the premature state could be a way for the brain tissue to create a more immature milieu suitable for remodeling and repair.

Large-scale screening of gene expression in traumatic brain injury is a fairly new approach in brain trauma research. A search on PubMed with ‘traumatic brain injury and microarray only resulted in 27 reports at the time of writing this thesis. Although this number is growing, the opportunity to use microarrays is new. The approach is suitable for the complexity of trauma and allows a direct assessment of a large number of simultaneous reactions. This technology presents a more complete picture of the genetic events after injury than methods designed to study single genes. Even if a gene regulation description necessarily omits other relevant aspects, such as protein actions, ions and energy levels, an imprint of a large number of characteristic changes allows much broader analyses of different traumatic mechanisms, timepoints and opposing reactions than previously. The methodology allows hypotheses concerning patterns of gene regulation. An example was our hypothesis regarding depolarisation as a probable mechanism in post-traumatic events following an experimental contusion; the resulting events were defined through their patterns of gene expression. The amount of reports that describe different aspects of biochemical pathogenesis by other methods is enormous and a huge number of different and relevant mechanisms are known. Their dynamics and interrelationship are, however, hardly known at all. With microarrays, such questions can be more easily addressed. We detected fundamental differences between 1 and 4 days after trauma; supporting the previous discussion regarding a temporal pattern for posttraumatic events. An unexpected difficulty arose from the mere amount of information that became available. The methods to evaluate such data are, however, constantly improving. While better computerised analyses will simplify data interpretation, a reductionist study of single pathways remains important. A broad and unbiased screening can identify hitherto unknown important mediators and pathways. The analyses of specific mRNA, their cellular localisation and resulting

proteins as we did for CD44, osteopontin and TIMPs produces detailed information that is necessary to understand their actions and relevance.

In the future, datasets generated from specified brain injuries and deposited in a common assembly would allow for a grand research resource. With such large amounts of data, patterns of gene regulation would appear more pronounced and therefore be easier detected and addressed. A brain trauma database would also provide a good tool to test an idea without the need of a lab bench and experimental animals. Improved data-mining will be useful to explore common regulators of regulated genes: particular transcription factors, calcium dependence, and exploration of genes regulated by metal ions. Many of our findings need to be interpreted against a background of data that would reflect variations of our experimental conditions. Different time-points, injury mechanisms, energy transfer and therapeutic interventions are necessary to form and test further hypotheses regarding relevant molecular changes.

6 CONCLUSION

➢Our results corroborate that depolarisation is a mechanism of gene induction in trauma. Experimental contusion and depolarisation shared several genomic responses in the delayed phase after injury. In line with this is that:

- depolarisation-induced nestin expression resembled nestin expression after experimental trauma,
- expression patterns of MMP-TIMP system were similarly triggered by both depolarisation and experimental contusion,
- traumatical osteopontin and CD44 expression resembled the expression of depolarisation induced gene expression of osteopontin and CD44

➢ Early and delayed alterations in gene expression had little overlapping genomic responses. The early posttraumatic phase mounted a larger response in number of genes compared to the late phase. Secondary injury mechanisms in a delayed phase was likely represented by genes involved in the inflammation and proteolysis

➢ Intervention with NMDA receptors after depolarisation and experimental contusion did not have a significant effect on gene expression

➢ Nestin protein expression was induced in reactive astrocytes by depolarisation

➢ MMP-9 and TIMP-1 displayed time-dependent expression patterns after depolarisation and experimental contusion

➢ Osteopontin and its receptor CD44 were locally upregulated at the injury site with a correlating expression at protein level

➢ Microarray technology allowed a new twofold approach to study brain trauma: Arrays were used in a wide and unbiased search for possibly relevant genetic events, and to produce an image of the totality of genetic events at a given time. Both approaches allowed for generation of hypotheses that would have been difficult to test with previously available methods.

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8 REFERENCES

Aarts, M., Y. Liu, et al. (2002). "Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions." Science. **298**(5594): 846-50.

Abilleira, S., J. Montaner, et al. (2003). "Matrix metalloproteinase-9 concentration after spontaneous intracerebral hemorrhage

Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke

Biochemical changes and inflammatory response as markers for brain ischaemia: molecular markers of diagnostic utility and prognosis in human clinical practice." J Neurosurg. **99**(1): 65-70.

Altman, J. and G. D. Das (1965). "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." J Comp Neurol. **124**(3): 319-35.

Anderova, M., S. Kubinova, et al. (2001). "Effect of elevated K(+), hypotonic stress, and cortical spreading depression on astrocyte swelling in GFAP-deficient mice." Glia. **35**(3): 189-203.

Anderson, C. V. and E. D. Bigler (1995). "Ventricular dilation, cortical atrophy, and neuropsychological outcome following traumatic brain injury." J Neuropsychiatry Clin Neurosci. **7**(1): 42-8.

Anderson, T. R., C. R. Jarvis, et al. (2005). "Blocking the anoxic depolarization protects without functional compromise following simulated stroke in cortical brain slices." J Neurophysiol. **93**(2): 963-79. Epub 2004 Sep 29.

Arundine, M. and M. Tymianski (2004). "Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury." Cell Mol Life Sci. **61**(6): 657-68.

Asahi, M., X. Wang, et al. (2001). "Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia." J Neurosci **21**(19): 7724-32.

Azcoitia, I., L. L. Doncarlos, et al. (2002). "Estrogen and brain vulnerability." Neurotox Res. **4**(3): 235-45.

Azcoitia, I., A. Sierra, et al. (2001). "Brain aromatase is neuroprotective." J Neurobiol. **47**(4): 318-29.

Baker, A. J., R. J. Moulton, et al. (1993). "Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans." J Neurosurg. **79**(3): 369-72.

Balestrino, M. (1995). "Pathophysiology of anoxic depolarization: new findings and a working hypothesis." J Neurosci Methods. **59**(1): 99-103.

Basarsky, T. A., S. N. Duffy, et al. (1998). "Imaging spreading depression and associated intracellular calcium waves in brain slices." J Neurosci. **18**(18): 7189-99.

Basarsky, T. A., D. Feighan, et al. (1999). "Glutamate release through volume-activated channels during spreading depression." J Neurosci. **19**(15): 6439-45.

Bayir, H., D. W. Marion, et al. (2004). "Marked gender effect on lipid peroxidation after severe traumatic brain injury in adult patients." J Neurotrauma. **21**(1): 1-8.

Bellail, A. C., S. B. Hunter, et al. (2004). "Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion." Int J Biochem Cell Biol **36**(6): 1046-69.

Bellander, B. M. (2004). On the role of complement activation following traumatic brain injury. Department of Clinical Neuroscience. Stockholm, Karolinska Institutet.

Bellander, B. M., S. K. Singhrao, et al. (2001). "Complement activation in the human brain after traumatic head injury." J Neurotrauma **18**(12): 1295-311.

Bellander, B. M., H. von Holst, et al. (1996). "Activation of the complement cascade and increase of clusterin in the brain following a cortical contusion in the adult rat." J Neurosurg **85**(3): 468-75.

Benveniste, H., J. Drejer, et al. (1984). "Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis." J Neurochem. **43**(5): 1369-74.

Biegon, A., P. A. Fry, et al. (2004). "Dynamic changes in N-methyl-D-aspartate receptors after closed head injury in mice: Implications for treatment of

neurological and cognitive deficits." Proc Natl Acad Sci U S A. **101**(14): 5117-22. Epub 2004 Mar 24.
Bito, H. and S. Takemoto-Kimura (2003). "Ca(2+)/CREB/CBP-dependent gene regulation: a shared mechanism critical in long-term synaptic plasticity and neuronal survival." Cell Calcium. **34**(4-5): 425-30.
Bonthius, D. J. and O. Steward (1993). "Induction of cortical spreading depression with potassium chloride upregulates levels of messenger RNA for glial fibrillary acidic protein in cortex and hippocampus: inhibition by MK-801." Brain Res **618**(1): 83-94.
Bramlett, H. M. and W. D. Dietrich (2002). "Quantitative structural changes in white and gray matter 1 year following traumatic brain injury in rats." Acta Neuropathol (Berl). **103**(6): 607-14. Epub 2002 Mar 20.
Bramlett, H. M. and W. D. Dietrich (2004). "Pathophysiology of cerebral ischemia and brain trauma: similarities and differences." J Cereb Blood Flow Metab. **24**(2): 133-50.
Braunewell, K., P. Riederer, et al. (2001). "Abnormal localization of two neuronal calcium sensor proteins, visinin-like proteins (vilips)-1 and -3, in neocortical brain areas of Alzheimer disease patients." Dement Geriatr Cogn Disord. **12**(2): 110-6.
Brenman, J. E., D. S. Chao, et al. (1996). "Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains." Cell. **84**(5): 757-67.
Bruns, J., Jr. and W. A. Hauser (2003). "The epidemiology of traumatic brain injury: a review." Epilepsia. **44**(Suppl 10): 2-10.
Buki, A., O. Farkas, et al. (2003). "Preinjury administration of the calpain inhibitor MDL-28170 attenuates traumatically induced axonal injury." J Neurotrauma. **20**(3): 261-8.
Castellanos, M., R. Leira, et al. (2003). "Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke." Stroke **34**(1): 40-6.
Castillo, J. and I. Rodriguez (2004). "Biochemical changes and inflammatory response as markers for brain ischaemia: molecular markers of diagnostic utility and prognosis in human clinical practice." Cerebrovasc Dis. **17**(Suppl 1): 7-18.
Cernak, I. (2005). "Animal models of head trauma." NeuroRx. **2**(3): 410-22.
Cernak, I., R. Vink, et al. (2004). "The pathobiology of moderate diffuse traumatic brain injury as identified using a new experimental model of injury in rats." Neurobiol Dis. **17**(1): 29-43.
Chaillan, F. A., S. Rivera, et al. (2006). "Involvement of tissue inhibition of metalloproteinases-1 in learning and memory in mice." Behav Brain Res **21**: 21.
Chazot, P. L., O. V. Godukhin, et al. (2002). "Spreading depression-induced preconditioning in the mouse cortex: differential changes in the protein expression of ionotropic nicotinic acetylcholine and glutamate receptors." J Neurochem. **83**(5): 1235-8.
Chen, Y. and R. A. Swanson (2003). "Astrocytes and brain injury." J Cereb Blood Flow Metab. **23**(2): 137-49.
Chow, A. K., C. S. Thompson, et al. (2002). "Cortical spreading depression transiently activates MAP kinases." Brain Res Mol Brain Res. **99**(1): 75-81.
Chung, R. S., G. H. McCormack, et al. (2005). "Glutamate induces rapid loss of axonal neurofilament proteins from cortical neurons in vitro." Exp Neurol. **193**(2): 481-8.
Church, A. J. and R. D. Andrew (2005). "Spreading depression expands traumatic injury in neocortical brain slices." J Neurotrauma. **22**(2): 277-90.
Clark, R. S., P. M. Kochanek, et al. (1996). "Inducible nitric oxide synthase expression in cerebrovascular smooth muscle and neutrophils after traumatic brain injury in immature rats." Pediatr Res. **39**(5): 784-90.
Clayton, D. A. and M. D. Browning (2001). "Deficits in the expression of the NR2B subunit in the hippocampus of aged Fisher 344 rats." Neurobiol Aging. **22**(1): 165-8.

Clayton, D. A., M. H. Mesches, et al. (2002). "A hippocampal NR2B deficit can mimic age-related changes in long-term potentiation and spatial learning in the Fischer 344 rat." J Neurosci. **22**(9): 3628-37.
Coimbra, R., D. B. Hoyt, et al. (2003). "Does sexual dimorphism influence outcome of traumatic brain injury patients? The answer is no!" J Trauma. **54**(4): 689-700.
Conti, A. C., R. Raghupathi, et al. (1998). "Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period." J Neurosci. **18**(15): 5663-72.
Crocker, S. J., A. Pagenstecher, et al. (2004). "The TIMPs tango with MMPs and more in the central nervous system." J Neurosci Res. **75**(1): 1-11.
Cull-Candy, S., S. Brickley, et al. (2001). "NMDA receptor subunits: diversity, development and disease." Curr Opin Neurobiol. **11**(3): 327-35.
Dahlstrand, J., M. Lardelli, et al. (1995). "Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system." Brain Res Dev Brain Res. **84**(1): 109-29.
Davis, D. P., D. J. Douglas, et al. (2006). "Traumatic brain injury outcomes in pre- and post- menopausal females versus age-matched males." J Neurotrauma. **23**(2): 140-8.
Denhardt, D. T., M. Noda, et al. (2001). "Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival." J Clin Invest **107**(9): 1055-61.
Dhillon, H. S., H. M. Carman, et al. (1999). "Regional activities of phospholipase C after experimental brain injury in the rat." Neurochem Res. **24**(6): 751-5.
Dietrich, W. D., O. Alonso, et al. (1994). "Early microvascular and neuronal consequences of traumatic brain injury: a light and electron microscopic study in rats." J Neurotrauma. **11**(3): 289-301.
Dijkhuizen, R. M., J. P. Beekwilder, et al. (1999). "Correlation between tissue depolarizations and damage in focal ischemic rat brain." Brain Res. **840**(1-2): 194-205.
Dixon, C. E., G. L. Clifton, et al. (1991). "A controlled cortical impact model of traumatic brain injury in the rat." J Neurosci Methods. **39**(3): 253-62.
Dixon, C. E., B. G. Lyeth, et al. (1987). "A fluid percussion model of experimental brain injury in the rat." J Neurosurg. **67**(1): 110-9.
Doherty, T. M., K. Asotra, et al. (2002). "Therapeutic developments in matrix metalloproteinas inhibition." Expert Opinion **12**(5): 665-707.
Douen, A. G., L. Dong, et al. (2004). "Regulation of nestin expression after cortical ablation in adult rat brain." Brain Res. **1008**(2): 139-46.
Dubal, D. B., S. W. Rau, et al. (2006). "Differential modulation of estrogen receptors (ERs) in ischemic brain injury: a role for ERalpha in estradiol-mediated protection against delayed cell death." Endocrinology. **147**(6): 3076-84. Epub 2006 Mar 9.
Dudoit S., Y. Y. H., Callow M.J. Speed T.P. (2000). Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. Berkeley, Statistics Department, University of California.
Ehlers, M. D., E. T. Fung, et al. (1998). "Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments." J Neurosci. **18**(2): 720-30.
Elf, K., P. Nilsson, et al. (2002). "Outcome after traumatic brain injury improved by an organized secondary insult program and standardized neurointensive care." Crit Care Med. **30**(9): 2129-34.
Ellison, J. A., F. C. Barone, et al. (1999). "Matrix remodeling after stroke. De novo expression of matrix proteins and integrin receptors." Ann N Y Acad Sci **890**: 204-22.
Ellison, J. A., J. J. Velier, et al. (1998). "Osteopontin and its integrin receptor alpha(v)beta3 are upregulated during formation of the glial scar after focal stroke
Delayed expression of osteopontin after focal stroke in the rat." Stroke **29**(8): 1698-706; discussion 1707.

Ernst, C. and B. R. Christie (2005). "Nestin-expressing cells and their relationship to mitotically active cells in the subventricular zones of the adult rat." Eur J Neurosci. **22**(12): 3059-66.
Ernst, C. and B. R. Christie (2006). "The putative neural stem cell marker, nestin, is expressed in heterogeneous cell types in the adult rat neocortex." Neuroscience. **138**(1): 183-8. Epub 2005 Dec 15.
Esteller, M., P. G. Corn, et al. (2001). "A gene hypermethylation profile of human cancer." Cancer Res. **61**(8): 3225-9.
Fabricius, M., S. Fuhr, et al. (2006). "Cortical spreading depression and peri-infarct depolarization in acutely injured human cerebral cortex." Brain. **129**(Pt 3): 778-90. Epub 2005 Dec 19.
Faden, A. I. (2002). "Neuroprotection and traumatic brain injury: theoretical option or realistic proposition." Curr Opin Neurol. **15**(6): 707-12.
Faden, A. I., P. Demediuk, et al. (1989). "The role of excitatory amino acids and NMDA receptors in traumatic brain injury." Science. **244**(4906): 798-800.
Farin, A., R. Deutsch, et al. (2003). "Sex-related differences in patients with severe head injury: greater susceptibility to brain swelling in female patients 50 years of age and younger." J Neurosurg. **98**(1): 32-6.
Fawcett, J. W. R., A.E. Dunnett, S.B. (2001). Neuroprotection. Brain damage, brain repair. New York, Oxford University Press, Inc.
Feeney, D. M., M. G. Boyeson, et al. (1981). "Responses to cortical injury: I. Methodology and local effects of contusions in the rat." Brain Res **211**(1): 67-77.
Flores-Morales, A., N. Stahlberg, et al. (2001). "Microarray analysis of the in vivo effects of hypophysectomy and growth hormone treatment on gene expression in the rat." Endocrinology **142**(7): 3163-76.
Friedman, G., P. Froom, et al. (1999). "Apolipoprotein E-epsilon4 genotype predicts a poor outcome in survivors of traumatic brain injury." Neurology. **52**(2): 244-8.
Frisen, J., C. B. Johansson, et al. (1995). "Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury." J Cell Biol. **131**(2): 453-64.
Furuta, Y., M. Kobayashi, et al. (1999). "Age-related changes in expression of hippocalcin and NVP2 in rat brain." Neurochem Res. **24**(5): 651-8.
Gahm, C., S. Holmin, et al. (2000). "Temporal profiles and cellular sources of three nitric oxide synthase isoforms in the brain after experimental contusion." Neurosurgery. **46**(1): 169-77.
Garcia-Ovejero, D., S. Veiga, et al. (2002). "Glial expression of estrogen and androgen receptors after rat brain injury." J Comp Neurol. **450**(3): 256-71.
Geddes-Klein, D. M., K. B. Schiffman, et al. (2006). "Mechanisms and consequences of neuronal stretch injury in vitro differ with the model of trauma." J Neurotrauma. **23**(2): 193-204.
Gibbons, S. J., J. R. Brorson, et al. (1993). "Calcium influx and neurodegeneration." Ann N Y Acad Sci. **679**: 22-33.
Gorelova, N. A., V. I. Koroleva, et al. (1987). "Ketamine blockade of cortical spreading depression in rats." Electroencephalogr Clin Neurophysiol. **66**(4): 440-7.
Gorji, A. (2001). "Spreading depression: a review of the clinical relevance." Brain Res Brain Res Rev. **38**(1-2): 33-60.
Graham, D. I. (1995). Neuropathology of head injury. Neurotrauma. R. K. Narayan, Wilberger, J.E., Povlishock, J.T. The McGraw-Hill Companies,Inc.**:** 43-59.
Groswasser, Z., M. Cohen, et al. (1998). "Female TBI patients recover better than males." Brain Inj. **12**(9): 805-8.
Gursoy-Ozdemir, Y., J. Qiu, et al. (2004). "Cortical spreading depression activates and upregulates MMP-9." J Clin Invest. **113**(10): 1447-55.
Haas, J. and S. L. Erdo (1991). "Quisqualate-induced excitotoxic death of glial cells: transient vulnerability of cultured astrocytes." Glia. **4**(1): 111-4.
Hans, V. H., T. Kossmann, et al. (1999). "Experimental axonal injury triggers interleukin-6 mRNA, protein synthesis and release into cerebrospinal fluid." J Cereb Blood Flow Metab. **19**(2): 184-94.

Hartings, J. A., M. L. Rolli, et al. (2003). "Delayed secondary phase of peri-infarct depolarizations after focal cerebral ischemia: relation to infarct growth and neuroprotection." J Neurosci. **23**(37): 11602-10.
Hashimoto, Y., H. Jiang, et al. (2000). "Neuronal apoptosis by apolipoprotein E4 through low-density lipoprotein receptor-related protein and heterotrimeric GTPases." J Neurosci. **20**(22): 8401-9.
Hasselblatt, M., H. Ehrenreich, et al. (2006). "The brain erythropoietin system and its potential for therapeutic exploitation in brain disease." J Neurosurg Anesthesiol. **18**(2): 132-8.
Hauptschein, R. S., K. E. Sloan, et al. (2005). "Functional proteomic screen identifies a modulating role for CD44 in death receptor-mediated apoptosis." Cancer Res **65**(5): 1887-96.
Hayakawa, T., K. Yamashita, et al. (1994). "Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2)." J Cell Sci **107**(Pt 9): 2373-9.
Hayakawa, T., K. Yamashita, et al. (1992). "Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum." FEBS Lett **298**(1): 29-32.
Hayashi, Y., M. Nishio, et al. (1999). "Regulation of neuronal nitric-oxide synthase by calmodulin kinases." J Biol Chem. **274**(29): 20597-602.
Heese, K., C. Hock, et al. (1998). "Inflammatory signals induce neurotrophin expression in human microglial cells." J Neurochem. **70**(2): 699-707.
Hermann, D. M., G. Mies, et al. (1999). "Expression of c-fos, junB, c-jun, MKP-1 and hsp72 following traumatic neocortical lesions in rats--relation to spreading depression." Neuroscience **88**(2): 599-608.
Hetman, M. and G. Kharebava (2006). "Survival signaling pathways activated by NMDA receptors." Curr Top Med Chem. **6**(8): 787-99.
Hirai, S., H. Kawasaki, et al. (1991). "Degradation of transcription factors, c-Jun and c-Fos, by calpain." FEBS Lett. **287**(1-2): 57-61.
Holmin, S., P. Almqvist, et al. (1997). "Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury." Eur J Neurosci. **9**(1): 65-75.
Holmin, S. and T. Mathiesen (1999). "Long-term intracerebral inflammatory response after experimental focal brain injury in rat." Neuroreport. **10**(9): 1889-91.
Holmin, S. and T. Mathiesen (2000). "Intracerebral administration of interleukin-1beta and induction of inflammation, apoptosis, and vasogenic edema." J Neurosurg. **92**(1): 108-20.
Holmin, S., T. Mathiesen, et al. (1995). "Intracerebral inflammatory response to experimental brain contusion." Acta Neurochir **132**(1-3): 110-9.
Holmin, S., M. Schalling, et al. (1997). "Delayed cytokine expression in rat brain following experimental contusion." J Neurosurg **86**(3): 493-504.
Holmin, S., J. Soderlund, et al. (1998). "Intracerebral inflammation after human brain contusion." Neurosurgery **42**(2): 291-8; discussion 298-9.
Huang, Y., X. Q. Liu, et al. (2001). "Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons." Proc Natl Acad Sci U S A. **98**(15): 8838-43. Epub 2001 Jul 10.
Ikeda, R., M. S. Kurokawa, et al. (2005). "Transplantation of neural cells derived from retinoic acid-treated cynomolgus monkey embryonic stem cells successfully improved motor function of hemiplegic mice with experimental brain injury." Neurobiol Dis. **20**(1): 38-48.
Ikonomidou, C., V. Stefovska, et al. (2000). "Neuronal death enhanced by N-methyl-D-aspartate antagonists." Proc Natl Acad Sci U S A. **97**(23): 12885-90.
Itoh, T., T. Satou, et al. (2005). "Isolation of neural stem cells from damaged rat cerebral cortex after traumatic brain injury." Neuroreport. **16**(15): 1687-91.
Ivanov, A., C. Pellegrino, et al. (2006). "Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons." J Physiol. **572**(Pt 3): 789-98.
Jander, S., S. Bussini, et al. (2002). "Osteopontin: a novel axon-regulated Schwann cell gene." J Neurosci Res **67**(2): 156-66.

Jander, S., M. Schroeter, et al. (2001). "Cortical spreading depression induces proinflammatory cytokine gene expression in the rat brain." J Cereb Blood Flow Metab **21**(3): 218-25.
Jarvis, C. R., T. R. Anderson, et al. (2001). "Anoxic depolarization mediates acute damage independent of glutamate in neocortical brain slices." Cereb Cortex. **11**(3): 249-59.
Jaworski, D. M. (2000). "Differential regulation of tissue inhibitor of metalloproteinase mRNA expression in response to intracranial injury." Glia **30**(2): 199-208.
Jourquin, J., E. Tremblay, et al. (2005). "Tissue inhibitor of metalloproteinases-1 (TIMP-1) modulates neuronal death, axonal plasticity, and learning and memory." Eur J Neurosci. **22**(10): 2569-78.
Kampfl, A., R. M. Posmantur, et al. (1997). "Mechanisms of calpain proteolysis following traumatic brain injury: implications for pathology and therapy: implications for pathology and therapy: a review and update." J Neurotrauma. **14**(3): 121-34.
Kariko, K., V. A. Harris, et al. (1998). "Effect of cortical spreading depression on the levels of mRNA coding for putative neuroprotective proteins in rat brain." J Cereb Blood Flow Metab **18**(12): 1308-15.
Kato, H. and W. Walz (2000). "The initiation of the microglial response." Brain Pathol. **10**(1): 137-43.
Kawahara, N., C. A. Ruetzler, et al. (1999). "Cortical spreading depression increases protein synthesis and upregulates basic fibroblast growth factor." Exp Neurol **158**(1): 27-36.
Kernie, S. G., T. M. Erwin, et al. (2001). "Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice." J Neurosci Res **66**(3): 317-26.
Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer. **26**(4): 239-57.
Kim, M. D., H. J. Cho, et al. (2004). "Expression of osteopontin and its ligand, CD44, in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis." J Neuroimmunol **151**(1-2): 78-84.
Kimura, Y., M. Madhavan, et al. (2003). "Expression of complement 3 and complement 5 in newt limb and lens regeneration." J Immunol. **170**(5): 2331-9.
Kobayashi, M., T. Masaki, et al. (2005). "Hippocalcin-deficient mice display a defect in cAMP response element-binding protein activation associated with impaired spatial and associative memory." Neuroscience. **133**(2): 471-84.
Kobayashi, S., V. A. Harris, et al. (1995). "Spreading depression induces tolerance of cortical neurons to ischemia in rat brain." J Cereb Blood Flow Metab **15**(5): 721-7.
Kobori, N., G. L. Clifton, et al. (2002). "Altered expression of novel genes in the cerebral cortex following experimental brain injury." Brain Res Mol Brain Res **104**(2): 148-58.
Korhonen, L., I. Hansson, et al. (2005). "Hippocalcin protects against caspase-12-induced and age-dependent neuronal degeneration." Mol Cell Neurosci. **28**(1): 85-95.
Kossmann, T., V. Hans, et al. (1996). "Interleukin-6 released in human cerebrospinal fluid following traumatic brain injury may trigger nerve growth factor production in astrocytes." Brain Res. **713**(1-2): 143-52.
Krapivinsky, G., L. Krapivinsky, et al. (2003). "The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1." Neuron. **40**(4): 775-84.
Kraus, J. F., McArthur, D.L. (1993). Epidemiology of head injury. Head Injury. P. R. Cooper. Baltimore, Williams & Wilkins**:** 1-26.
Kumar, A., L. Zou, et al. (2002). "N-methyl-D-aspartate receptors: transient loss of NR1/NR2A/NR2B subunits after traumatic brain injury in a rodent model." J Neurosci Res. **67**(6): 781-6.
Kupina, N. C., M. R. Detloff, et al. (2003). "Cytoskeletal protein degradation and neurodegeneration evolves differently in males and females following experimental head injury." Exp Neurol **180**(1): 55-73.

Kuppner, M. C., E. Van Meir, et al. (1992). "Differential expression of the CD44 molecule in human brain tumours." Int J Cancer. **50**(4): 572-7.
Kurca, E., S. Sivak, et al. (2006). "Impaired cognitive functions in mild traumatic brain injury patients with normal and pathologic magnetic resonance imaging." Neuroradiology **20**: 20.
Lander, E. S., L. M. Linton, et al. (2001). "Initial sequencing and analysis of the human genome." Nature. **409**(6822): 860-921.
Larrosa, B., J. Pastor, et al. (2006). "A role for glutamate and glia in the fast network oscillations preceding spreading depression." Neuroscience. **141**(2): 1057-68. Epub 2006 May 18.
Leao, A. (1944). "Spreading depression of activity in the cerebral cortex." J Neurophysiol **7**: 359-90.
Lendahl, U., L. B. Zimmerman, et al. (1990). "CNS stem cells express a new class of intermediate filament protein." Cell. **60**(4): 585-95.
Lenzlinger, P. M., M. C. Morganti-Kossmann, et al. (2001). "The duality of the inflammatory response to traumatic brain injury." Mol Neurobiol **24**(1-3): 169-81.
Lepekhin, E. A., C. Eliasson, et al. (2001). "Intermediate filaments regulate astrocyte motility." J Neurochem. **79**(3): 617-25.
Li, H., S. Pin, et al. (2005). "Sex differences in cell death." Ann Neurol. **58**(2): 317-21.
Li, H. H., S. M. Lee, et al. (2004). "Differential gene expression in hippocampus following experimental brain trauma reveals distinct features of moderate and severe injuries." J Neurotrauma **21**(9): 1141-53.
Liem, R. K. (1993). "Molecular biology of neuronal intermediate filaments." Curr Opin Cell Biol. **5**(1): 12-6.
Lindsey, J. C., M. E. Lusher, et al. (2004). "Identification of tumour-specific epigenetic events in medulloblastoma development by hypermethylation profiling." Carcinogenesis. **25**(5): 661-8. Epub 2003 Dec 19.
Loddick, S. A. and N. J. Rothwell (1996). "Neuroprotective effects of human recombinant interleukin-1 receptor antagonist in focal cerebral ischaemia in the rat." J Cereb Blood Flow Metab. **16**(5): 932-40.
Longhi, L., K. E. Saatman, et al. (2001). "A review and rationale for the use of genetically engineered animals in the study of traumatic brain injury." J Cereb Blood Flow Metab **21**(11): 1241-58.
Lu, J., A. Marmarou, et al. (2005). "Mortality from traumatic brain injury." Acta Neurochir Suppl. **95**: 281-5.
Lucas, D. R. and J. P. Newhouse (1957). "The toxic effect of sodium L-glutamate on the inner layers of the retina." AMA Arch Ophthalmol. **58**(2): 193-201.
Lye, T. C. and E. A. Shores (2000). "Traumatic brain injury as a risk factor for Alzheimer's disease: a review." Neuropsychol Rev. **10**(2): 115-29.
Maas, A. I. (2001). "Neuroprotective agents in traumatic brain injury." Expert Opin Investig Drugs. **10**(4): 753-67.
Magnusson, K. R., S. E. Nelson, et al. (2002). "Age-related changes in the protein expression of subunits of the NMDA receptor." Brain Res Mol Brain Res. **99**(1): 40-5.
Mahley, R. W. and S. C. Rall, Jr. (2000). "Apolipoprotein E: far more than a lipid transport protein." Annu Rev Genomics Hum Genet. **1**: 507-37.
Mali, R. S., M. Cheng, et al. (2005). "Intravitreous injection of a membrane depolarization agent causes retinal degeneration via matrix metalloproteinase-9." Invest Ophthalmol Vis Sci. **46**(6): 2125-32.
Marik, P. E., J. Varon, et al. (2002). "Management of head trauma." Chest. **122**(2): 699-711.
Marmarou, A., P. Fatouros, et al. (1990). "In vivo measurement of brain water by MRI." Acta Neurochir Suppl (Wien). **51**: 123-4.
Marmarou, A., M. A. Foda, et al. (1994). "A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics." J Neurosurg. **80**(2): 291-300.
Martins-Ferreira, H., M. Nedergaard, et al. (2000). "Perspectives on spreading depression." Brain Res Brain Res Rev. **32**(1): 215-34.

Marvin, M. J., J. Dahlstrand, et al. (1998). "A rod end deletion in the intermediate filament protein nestin alters its subcellular localization in neuroepithelial cells of transgenic mice." J Cell Sci. **111**(Pt 14): 1951-61.
Mathew, P., R. Bullock, et al. (1996). "Changes in local microvascular permeability and in the effect of intervention with 21-aminosteroid (Tirilazad) in a new experimental model of focal cortical injury in the rat." J Neurotrauma. **13**(8): 465-72.
Mathisen, P. M., J. M. Johnson, et al. (1999). "Visinin-like protein (VILIP) is a neuron-specific calcium-dependent double-stranded RNA-binding protein." J Biol Chem. **274**(44): 31571-6.
Matute, C., M. V. Sanchez-Gomez, et al. (1997). "Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes." Proc Natl Acad Sci U S A. **94**(16): 8830-5.
McIntosh, T. K., L. Noble, et al. (1987). "Traumatic brain injury in the rat: characterization of a midline fluid-percussion model." Cent Nerv Syst Trauma. **4**(2): 119-34.
McIntosh, T. K., R. Vink, et al. (1989). "Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model." Neuroscience. **28**(1): 233-44.
Meller, R., S. L. Stevens, et al. (2005). "Neuroprotection by osteopontin in stroke." J Cereb Blood Flow Metab **25**(2): 217-25.
Menet, V., M. Gimenez y Ribotta, et al. (2001). "Inactivation of the glial fibrillary acidic protein gene, but not that of vimentin, improves neuronal survival and neurite growth by modifying adhesion molecule expression." J Neurosci. **21**(16): 6147-58.
Menet, V., Y. R. M. Gimenez, et al. (2000). "GFAP null astrocytes are a favorable substrate for neuronal survival and neurite growth." Glia. **31**(3): 267-72.
Mielke, R., K. Zerres, et al. (1998). "Apolipoprotein E polymorphism influences the cerebral metabolic pattern in Alzheimer's disease." Neurosci Lett. **254**(1): 49-52.
Mira, E., S. Manes, et al. (1999). "Insulin-like growth factor I-triggered cell migration and invasion are mediated by matrix metalloproteinase-9." Endocrinology. **140**(4): 1657-64.
Moon, C., M. Ahn, et al. (2004). "Temporal patterns of the embryonic intermediate filaments nestin and vimentin expression in the cerebral cortex of adult rats after cryoinjury." Brain Res. **1028**(2): 238-42.
Morales, D. M., N. Marklund, et al. (2005). "Experimental models of traumatic brain injury: do we really need to build a better mousetrap?" Neuroscience. **136**(4): 971-89. Epub 2005 Oct 20.
Morita-Fujimura, Y., M. Fujimura, et al. (2000). "Overexpression of copper and zinc superoxide dismutase in transgenic mice prevents the induction and activation of matrix metalloproteinases after cold injury-induced brain trauma." J Cereb Blood Flow Metab **20**(1): 130-8.
Morrison, B., 3rd, J. H. Eberwine, et al. (2000). "Traumatic injury induces differential expression of cell death genes in organotypic brain slice cultures determined by complementary DNA array hybridization." Neuroscience **96**(1): 131-9.
Morshead, C. M., B. A. Reynolds, et al. (1994). "Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells." Neuron. **13**(5): 1071-82.
Mukherjee, P. and G. M. Pasinetti (2001). "Complement anaphylatoxin C5a neuroprotects through mitogen-activated protein kinase-dependent inhibition of caspase 3." J Neurochem. **77**(1): 43-9.
Myer, D. J., G. G. Gurkoff, et al. (2006). "Essential protective roles of reactive astrocytes in traumatic brain injury." Brain **5**: 5.
Myers, W. A., J. D. Churchill, et al. (2000). "Role of NMDA receptors in adult primate cortical somatosensory plasticity." J Comp Neurol. **418**(4): 373-82.
Nakamura, T., Y. Hua, et al. (2005). "Estrogen therapy for experimental intracerebral hemorrhage in rats." J Neurosurg. **103**(1): 97-103.
Nakamura, T., G. Xi, et al. (2006). "Effects of endogenous and exogenous estrogen on intracerebral hemorrhage-induced brain damage in rats." Acta Neurochir Suppl. **96**: 218-21.

Nallet, H., E. T. MacKenzie, et al. (1999). "The nature of penumbral depolarizations following focal cerebral ischemia in the rat." Brain Res. **842**(1): 148-58.
Narayan, R. K., M. E. Michel, et al. (2002). "Clinical trials in head injury." J Neurotrauma. **19**(5): 503-57.
Narita, M., G. Bu, et al. (1997). "The low-density lipoprotein receptor-related protein, a multifunctional apolipoprotein E receptor, modulates hippocampal neurite development." J Neurochem. **68**(2): 587-95.
Nedergaard, M. and A. J. Hansen (1988). "Spreading depression is not associated with neuronal injury in the normal brain." Brain Res. **449**(1-2): 395-8.
Newcomb, J. K., X. Zhao, et al. (1999). "Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat." Exp Neurol. **158**(1): 76-88.
Obeidat, A. S., C. R. Jarvis, et al. (2000). "Glutamate does not mediate acute neuronal damage after spreading depression induced by O2/glucose deprivation in the hippocampal slice." J Cereb Blood Flow Metab. **20**(2): 412-22.
Oh, D. Y., C. Yon, et al. (2006). "Hippocalcin increases phospholipase D2 expression through extracellular signal-regulated kinase activation and lysophosphatidic acid potentiates the hippocalcin-induced phospholipase D2 expression." J Cell Biochem. **97**(5): 1052-65.
Oikawa, K., S. Kimura, et al. (2004). "Neuronal calcium sensor protein visinin-like protein-3 interacts with microsomal cytochrome b5 in a Ca2+-dependent manner." J Biol Chem. **279**(15): 15142-52. Epub 2004 Jan 22.
Olney, J. W. (1969). "Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate." Science. **164**(880): 719-21.
Ontl, T., Y. Xing, et al. (2004). "Development and aging of N-methyl-D-aspartate receptor expression in the prefrontal/frontal cortex of mice." Neuroscience. **123**(2): 467-79.
Osteen, C. L., C. C. Giza, et al. (2004). "Injury-induced alterations in N-methyl-D-aspartate receptor subunit composition contribute to prolonged 45calcium accumulation following lateral fluid percussion." Neuroscience. **128**(2): 305-22.
Oyesiku, N. M., C. O. Evans, et al. (1999). "Regional changes in the expression of neurotrophic factors and their receptors following acute traumatic brain injury in the adult rat brain." Brain Res **833**(2): 161-72.
Parker, G. R., H. M. Cathcart, et al. (2005). "Apolipoprotein gene E4 allele promoter polymorphisms as risk factors for Alzheimer's disease." Psychiatr Genet. **15**(4): 271-5.
Parsons, A. A. (1998). "Recent advances in mechanisms of spreading depression." Curr Opin Neurol **11**(3): 227-31.
Pekny, M., C. B. Johansson, et al. (1999). "Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin." J Cell Biol. **145**(3): 503-14.
Penkowa, M., J. Carrasco, et al. (2000). "Altered central nervous system cytokine-growth factor expression profiles and angiogenesis in metallothionein-I+II deficient mice." J Cereb Blood Flow Metab. **20**(8): 1174-89.
Penkowa, M., J. Carrasco, et al. (1999). "CNS wound healing is severely depressed in metallothionein I- and II-deficient mice." J Neurosci. **19**(7): 2535-45.
Penkowa, M., T. Moos, et al. (1999). "Strongly compromised inflammatory response to brain injury in interleukin-6-deficient mice." Glia. **25**(4): 343-57.
Petzold, G. C., O. Windmuller, et al. (2005). "Increased extracellular K+ concentration reduces the efficacy of N-methyl-D-aspartate receptor antagonists to block spreading depression-like depolarizations and spreading ischemia." Stroke. **36**(6): 1270-7. Epub 2005 May 5.
Pfister, B., G. Oyler, et al. (2004). "The effects of BclXL and Bax over-expression on stretch-injury induced neural cell death." Mech Chem Biosyst. **1**(4): 233-43.
Picard-Riera, N., B. Nait-Oumesmar, et al. (2004). "Endogenous adult neural stem cells: limits and potential to repair the injured central nervous system." J Neurosci Res. **76**(2): 223-31.
Pierce, J. E., D. H. Smith, et al. (1998). "Enduring cognitive, neurobehavioral and histopathological changes persist for up to one year following severe experimental brain injury in rats." Neuroscience. **87**(2): 359-69.

Ploner, A., S. Calza, et al. (2006). "Multidimensional local false discovery rate for microarray studies." Bioinformatics. **22**(5): 556-65. Epub 2005 Dec 20.
Pontén U., P. L., Hillered L. (1995). Traumatiska hjärnskador. En bok om hjärnan. W. L., Gummerus printing.
Posmantur, R., A. Kampfl, et al. (1997). "A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental traumatic brain injury in the rat." Neuroscience. **77**(3): 875-88.
Povlishock, J. T. (1995). An overview of brain injury models. Neurotrauma. R. K. Narayan, Wilberger, J.E., Povlishock, J.T., The McGraw-Hill Companies,Inc.**:** 1325-1336.
Povlishock, J. T., A. Buki, et al. (1999). "Initiating mechanisms involved in the pathobiology of traumatically induced axonal injury and interventions targeted at blunting their progression." Acta Neurochir Suppl. **73**: 15-20.
Qiu, Z., K. A. Crutcher, et al. (2003). "ApoE isoforms affect neuronal N-methyl-D-aspartate calcium responses and toxicity via receptor-mediated processes." Neuroscience. **122**(2): 291-303.
Qiu, Z., D. K. Strickland, et al. (2002). "alpha 2-Macroglobulin exposure reduces calcium responses to N-methyl-D-aspartate via low density lipoprotein receptor-related protein in cultured hippocampal neurons." J Biol Chem. **277**(17): 14458-66. Epub 2002 Feb 11.
Quackenbush, J. (2002). "Microarray data normalization and transformation." Nat Genet **32**(Suppl): 496-501.
Raghupathi, R., A. C. Conti, et al. (2002). "Mild traumatic brain injury induces apoptotic cell death in the cortex that is preceded by decreases in cellular Bcl-2 immunoreactivity." Neuroscience. **110**(4): 605-16.
Raghupathi, R., K. I. Strauss, et al. (2003). "Temporal alterations in cellular Bax:Bcl-2 ratio following traumatic brain injury in the rat." J Neurotrauma. **20**(5): 421-35.
Rahpeymai, Y., M. A. Hietala, et al. (2006). "Complement: a novel factor in basal and ischemia-induced neurogenesis." Embo J. **25**(6): 1364-74. Epub 2006 Feb 23.
Ray, S. K., S. Karmakar, et al. (2006). "Inhibition of calpain and caspase-3 prevented apoptosis and preserved electrophysiological properties of voltage-gated and ligand-gated ion channels in rat primary cortical neurons exposed to glutamate." Neuroscience. **139**(2): 577-95. Epub 2006 Feb 28.
Redmond, L., A. H. Kashani, et al. (2002). "Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription." Neuron. **34**(6): 999-1010.
Reilly, P. L. (2001). "Brain injury: the pathophysiology of the first hours.'Talk and Die revisited'." J Clin Neurosci. **8**(5): 398-403.
Reilly, P. L., D. I. Graham, et al. (1975). "Patients with head injury who talk and die." Lancet. **2**(7931): 375-7.
Rice, A. C., A. Khaldi, et al. (2003). "Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury." Exp Neurol **183**(2): 406-17.
Robertson, C. L. (2004). "Mitochondrial dysfunction contributes to cell death following traumatic brain injury in adult and immature animals." J Bioenerg Biomembr. **36**(4): 363-8.
Robison, A. J., R. K. Bartlett, et al. (2005). "Differential modulation of Ca2+/calmodulin-dependent protein kinase II activity by regulated interactions with N-methyl-D-aspartate receptor NR2B subunits and alpha-actinin." J Biol Chem. **280**(47): 39316-23. Epub 2005 Sep 19.
Romner, B., T. Ingebrigtsen, et al. (2000). "[Scandinavian guidelines for management of head injuries. Evidence-based management of minimal, mild and moderate head injuries]." Lakartidningen. **97**(26-27): 3186-92.
Rosenberg, G. A. and M. Navratil (1997). "Metalloproteinase inhibition blocks edema in intracerebral hemorrhage in the rat." Neurology **48**(4): 921-6.
Rosenberg, G. A., M. Navratil, et al. (1996). "Proteolytic cascade enzymes increase in focal cerebral ischemia in rat." J Cereb Blood Flow Metab **16**(3): 360-6.
Rudehill, A., B. M. Bellander, et al. (2002). "Outcome of traumatic brain injuries in 1,508 patients: impact of prehospital care." J Neurotrauma. **19**(7): 855-68.
Ryan, L. M. and D. L. Warden (2003). "Post concussion syndrome." Int Rev Psychiatry. **15**(4): 310-6.

Rycroft, B. K. and A. J. Gibb (2004). "Regulation of single NMDA receptor channel activity by alpha-actinin and calmodulin in rat hippocampal granule cells." J Physiol. **557**(Pt 3): 795-808. Epub 2004 Apr 8.
Saatman, K. E., H. Murai, et al. (1996). "Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat." Proc Natl Acad Sci U S A. **93**(8): 3428-33.
Sailor, K. A., V. K. Dhodda, et al. (2003). "Osteopontin infusion into normal adult rat brain fails to increase cell proliferation in dentate gyrus and subventricular zone." Acta Neurochir Suppl. **86**: 181-5.
Schalen, W., L. Hansson, et al. (1994). "Psychosocial outcome 5-8 years after severe traumatic brain lesions and the impact of rehabilitation services." Brain Inj. **8**(1): 49-64.
Scheff, S. W., S. A. Baldwin, et al. (1997). "Morris water maze deficits in rats following traumatic brain injury: lateral controlled cortical impact." J Neurotrauma. **14**(9): 615-27.
Scherbel, U., R. Raghupathi, et al. (1999). "Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury." Proc Natl Acad Sci U S A. **96**(15): 8721-6.
Schinder, A. F., E. C. Olson, et al. (1996). "Mitochondrial dysfunction is a primary event in glutamate neurotoxicity." J Neurosci. **16**(19): 6125-33.
Schmued, L. C., C. Albertson, et al. (1997). "Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration." Brain Res. **751**(1): 37-46.
Schnurra, I., H. G. Bernstein, et al. (2001). "The neuronal calcium sensor protein VILIP-1 is associated with amyloid plaques and extracellular tangles in Alzheimer's disease and promotes cell death and tau phosphorylation in vitro: a link between calcium sensors and Alzheimer's disease?" Neurobiol Dis. **8**(5): 900-9.
Schroeter, M., P. Zickler, et al. (2006). "Increased thalamic neurodegeneration following ischaemic cortical stroke in osteopontin-deficient mice." Brain. **129**(Pt 6): 1426-37. Epub 2006 Apr 24.
Selvaraju, R., L. Bernasconi, et al. (2004). "Osteopontin is upregulated during in vivo demyelination and remyelination and enhances myelin formation in vitro." Mol Cell Neurosci **25**(4): 707-21.
Sergent-Tanguy, S., D. C. Michel, et al. (2006). "Long-lasting coexpression of nestin and glial fibrillary acidic protein in primary cultures of astroglial cells with a major participation of nestin(+)/GFAP(-) cells in cell proliferation." J Neurosci Res. **83**(8): 1515-24.
Shin, T., M. Ahn, et al. (2005). "Temporal expression of osteopontin and CD44 in rat brains with experimental cryolesions." Brain Res **1041**(1): 95-101.
Siesjo, B. K. and F. Bengtsson (1989). "Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis." J Cereb Blood Flow Metab. **9**(2): 127-40.
Smith, D. H., X. H. Chen, et al. (1997). "Progressive atrophy and neuron death for one year following brain trauma in the rat." J Neurotrauma. **14**(10): 715-27.
Sofroniew, M. V. (2005). "Reactive astrocytes in neural repair and protection." Neuroscientist. **11**(5): 400-7.
Stein, D. G. (2001). "Brain damage, sex hormones and recovery: a new role for progesterone and estrogen?" Trends Neurosci **24**(7): 386-91.
Streit, W. J., R. E. Mrak, et al. (2004). "Microglia and neuroinflammation: a pathological perspective." J Neuroinflammation **1**(1): 14.
Strong, A. J., M. Fabricius, et al. (2002). "Spreading and synchronous depressions of cortical activity in acutely injured human brain." Stroke. **33**(12): 2738-43.
Stulemeijer, M., S. van der Werf, et al. (2006). "Recovery from mild traumatic brain injury : A focus on fatigue." J Neurol **17**: 17.
Stylli, S. S., A. H. Kaye, et al. (2000). "Induction of CD44 expression in stab wounds of the brain: long term persistence of CD44 expression." J Clin Neurosci **7**(2): 137-40.

Sullivan, P. G., A. J. Bruce-Keller, et al. (1999). "Exacerbation of damage and altered NF-kappaB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury." J Neurosci. **19**(15): 6248-56.
Sun, D., M. Tani, et al. (2000). "Role of chemokines, neuronal projections, and the blood-brain barrier in the enhancement of cerebral EAE following focal brain damage." J Neuropathol Exp Neurol. **59**(12): 1031-43.
Sun, F. Y. and A. I. Faden (1995). "Pretreatment with antisense oligodeoxynucleotides directed against the NMDA-R1 receptor enhances survival and behavioral recovery following traumatic brain injury in rats." Brain Res. **693**(1-2): 163-8.
Tagliaferri, F., C. Compagnone, et al. (2006). "A systematic review of brain injury epidemiology in Europe." Acta Neurochir (Wien). **148**(3): 255-68; discussion 268.
Tamagno, I. and D. Schiffer (2006). "Nestin expression in reactive astrocytes of human pathology." J Neurooncol **7**: 7.
Tan, H. K., D. Heywood, et al. (2003). "Tissue inhibitor of metalloproteinase 1 inhibits excitotoxic cell death in neurons." Mol Cell Neurosci. **22**(1): 98-106.
Tang, Y., H. Zou, et al. (2006). "Paradoxical effects of very low dose MK-801." Eur J Pharmacol. **537**(1-3): 77-84. Epub 2006 Mar 20.
Tang, Y. P., E. Shimizu, et al. (1999). "Genetic enhancement of learning and memory in mice." Nature. **401**(6748): 63-9.
Theis, M., R. Jauch, et al. (2003). "Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin43." J Neurosci. **23**(3): 766-76.
Thompson, C. S. and A. M. Hakim (2005). "Cortical spreading depression modifies components of the inflammatory cascade." Mol Neurobiol. **32**(1): 51-7.
Tolar, M., J. N. Keller, et al. (1999). "Truncated apolipoprotein E (ApoE) causes increased intracellular calcium and may mediate ApoE neurotoxicity." J Neurosci. **19**(16): 7100-10.
Tong, C. K. and M. Chesler (2000). "Modulation of spreading depression by changes in extracellular pH." J Neurophysiol. **84**(5): 2449-57.
Truettner, J., R. Schmidt-Kastner, et al. (1999). "Expression of brain-derived neurotrophic factor, nerve growth factor, and heat shock protein HSP70 following fluid percussion brain injury in rats." J Neurotrauma **16**(6): 471-86.
Tusher, V. G., R. Tibshirani, et al. (2001). "Significance analysis of microarrays applied to the ionizing radiation response." Proc Natl Acad Sci U S A **98**(9): 5116-21. Epub 2001 Apr 17.
Tymianski, M., M. P. Charlton, et al. (1993). "Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons." J Neurosci. **13**(5): 2085-104.
Wagner, A. K., A. Fabio, et al. (2005). "Gender associations with cerebrospinal fluid glutamate and lactate/pyruvate levels after severe traumatic brain injury." Crit Care Med. **33**(2): 407-13.
van der Hel, W. S., W. M. van den Bergh, et al. (1998). "Suppression of cortical spreading depressions after magnesium treatment in the rat." Neuroreport. **9**(10): 2179-82.
Wang, X., J. Jung, et al. (2000). "Effects of matrix metalloproteinase-9 gene knock-out on morphological and motor outcomes after traumatic brain injury." J Neurosci **20**(18): 7037-42.
Wang, X., S. R. Lee, et al. (2003). "Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator." Nat Med. **9**(10): 1313-7. Epub 2003 Sep 7.
Wang, X., C. Louden, et al. (1998). "Delayed expression of osteopontin after focal stroke in the rat." J Neurosci **18**(6): 2075-83.
Wang, X., L. Xu, et al. (2002). "CD44 deficiency in mice protects brain from cerebral ischemia injury." J Neurochem **83**(5): 1172-9.
Vecil, G. G., P. H. Larsen, et al. (2000). "Interleukin-1 is a key regulator of matrix metalloproteinase-9 expression in human neurons in culture and following mouse brain trauma in vivo." J Neurosci Res **61**(2): 212-24.
Wennersten, A., S. Holmin, et al. (2006). "Sustained survival of xenografted human neural stem/progenitor cells in experimental brain trauma despite

discontinuation of immunosuppression." Exp Neurol. **199**(2): 339-47. Epub 2006 Feb 21.
Wennersten, A., S. Holmin, et al. (2003). "Characterization of Bax and Bcl-2 in apoptosis after experimental traumatic brain injury in the rat." Acta Neuropathol (Berl) **105**(3): 281-8.
Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." Science. **291**(5507): 1304-51.
Westerlund, U., M. Svensson, et al. (2005). "Endoscopically harvested stem cells: a putative method in future autotransplantation." Neurosurgery. **57**(4): 779-84; discussion 779-84.
Wetzel, M., J. Tibbitts, et al. (2004). "Vulnerability of mouse cortical neurons to doxorubicin-induced apoptosis is strain-dependent and is correlated with mRNAs encoding Fas, Fas-Ligand, and metalloproteinases." Apoptosis. **9**(5): 649-56.
Wilhelmsson, U., L. Li, et al. (2004). "Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration." J Neurosci. **24**(21): 5016-21.
Vink, R., P. G. Mullins, et al. (2001). "Small shifts in craniotomy position in the lateral fluid percussion injury model are associated with differential lesion development." J Neurotrauma. **18**(8): 839-47.
Wong, E. H., J. A. Kemp, et al. (1986). "The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist." Proc Natl Acad Sci U S A. **83**(18): 7104-8.
Yamakura, T. and K. Shimoji (1999). "Subunit- and site-specific pharmacology of the NMDA receptor channel." Prog Neurobiol. **59**(3): 279-98.
Ye, Q., X. Li, et al. (2006). "Structure of calmodulin bound to a calcineurin peptide: a new way of making an old binding mode." Biochemistry. **45**(3): 738-45.
Yepes, M., M. Sandkvist, et al. (2003). "Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein." J Clin Invest. **112**(10): 1533-40.
Yin, X. H., Q. G. Zhang, et al. (2005). "Neuroprotective effects of preconditioning ischaemia on ischaemic brain injury through inhibition of mixed-lineage kinase 3 via NMDA receptor-mediated Akt1 activation." J Neurochem. **93**(4): 1021-9.
Ylagan, L. R. and B. Quinn (1997). "CD44 expression in astrocytic tumors." Mod Pathol. **10**(12): 1239-46.
Yoshioka, A., M. Hardy, et al. (1995). "Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage." J Neurochem. **64**(6): 2442-8.
Yu, Q. and I. Stamenkovic (1999). "Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion." Genes Dev. 13(1): 35-48.
Zhang, L., J. C. Hsu, et al. (1997). "Transient global ischemia alters NMDA receptor expression in rat hippocampus: correlation with decreased immunoreactive protein levels of the NR2A/2B subunits, and an altered NMDA receptor functionality." J Neurochem. 69(5): 1983-94.