# REACTIVE GLIOSIS IN THE INJURED BRAIN:
# The effect of cell communication and Nrf2-mediated cellular defence

**Heléne Andersson**

---

Center for Brain Repair and Rehabilitation
Department of Clinical Neuroscience and Rehabilitation
Institute of Neuroscience and Physiology
at Sahlgrenska Academy
University of Gothenburg
Sweden
2011

*Tryck: Intellecta infolog*
ISBN: 978-91-628-8242-6

***Cover image**: Immunocytochemical staining of GFAP in cultured mouse astrocyte by Charlotta Lindwall*

***To my family,***

***for endless support and encouragement***

# ABSTRACT

Stroke and other brain injuries trigger an extensive glial cell response referred to as reactive gliosis. Reactive gliosis is characterized by hypertrophic and proliferating astrocytes, proliferating microglia and NG2-positive cells, which eventually form a bordering glial scar around the damaged area. Although reactive gliosis may protect the injured brain initially, the resulting glial scar inhibits neuronal regeneration. This thesis focuses on the role of intercellular communication and endogenous oxidative defence systems on reactive gliosis after injury.

Neural cells frequently utilize gap junction channels to transport molecules between cells. We hypothesised that blocking gap junction communication would limit reactive gliosis. Two different gap junction channel blockers, octanol and carbenoxolone, were given to rats 30 min before a minor traumatic brain injury. Two days after injury, octanol decreased the extent of reactive astrocytes and NG2-positive cells, and reduced the number of reactive microglia around the wound. Carbonoxolone did not affect reactive astrocytes, but both octanol and carbenoxolone significantly decreased cell proliferation. Thus, blocking gap junction communication may attenuate the progression of reactive gliosis.

Astrocytes play an essential role in antioxidant defence, much of which is regulated by the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is activated by xenobiotics like sulforaphane which provides long-term protection against radical damage, even though sulforaphane is cleared from the body within a few hours. We hypothesized that this brief sulforaphane stimulation would be sufficient to induce prolonged Nrf2-induced gene expression. In primary rat astrocyte cultures, brief exposure to sulforaphane increased Nrf2-dependent gene expression; mRNA and protein levels were elevated for up to 24 h and 48 h respectively. Moreover Nrf2-dependent mRNA and proteins accumulated after repeated exposure and sulforaphane-stimulated astrocytes were more resistant to oxidative damage. Thus, stimulation of the Nrf2 pathway with sulforaphane results in prolonged elevation of endogenous antioxidants.

We further hypothesised that sulforaphane-induced Nrf2 stimulation would modify stroke outcome when given after permanent focal ischaemia. Sulforaphane (a single dose or repeated dose starting 15 min after injury) did not significantly affect motor-function, infarct volume, proliferation, or glial cell activation 1 and 3 days after photothrombosis in mice. Thus, sulforaphane does not provide neuroprotection in the photothrombotic stroke model in mice when given 15 min after stroke onset.

In summary, this thesis describes the kinetics of Nrf2-mediated gene expression in cultured astrocytes, and the role of intercellular communication and Nrf2 activation on aspects of reactive gliosis after brain injury.

**Keywords:** astrocyte, gap junction, Hmox1, microglia, Nrf2, Nqo1, oxidative stress, reactive gliosis

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Konsekvenserna av stroke eller traumatisk hjärnskada är ofta betydande. För den enskilde leder dessa tillstånd ofta till genomgripande förändringar i livet vilket ofta involverar permanenta fysiska och kognitiva funktions-nedsättningar. I hjärnan finner man att mycket av den skadade nervvävnaden och de neurologiska besvär som följer på en skada inte direkt är orsakade av infarkten eller skadan i sig, utan av de omfattande fördröjda biokemiska reaktioner som senare uppstår i vävnaden. Dessa reaktioner är en konsekvens av den omfattande cellulära respons som följer efter skadan, inkluderande inflammation, vävnadssvullnad, syrebrist och överproduktion av fria radikaler. Idag finns det enbart begränsade möjligheter att i akutskedet behandla dessa patienter och intresset är stort inom forskningen för att finna nya behandlingsmetoder som kan minimera konsekvenserna av dessa tillstånd.

Det centrala nervsystemet är uppbyggt av nervceller, gliaceller och ett mycket väl utvecklat kärlträd. Till familjen gliaceller hör astrocyter, mikroglia och NG2-celler. Stroke och andra skador som drabbar hjärnan, resulterar i en omfattande aktivering av gliacellerna, en process som kallas reaktiv glios. Den reaktiva gliosen karaktäriseras av att gliacellerna ändrar utseende och sina funktionella egenskaper. En nybildning av gliaceller sker också. Reaktiv glios leder ofta i slutändan till att ärrvävnad bildas runt det skadade området. I det inledande skedet efter skada är den reaktiva gliosen sannolikt mest fördelaktig då cellerna försöker kompensera för störningar i hjärnans mikromiljö. I senare skeden utgör dock den slutliga ärrvävnaden ett hinder för reparation och återväxt av nya nervceller.

Kunskapen om nervcellernas funktion i hjärnan är betydligt mer omfattande i relation till vad man vet om gliacellernas roller och funktioner. Således föreligger ett mycket stort behov av att erhålla mer kunskap om gliacellernas betydelse i det normala nervsystemet såväl som i det av skada eller sjukdom drabbade nervsystemet. Denna avhandling fokuserar på hur den intercellulära kommunikationen och delar av det inre cellulära skyddet mot fria radikaler i hjärnan involverar aktivering av gliaceller, och senare det skydd mot de generella cellskador som uppstår efter inverkan av fria radikaler, så kallad oxidativ stress.

Gliaceller, och då främst astrocyterna, använder vanligen så kallade gap junction kanaler för att transportera små molekyler mellan sig. För de inledande studierna i avhandlingen var vår hypotes att blockad av gap junction kommunikationen efter en mindre traumatisk hjärnskada i råtta skulle kunna leda till en minskad reaktiv glios, och därmed på så sätt underlätta reparations- processen i ett senare skede. För att studera detta, använde vi två olika gap junction-blockerare, octanol och carbenoxolone. Vi

fann att behandlingen med octanol påverkade den reaktiva gliosen genom att minska reaktiviteten av astrocyter, mikroglia och NG2 celler runt det skadade området. Dessutom minskade både carbenoxolone och octanol signifikant antalet nybildande celler. Detta tyder på kommunikationen genom gap junction kanalerna kan ha betydelse för aktivering av gliaceller efter en hjärnskada samt att en blockering av dessa kanaler kan reducera utvecklingen av den reaktiva gliosen.

Vid en hjärnskada, till exempel en stroke, bildas snabbt reaktiva fria syreradikaler. Dessa reaktiva molekyler leder till oxidativ stress och bidrar starkt till cellskada och senare celldöd. Astrocyterna spelar en stor roll i försvaret mot fria radikaler i hjärnan genom att de producerar och frisätter potenta antioxidanter. Produktionen av dessa substanser regleras till stor del av transkriptionsfaktorer, och en särskilt viktigt sådan faktor är Nrf2. Nrf2 kan aktiveras av xenobiotika, kroppsfrämmande ämnen. Sulforafan är ett sådant ämne och det finns bl.a. i höga koncentrationer i olika kålsorter såsom broccoli och brysselkål. Sulforafan kan ge långtidsskydd mot de negativa effekterna av fria radikaler trots att sulforafan elimineras från kroppen inom några timmar. Vår hypotes för avhandlingens andra arbete var att det långvariga skyddet mot fria radikaler som observerats efter stimulering med sulforafan kan förklaras med att viktiga antioxidanter anrikas efter en kort stimulering av Nrf2-sytemet och att nedbrytning av de antioxidanter som bildas sker långsamt. För att undersöka detta använde vi astrocyter som odlats i cellkulturer, vilka utsattes för kortvarig exponering för sulforafan. Försöken visade en ökning av antioxidanter i astrocyterna som både var långvarig och gradvis kunde byggas upp av upprepade sulforafan exponeringar. Dessutom visade sig de astrocyter som exponerats för sulforafan vara mer motståndskraftiga mot skador inducerade av fria radikaler. Kortvarig sulforafan aktivering av astrocyternas Nrf2-system i den använda modellen kan således resultera i en produktionsökning av cellernas egna antioxidanter över tiden och ett förstärkt skydd mot exponering av fria radikaler.

För att vidare undersöka de skyddande effekterna av Nrf2 aktivering, undersökte vi om sulforafan kunde reducera hjärnskadan och reaktiv glios efter experimentell stroke. Till dessa försök använde vi möss som efter en stroke behandlades med sulforafan i enstaka dos eller upprepade gånger. Efter skadan utfördes analyser avseende motorisk funktion, infarkt volym och utveckling av reaktiv glios. Resultaten från denna studie visade att under dessa experimentella omständigheter hade sulforafan ingen inverkan på någon av de parametrar som undersöktes.

Sammanfattningsvis har de arbeten som redovisats i denna avhandling bidragit till ökad kunskap om Nrf2-systemets funktioner i astrocyter *in vitro* samt efter experimentell stroke *in vivo.* Studierna har också belyst betydelsen

av intercellulär kommunikation mellan gliaceller i hjärnan för utveckling och kontroll av reaktiv glios efter hjärnskada.

# LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred in the text by their Roman numerals

I. **Trauma-induced reactive gliosis is reduced after treatment with octanol and carbenoxolone**
Heléne C. Andersson, Michelle F. Anderson, Michelle J. Porritt, Christina Nodin, Fredrik Blomstrand, Michael Nilsson
*Neurological Research 2011, in press*

II. **Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage.**
Petra Bergström*, Heléne C. Andersson*, Yue Gao, Jan-Olof Karlsson, Christina Nodin, Michelle F. Anderson, Michael Nilsson, Ola Hammarsten
*Neuropharmacology 2011 Feb-Mar;60 (2-3):343-53*
* Equal contribution of these two authors

III. **The effect of sulforaphane on infarct size, glial activation, cell proliferation and functional outcome following photothrombotic stroke in mice.**
Heléne C. Andersson, Linda Hou, Åsa Nilsson, Marcela Pekna, Milos Pekny, Michelle J. Porritt, Michael Nilsson
*Manuscript*

# TABLE OF CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ARE | Antioxidant responsive element |
| ATP | Adenosine triphosphate |
| BBB | Blood-brain barrier |
| BrdU | 5-Bromo-2-deoxyuridine |
| Cbx | Carbenoxolone |
| CNS | Central nervous system |
| DAB | 3, 3´-diamino-benzidine tetrahydrochloride |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| GFAP | Glial fibrillary acidic protein |
| GSH | Glutathione |
| HBSS | Hank´s buffered salt solution |
| Hmox1 | Heme oxygenase 1 |
| $H_2O_2$ | Hydrogen peroxide |
| i.p. | Intraperitoneally |
| kDa | Kilo Dalton |
| Keap1 | Kelch-like ECH associated protein 1 |
| MCAO | Middle cerebral artery occlusion |
| MCB | Monochlorobimane |
| mRNA | messenger ribonucleic acid |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| Nrf2 | Nuclear transcription factor erythroid derived 2, like 2 |
| Nqo1 | NAD(P)H quionone oxidoreductase 1 |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase Chain Reaction |
| PI | Propidium Iodide |
| ROS | Reactive oxygen species |
| RNS | Reactive nitrogen species |
| siRNA | small interfering ribonucleic acid |
| TBI | Traumatic brain injury |
| TBS | Tris-buffered saline |

# INTRODUCTION

## Stroke and traumatic brain injury

Stroke or traumatic brain injury (TBI) often leads to devastating life-changes for the patients, including physical, behavioural and cognitive disabilities. Today, there is very little that can be done to treat these patients in the early stages. Researchers in the neuroscience field are constantly searching for neuroprotective agents to treat patients with stroke and trauma.

Stroke constitutes the third highest cause of death and the major cause of adult disability in the western world. In Sweden, more than 30 000 cases are diagnosed each year. Stroke is most often due to reduced or blocked blood flow of a major blood vessel in the brain. If the occlusion is not rapidly reversed, the area will become ischaemic. That is oxygen and nutrients become deficient in the brain tissue due to a shortage of blood supply. If ischaemia is prolonged, it can lead to accumulation of metabolic-waste products, generation of free radicals and extensive cell loss (infarction). In the ischaemic core, cell death occurs within minutes and is considered to be beyond rescue. The infarct evolves over time and expands to include the areas surrounding the ischaemic core, the ischaemic penumbra. The penumbra is more moderately ischaemic due to collateral blood flow resulting in more delayed cell death in these regions.

TBI is a major cause of death and disabilities, especially among young adults and children, in both industrialized and developing countries. TBI is caused by an external force that, in different degrees, damages the scull, blood vessels and brain tissue (Gentleman et al., 1995; Povlishock and Christman, 1995). Most of the patients deteriorate over time due to the complex cascade of molecular and cellular events that occur minutes to days after the initial injury, resulting in an expansion of the tissue damage (reviewed in (Kochanek et al., 2000). This secondary damage often includes oedema, ischaemia, inflammation and overproduction of free radicals (Park et al., 2008). The expansion of the injury is also the major cause of death occurring in hospitals following a TBI (Ghajar, 2000).

In stroke and TBI the heterogeneity and complexity of the injuries and the plethora of molecular events affected, complicate the attempts to identify agents that potentially can protect or repair the brain tissue after such conditions. This partly explains why the current treatments are limited after a severe injury to the central nervous system (CNS). However, with time, most

patients do partially recover after stroke or traumatic brain injuries and the CNS is more prone to plastic changes than previously thought (Albright et al., 2000). New neurons are born throughout life (Kuhn et al., 1996; Eriksson et al., 1998). Despite this, the re-growth and repair of damage tissue in the CNS is not as extensive as after an injury in the peripheral nervous system. The limited regeneration is mainly due to inhibitory factors from surrounding non-neuronal cells and the extracellular environment. Further knowledge of the molecular and cellular mechanisms behind the cellular response and how to manipulate it, may lead to possible treatment approaches that could be of great clinical relevance.

The CNS consists of neurons and glial cells. The traditional view has been that neurons are the main unit for transmitting and processing information while the glial cells, have been considered as passive supportive cells. However, more recent studies suggest a gradually more complex and active role for glial cells in brain function, and particularly for astrocytes (Allen and Barres, 2009). Although it is now known that glial cells contribute at different levels to the evolving tissue damage and in subsequent attempts to repair the damaged or injured areas (Fitch and Silver, 2008), there is still much to learn about their role after an injury to the brain. The studies in this thesis focused on the role of intercellular communication and the Nrf2-induced endogenous antioxidant system on reactive gliosis and cellular protection in two different *in vivo* models of stroke and TBI and in an *in vitro* model of free radical-induced cellular stress.

## Glial cells

Glial cells comprise most of the cells in the brain and outnumber the neurons about 10-50 times. Glial cells include astrocytes, microglia, oligodendrocytes, and NG2 expressing cells. The different glial cells have specific unique functions of their own that involve supporting neurotransmission, maintaining ion homeostasis in the extracellular space and myelinating the axons (Fig. 1).

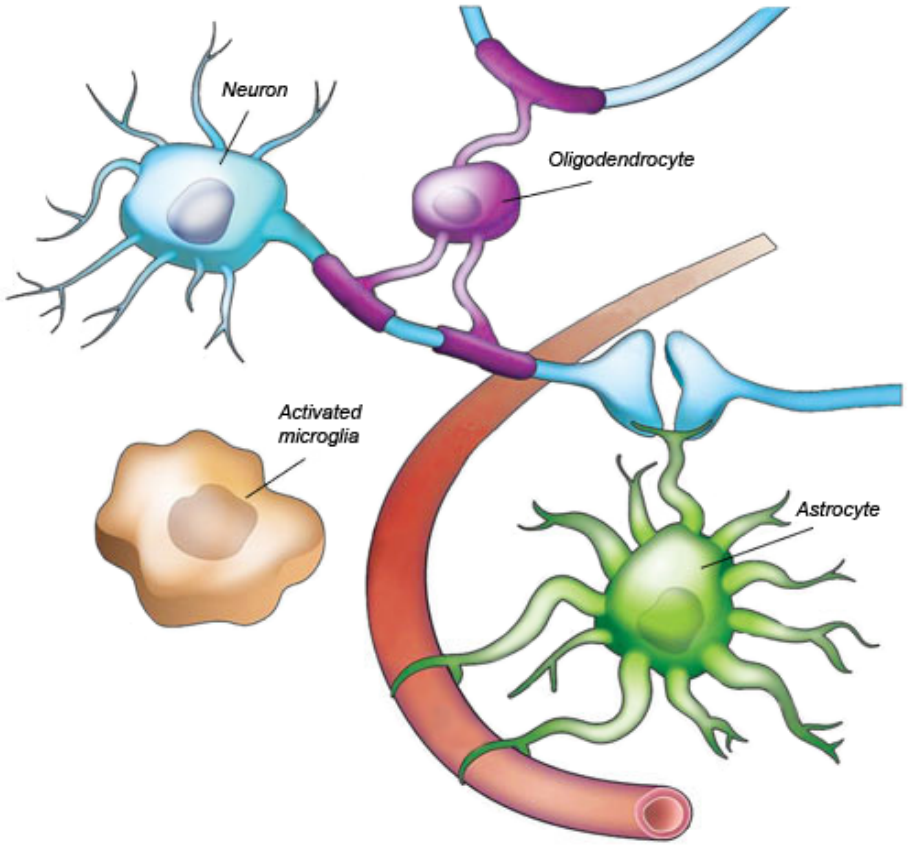


**Figure 1**. Illustration of glial-neuronal interaction. Oligodendrocytes wrap the myelin around the neuronal axon to isolate and speed up the neurotransmission. Astrocyte processes make contact with the neuronal synapses and the blood vessel. Activated microglia survey the environment for damage or intruders. Adapted from Allen and Barres 2009 (Allen and Barres, 2009)

## Astrocytes

Astrocytes are the most abundant cell type in the brain. They constitute a heterogeneous cell population with varying complexity and diversity within different brain regions as well as among species. The size and complexity of astrocytes increase in proportion to intelligence (Nedergaard et al., 2003; Oberheim et al., 2009). Astrocytes are classically divided into two main categories based on their location and morphology. *Protoplasmic* astrocytes are mainly found in the grey matter and exhibit branched processes while *fibrous* astrocytes have long and fiber-like processes and are mainly found in the white matter (Privat et al., 1995; Sofroniew and Vinters, 2010). However, it has been demonstrated that types of protoplasmic, and most likely also fibrous astrocytes, differ between regions and even within a region, although the specific functional differences are still not known (Allen and Barres, 2009).

The most common way to identify astrocytes in the brain is through expression of their main intermediate filament, glial fibrillary acidic protein (GFAP). When astrocytes were first visualised they appeared as stars and it was this feature that, gave rise to their name, “astro” which means star in Latin. However, after microinjecting dye into a single cell, it was revealed that astrocytes are actually more bush-like with many fine processes (Bushong et al., 2002; Wilhelmsson et al., 2004). The processes with non-overlapping domains and their strategic location close to other glial cells, neurons and blood vessels, enable them to influence and respond to changes in the environment and to be a part of a broad range of actions in the CNS (Araque et al., 2001; Fields and Stevens-Graham, 2002; Volterra and Meldolesi, 2005).

Astrocytes were previously considered solely as structural and chemical padding for the neurons. Nowadays, astrocytes are acknowledged as active participants contributing to various essential functions both in the developing and mature brain (Araque et al., 2001; Haydon, 2001; Kirchhoff et al., 2001). During development, astrocytes participate in the formation of synapses (Christopherson et al., 2005; Barres, 2008) and in the guidance of migrating axons (Powell and Geller, 1999). In the mature brain, astrocytes play essential roles for normal CNS functions, including providing energy metabolites to the neurons, participating in synaptic function, regulating blood flow, maintaining neurotransmitter and ion homeostasis in the extracellular space and being key players in the cellular defence against oxidative stress (Wilson, 1997; Dringen, 2000; Dringen et al., 2000;

Nedergaard et al., 2003; Ransom et al., 2003; Barres, 2008; Sofroniew and Vinters, 2010).

Characteristic for astrocytes is their extensive coupling to each other via so called gap junction channels which enables them to form large glial networks. As a consequence, astrocytes can function more as a group rather than as single cells (Giaume and McCarthy, 1996). These astrocytic networks play important roles in the normal brain. They can provide long-range signalling in the brain and enable the transport of molecules along a concentration gradient, a phenomenon referred to as spatial buffering (Dermietzel and Spray, 1993; Houades et al., 2006). These astrocytic networks also facilitate maintenance of homeostasis in the brain including regulation of the extracellular pH, and uptake and distribution of glutamate and potassium (Anderson and Swanson, 2000; Anderson et al., 2003; Ransom et al., 2003).

Unlike neurons, astrocytes do not respond to stimuli by firing action potentials (Nedergaard et al., 2003; Seifert et al., 2006). Instead, astrocytes can for example, communicate via calcium waves that can be propagated from one astrocyte to another, triggered by diffusion of molecules via intercellular gap junctions channels (Charles, 1998; Giaume and Venance, 1998; Blomstrand et al., 1999b). The regulation of intercellular calcium concentration is important for the communication with other astrocytes as well as with neurons (Nedergaard et al., 2003; Volterra and Meldolesi, 2005; Sofroniew and Vinters, 2010).

The astrocytic networks are also important for energy supply. The position of the astrocytes, as a bridge between neurons and the blood stream, enables them to have a bi-directional interaction with the blood (Gordon et al., 2007). Astrocytes are therefore highly involved in neuronal metabolism (Zonta et al., 2003). Astrocytes take up glucose and its metabolites from the blood with specific glucose transporters, and via gap junctions it is distributed to neighbouring astrocytes and neurons (Giaume et al., 1997; Tabernero et al., 2006). Moreover, the close interaction with blood vessels make astrocytes important participants in the formation and regulation of the blood brain barrier (review in (Abbott, 2005) and the regulation of the blood flow (Parri and Crunelli, 2003; Gordon et al., 2007; Iadecola and Nedergaard, 2007; Attwell et al., 2010).

Through their presence around the synapses, astrocytes are able to regulate water, ion and neurotransmitter homeostasis (Ventura and Harris, 1999). Astrocytes express a number of aquaporin water channels to regulate the fluid

homeostasis (Zador et al., 2009). Through their potassium channels they clear the extracellular space from excess potassium during neuronal activity to prevent depolarization (Giaume et al., 2007). Astrocytes normalize the extracellular space and protect neurons against glutamate toxicity after synaptic transmission by taking up excess glutamate, metabolising it and distributing it via the gap junction channels. They subsequently shunt metabolites back to the neurons as glutamine (Anderson and Swanson, 2000; Hansson et al., 2000; Broer and Brookes, 2001; Chaudhry et al., 2002). Astrocytes also actively participate in synaptic function by interacting with synaptic activity and by releasing transmitters in response to neuronal activity (Nedergaard et al., 2003; Andersson et al., 2007; Andersson and Hanse, 2010). The synapse thus consist of three units, the neuronal pre- and post-synaptic elements and now, recently added, also the astrocyte, that have given the rise to the" tripartite synapse" theory (Halassa et al., 2007; Perea et al., 2009; Perea and Araque, 2010).

## Microglia

Microglia are characterized for their function as the brain guardians and key players in the immune defence (Streit, 2002; Hanisch and Kettenmann, 2007). Microglias covers about 5-20% of the glial population in the mature brain and are most abundant in the grey matter (Lawson et al., 1990). During physiological conditions, microglia are recognised as highly branched cells with small processes and are distributed in non-overlapping domains throughout the brain (Kreutzberg, 1995). They possess ion channels and neurotransmitter receptors which enable them to sense changes in the CNS homeostasis (reviewed in (Farber and Kettenmann, 2005).

It is still not clear how microglia communicate. In contrast to astrocytes, functional gap junctions have only been demonstrated in activated microglia during pathological conditions (Eugenin et al., 2001). As microglial cells survey their own territory and maintain a distance from each other, auto- and paracrine mechanisms are suggested to be important for their communication (Graeber, 2010). By their constantly moving processes they survey the surroundings for damage or pathogens (Davalos et al., 2005; Nimmerjahn et al., 2005). They are extremely sensitive to micro-environmental alterations such as tissue damage or infections in the brain (Raivich, 2005). Microglia become activated within minutes in response to such micro-environmental alterations and can stay activated for a long time (Morioka et al., 1991). They transform into the macrophages of the brain and achieve phagyocytic and immunological functions. In response to injury, microglia also start to proliferate and migrate towards the site of injury (Graeber, 2010).

### Oligodendrocytes

Oligodendrocyte is the Greek name for "the cell with few branches". They are derived from oligodendrocyte precursor cells, also named NG2 expressing cells from their expression of the proteoglycan NG2. Oligodendrocytes are specialized cells that wrap tightly around axons with the own cell membrane, with the main assignment to provide neurons with myelin to speed up the electrical signal (action potential). Oligodendrocytes are able to myelinate several neuronal axons simultaneously (Nave, 2010). This also explains why oligodendrocytes are most abundant in the white matter.

### NG2-expressing cells

NG2-expressing cells are named and identified for their expression of the chondroitin sulfate proteoglycan NG2. NG2-expressing cells are relatively newly accepted members of the glial family and have recently been classified as the fourth type of glia cell (Peters, 2004; Trotter et al., 2010). They represent about 5-15% of the non-neuronal cells in the adult brain, are distributed in both white and grey matter (Staugaitis and Trapp, 2009; Trotter et al., 2010) and are morphologically highly branched. More recently, NG2-expressing cells have also been called polydendrocytes because of their satellite morphology (Nishiyama et al., 2009). The expression of NG2 is primarily linked to oligodendrocyte progenitor cells and the expression decreases during cell maturation (Levine, 1994; Nishiyama et al., 1996; Rhodes et al., 2006). However, recent studies reveal that NG2 cells also can give rise to neurons and astrocytes (Alonso, 2005; Tatsumi et al., 2005; Zhu et al., 2008). The function of the NG2 cells in the adult brain are still not well understood (Trotter et al., 2010). However, they possess neurotransmitter receptors and ion channels which enable them to interact with surrounding cells (Wigley and Butt, 2009; Bergles et al., 2010). In addition, NG2 cells are the only glial cells that have been observed to form synaptic contacts with neuronal axons (Bergles et al., 2010).

## Glial cell response to injury – reactive gliosis

CNS injury leads to cell death, cellular swelling, excitotoxicity (caused by increased glutamate release and impaired uptake systems) and the release of free radicals and nitric oxide. This triggers an extensive glial cell response and activation (Bonfoco et al., 1995; Back and Schuler, 2004). The glial response, collectively referred to as reactive gliosis, involves mainly activated microglia, NG2-cells and astrocytes (Giulian, 1993; Alonso, 2005;

Sofroniew, 2005; Fitch and Silver, 2008). Reactive gliosis is characterized by hypertrophic and proliferating astrocytes, and proliferating microglia and NG2-positive cells (Ridet et al., 1997; Fitch and Silver, 2008). Eventually this process results in a meshwork of tightly interwoven cell processes, that together with accumulation of activated microglia and various secreted molecules, form a bordering scar around the lesion, the glial scar. Reactive gliosis is observed following stroke and TBI, after many viral infections, tumours and neurodegenerative diseases, and in the aging brain. After injury, the degree of reactive gliosis often reflects the severity of the tissue damage.

Damage to the brain leads to cell death and alteration of the micro-environment. Cells at the site of injury secrete factors that commence and regulate the activation of glial cells, including growth factors, cytokines, neurotransmitters (glutamate, noradrenalin), nucleotides (ATP) and reactive oxygen species (Davalos et al., 2005; Fitch and Silver, 2008; Sofroniew and Vinters, 2010). Many of these factors can also be directly produced and released by astrocytes and infiltrating blood cells such as macrophages. These triggering factors, especially the inflammatory-mediated factors, initiate the activation of microglia, macrophages, NG2 positive cells and astrocytes (Fitch et al., 1999; Rhodes et al., 2006; Fitch and Silver, 2008).

## Activated microglia

Microglia are very sensitive to extracellular changes and can be detected as early as 24 h after injury with a maximum around 3 days after (Gehrmann et al., 1991; Kreutzberg, 1996). The first line of cellular defence against pathogens and cellular damage is mainly orchestrated by the microglia, that respond by becoming activated (Block et al., 2007; Hu et al., 2008). Upon activation, microglia transform from highly ramified resting cells to a more round compact form with retracted processes (Raivich, 2005). They start to proliferate and migrate to the site of injury (Streit et al., 1999). They also produce and release pro-inflammatory cytokines and chemokines and upregulate the expression of cell surface molecules and membrane proteins such as receptors and channels (Gebicke-Haerter et al., 1996; Streit et al., 1999). In their active state, microglia have the ability to phagocyte debris and dying cells (Davalos et al., 2005; Walter and Neumann, 2009) However, their response also includes the release of potentially harmful oxygen free radicals (peroxy-nitrite and superoxide) (Dringen, 2005). Microglia and infiltrating macrophages can be observed within 24 h after injury (Gehrmann et al., 1991) and precede the astrocytic response which commonly begins a day later (Norton, 1999). They are also probably a major triggering factor behind glial cell activation, including the initiation and development of activated

astrocytes and the glial scar formation (Fitch and Silver, 1997; Fitch et al., 1999; Rohl et al., 2007; Zhang et al., 2010).

## NG2 cell response

NG2 cells respond quickly after injury by upregulating the expression of the chondroitin sulfate proteoglycan NG2. They migrate to the site of injury and constitute most of the proliferating cells that can be observed during the first week after injury (Levine et al., 2001; Hampton et al., 2004). NG2 cells are observed in several pathological conditions exhibiting a changed morphology of shorter, thicker and fewer processes (Staugaitis and Trapp, 2009). Reactive macrophages can also express NG2 following injury (Jones et al., 2002). For example, NG2 immunoreactivity increases in response to stab wound in the brain (Hampton et al., 2004) ischaemic injury (Tanaka et al., 2001), and viral infection of motor neurons (Levine et al., 1998). In addition, the chondroitin sulfate side chain of NG2 is known to inhibit regeneration and constitute a component of the glial scar (Chen et al., 2002a; Tan et al., 2005).

## Reactive Astrocytes

Reactive astrocytes are commonly observed in basically all pathologies in the CNS (reviewed in detail by Sofroniew (Sofroniew, 2009)). Activation of astrocytes includes genetic, molecular, cellular and functional alterations (Ridet et al., 1997; Eng et al., 2000). Reactive astrocytes are characterized by cellular hypertrophy, an increase in number and upregulation of intermediate filament components, in particular GFAP and vimentin, (Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010). Antibodies against GFAP, which is contained within intermediate filaments, are commonly used to immunohistochemically identify reactive astrocytes. Although the exact function of this upregulation is unclear and probably includes multiple mechanisms, the expression of GFAP is a hallmark of the activation process of reactive astrocytes and for glial scar formation (Pekny et al., 1995; Pekny and Pekna, 2004; Li et al., 2007). During normal conditions, GFAP is not expressed in all astrocytes at levels detectable by immunohistochemistry and the expression may also vary anatomically (Sofroniew, 2009). However, in the injured brain the upregulation of GFAP is a reliable marker of reactive astrocytes. In their reactive state, astrocytes produce and release various growth factors and inflammatory agents. As important players in the defence against free radicals in the brain, reactive astrocytes also upregulate their production and release of antioxidants (Ridet et al., 1997; Wilson, 1997; Araque et al., 2001; Little and O'Callagha, 2001).

The mechanisms leading to astrocyte activation are far from clear. Reactive astrocytosis is not uniform and the progression varies depending on the severity of the pathological insult. Astrocytes respond to mild pathological insults such as virus infection or non-penetrating trauma by only some or no proliferation (Sofroniew and Vinters, 2010). In these cases, astrocytes often return to their normal appearance when the insult heals (Sofroniew, 2009). However, when astrocytes are triggered by more severe insults such as ischaemia, penetrating trauma or autoimmune inflammation, the proliferation and GFAP expression is more pronounced, and the hypertrophic processes overlap with neighbouring reactive astrocytes (Sofroniew and Vinters, 2010). The astrocytic response in severe injuries can proceed for days up to weeks, and frequently ends with the glial scar formation (Pekny et al., 1999; McGraw et al., 2001). The glial scar is composed of tightly interwoven cell processes of activated glial cells, primarily reactive astrocytes, bordering the lesion. Together with a host of extra cellular matrix protein, such as the chondroitin sulfate proteoglycan NG2 as an important element, the permanent glial scar is formed (Silver and Miller, 2004; Fitch and Silver, 2008; Zhu et al., 2008).

## The paradoxical role of reactive gliosis

In the injured brain, reactive gliosis and scar formation might have a complex dual role for the recovery process (Fawcett and Asher, 1999; Buffo et al., 2009; Sofroniew, 2009). Reactive gliosis is beneficial in the initial state after injury, and the process is most likely an attempt to protect and promote recovery after injury by re-establishing the environment both physically and chemically (Ridet et al., 1997; Buffo et al., 2009). However, in the brain, neuronal regeneration following injury is very limited and only few axons successful re-grow into the injured area. The failure is most likely due to the inhibiting environment that has been formed, where different components of reactive gliosis play a important role (Cafferty et al., 2007). Most likely, astrocytes, microglia, NG2 cells as well as infiltrating blood cells, all contribute to the non-permissive milieu that hinders regeneration after injury.

NG2-expressing cells respond to several types of injury by proliferating and migrating to the site of damage (Levine, 1994; Chen et al., 2002b). Their ability to give rise to not only oligodendrocytes but also neurons (Belachew et al., 2003) and astrocytes (Leoni et al., 2009) may be a possible way to replace damaged cells. However, accumulation of NG2 cells at the injury site contribute to the detrimental effect of the glial scar by producing inhibiting chondroitin sulfate proteoglycans, in particular NG2 (Chen et al., 2002b; Tan et al., 2005). It is also evident that infiltrating macrophages and other serum

molecules from the breakdown of the BBB are associated with the production of chondroitin sulfate proteoglycans at the injury site (Fitch and Silver, 1997; Fitch et al., 1999; Jones et al., 2002).

Activated microglia are involved in neuroprotection and neurogenesis by releasing neurotropic and anti-inflammatory molecules (Hanisch and Kettenmann, 2007). They detoxify and phagocyte toxic products and invading pathogens thereby removing dead cells and debris to promote neuroregeneration (Streit et al., 1999; Aldskogius, 2001). However, over-activated microglia can have a toxic effect by releasing cytotoxic substances and oxidative stress-related factors such as nitric oxide, hydrogen peroxide and superoxide, and pro-inflammatory agents such as interleukin-1 and tumour necrosis factor-α, (Block and Hong, 2005). The inflammatory responses play a major role in the initiation of the cascade of secondary tissue damage and formation of the glial scar (Fitch et al., 1999; Tian et al., 2007). The underlying mechanisms and the conditions that lead to activation or over-activation of the microglia are still not fully understood.

It is well known that reactive astrocytes can provide neuroprotection in various models of CNS injury, such as in spinal cord injury, and under conditions of oxidative stress such as ischaemia. Their protective effects are mediated via their ability to spatially buffer various potentially harmful molecules, remove excess neurotransmitters (Rothstein et al., 1996; Swanson et al., 2004), produce glutathione (Shih et al., 2003; Swanson et al., 2004; Vargas et al., 2008), participate in blood brain barrier repair and reduce oedema after injury (Bush et al., 1999; Faulkner et al., 2004). It has also been shown that mature astrocytes proliferate and acquire stem cell properties after injury suggesting they may have capacity to promote regeneration (Doetsch et al., 1999; Seri et al., 2004; Buffo et al., 2008). In addition, the resulting scar is a barrier that seals off the damaged area and prevents spreading of detrimental molecules to the still viable tissue.

Pathological conditions can result in altered or even reversed normal astrocytic functions (Rao et al., 1998; Takano et al., 2005). In addition, astrocytic swelling exacerbates the ischaemic damage by reducing the vascular perfusion (Sykova, 2001). A reduced extracellular space also alters the ion concentrations that in turn can affect the neuronal excitability. In humans, this is most likely one reason for the delayed cell death observed after stroke (Ayata and Ropper, 2002). In addition, produced and released inflammatory mediators from reactive astrocytes (Brambilla et al., 2005; Farina et al., 2007; Brambilla et al., 2009) and reactive oxygen species

(Swanson et al., 2004) can be involved in creating the detrimental environment.

## Modulation of reactive gliosis

Much research is aimed at elucidating the different underlying mechanisms for the progression of reactive gliosis in order to manipulate it and create a more favourable environment for regeneration.

One approach has been to completely or partially ablate reactive and dividing astrocytes. Studies where reactive astrocytes have been ablated have shown that reactive astrocytes are essential for the regulation of inflammation after injury. In these studies, the lack of reactive astrocytes in the injured brain resulted in increased neuro-degeneration and inflammation and repair failure of the BBB (Bush et al., 1999; Faulkner et al., 2004). This indicates a protective role for reactive astrocytes and the scar formation.

Another approach to study the involvement of reactive astrocytes after injury has been to focus on controlling the upregulation of the astroglial intermediate filaments, the most common hallmark of reactive gliosis. A mice model was generated, where the intermediate filaments GFAP and vimentin were ablated, thus leading to a reduced ability of the astrocytes to become reactive (Pekny et al., 1995; Eliasson et al., 1999; Pekny et al., 1999). These mice confirm the role of astrocytes in the scar formation by exhibiting an abnormal glial scar following injury. Combined with a less dense glial scar, the mice demonstrated a prolonged healing process, indicating an important role for astrocytic intermediate filaments for the successful wound healing (Pekny et al., 1999; Li et al., 2007). However, although healing was prolonged these transgenetic mice demonstrated improved synaptic regeneration (Wilhelmsson et al., 2004), again demonstrating the paradoxical role of reactive astrocytes in the brain.

There is also evidence that the chemical environment in the glial scar has a great impact on the inhibition of regeneration. Several studies demonstrate that the release of inhibitory molecules by reactive astrocytes and the dense composition of the glial scar are important aspects for inhibiting the recovery process (Fawcett and Asher, 1999; Buffo et al., 2009; Sofroniew, 2009). For instance, the chondroitin sulfate proteoglycan NG2 is increased after injury and is one component in the glial scar and a key inhibitory-molecule for axonal regeneration (Chen et al., 2002a; Sandvig et al., 2004; Tan et al., 2005). This was demonstrated by enhanced regeneration in a spinal cord injury using an antibody against NG2 (Tan et al., 2006). In addition, several

studies demonstrate that reducing the injury-induced cell proliferation, that mostly constitute NG2 cells and microglia, improves regeneration (Rhodes et al., 2003; Di Giovanni et al., 2005; Tian et al., 2007). In a number of injury models of inflammation the use of anti-inflammatory agents resulted in reduced activation of both microglia and astrocytes, and reduced neuronal cell death (Giovannini et al., 2002; Scali et al., 2003; Ryu et al., 2004).

Attenuation of reactive gliosis has also been demonstrated by the use of different pharmacological agents. In animal models of TBI, ribavirin, generally used as an anti-viral medication with anti-proliferating effect, decreased the number of reactive astrocytes (Pekovic et al., 2005) and simvastatin, a cholesterol synthesis inhibitor, reduced the activation of microglia and astrocytes (Li et al., 2009a; Wu et al., 2010).

## Gap junctions

Reactive gliosis can be observed at great distances from a brain lesion, and even in the contralateral hemisphere, (Moumdjian et al., 1991) indicating that long-distance signaling mechanisms are involved in the transformation of glial cells to their reactive states.

One form of cell-to-cell communication is mediated via gap junction channels. Gap junctions channels provide electrical as well as biochemical signaling and are vitally important for cellular functions in development, homeostasis, regulation and regeneration (Goodenough and Paul, 2009). Gap junctions are expressed in basically all tissues, except skeletal muscle and circulating blood cells (Bennett et al., 1991; Kumar and Gilula, 1996; De Maio et al., 2002) which attests their importance for cellular function.

Gap junctions are built up by microdomains of channels that are assembled on the cell membrane, called gap junction plaques (Laird, 2006). The channels are composed of small conduits that permit direct trafficking of small molecules from one cell to another. One connexon, also called a hemichannel, is formed by six connexin proteins named after their molecular weight in kilo Dalton (kDa) (Sohl and Willecke, 2004). The gap between the cells is usually about 2-3 nm wide, and two connexons create one channel between two adjacent cell membranes (fig. 2). A single hemichannel can also function as a passage for molecules to the extracellular space (Bennett et al., 2003). In mammals, about 20 connexin family members have been identified so far (Willecke et al., 2002; Laird, 2006). Cells usually express several different connexins, some that are generally expressed and others that are cell specific (Dermietzel, 1998; Rouach et al., 2002). The predominant connexin

proteins in astrocytes are connexin 43 and 30 (Nagy and Rash, 2000; Rouach et al., 2002; Theis et al., 2005) and in oligodendrocytes connexin 32 is the most common (Nagy and Rash, 2000). Microglia express connexin 36 and in their reactive state they also express connexin 43 (Eugenin et al., 2001; Dobrenis et al., 2005).

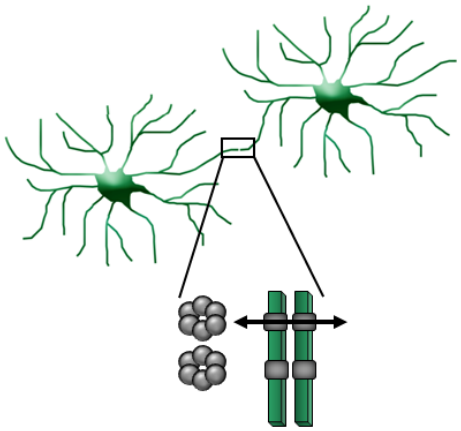

**Figure 2**. Illustration of gap junction communication. The channels are built up by connexons, consisting of six connexins. A gap junction channel is formed when connexons on one cell conjugate with a connexon on a neighbouring cell.

## Gap junction communication

A gap junction channel is 1.0-1.5 nm in diameter and allows the diffusion of molecules up to approximately 1.2 kDa (Bennett et al., 1991; Rouach et al., 2002). Molecules known to be able to pass through an open hemichannel or a gap junction channel include small molecules and second messengers such as ATP, glutathione, glutamate, and calcium (Cotrina et al., 1998b; Ye et al., 2003; Laird, 2006; Rana and Dringen, 2007).

It often takes a cluster of multiple gap junction channels to make functional cell-to-cell communication possible (Bukauskas et al., 2000; Contreras et al., 2004). The gap junction channels are very dynamic and the pathways can be regulated at several levels and differ for each connexin type. Different ways of altering the pathways include changing the properties of the channel (either mechanically or electrically), increasing or decreasing the protein expression or changing the connexin pore incorporation to the plasma membrane. Alteration of the transcription, translation and degradation of the connexin proteins is long time regulation that takes hours to days. Phosphorylation and translocation to the membrane is short-term regulation and takes seconds to minutes (Rouach et al., 2002; Houades et al., 2006).

The permeability of gap junctions varies depending on the connexins forming the channels. The astrocytic network that is mainly made up by connexin 43, is permeable to both positively and negatively charged molecules, wheras others are more charge specific (De Maio et al., 2002; Bennett et al., 2003). Gap junction channel permeability is modified by pH, intracellular second messengers and membrane potential. Increased neuronal activity and a large number of intra- and extra cellular molecules are able to alter the communication through the channels (Rouach et al., 2000). For example, increased extracellular concentrations of glutamate and potassium open the channels and increase calcium signaling (Enkvist and McCarthy, 1994; Blomstrand et al., 1999c), while elevated intracellular calcium concentrations or low pH inhibit the gap junction communication (Martinez and Saez, 2000; Rouach et al., 2002). Cytokines released during inflammatory conditions reduce gap junction communication while uncoupled connexons, the hemichannels, stay open (Hinkerohe et al., 2005; De Vuyst et al., 2007; Retamal et al., 2007).

In the CNS, neural cells utilize gap junctions to communicate with each other. The majority of astrocytes are highly coupled to each other via gap junction channels. The efficiency of the channels expressed by oligodendrocytes are considered to be very low in comparison to astrocytes (review in (Giaume et al., 2007). Functional gap junction channels have not been found on NG2 cells (Lin and Bergles, 2004) and microglia express functional gap junction channels only when reactive (Eugenin et al., 2001; Eugenin et al., 2003).

## Gap junction blockage during experimental conditions

Various substances have been used to modulate the communication through the gap junction channels. Commonly used gap junction blockers include glycyrrhetinic acid, a natural compound found in licorice and tobacco, and its synthetic analogue carbenoxolone, as well as alcohols such as octanol and heptanol (Davidson et al., 1986; Rozental et al., 2001; Juszczak and Swiergiel, 2009). Even if these compounds are strong gap junction blockers, neither one of them exhibits pharmacological specificity for this mechanism of action (Juszczak and Swiergiel, 2009). The use of connexin mimetic peptides to inhibit gap junctions has recently increased (Evans and Boitano, 2001). However, even if the peptides are suggested to be more effective than other blockers their specificity is also questioned (Wang et al., 2007).

### Function of gap junctions during pathological conditions

The role of gap junction communication during pathological conditions is not clear. Cell communication via gap junctions channels does persist during pathological conditions, although with reduced capacity (Cotrina et al., 1998a; Nodin et al., 2005). Following stroke, proapoptotic substances can diffuse through the network from dying cells in the ischaemic core, to still viable cells in the penumbra and cause cell death (Li et al., 1995b; Li et al., 1995a; Li et al., 1995c). Calcium and ATP are examples of molecules suggested to mediate cell death in the penumbra area (Budd and Lipton, 1998; Lin et al., 1998) . Calcium and ATP are also known to be involved in the activation of glial cells following injury, suggesting a role for gap junction communication in the activation of glial cells.

In some studies, alterations of gap junction channels have improved neuronal outcome and decreased cell death, while in other studies neuronal damage was increased (reviewed in (Giaume et al., 2007). Gap junction blockage with octanol or carbenoxolone decreases infarct volume and cell death after brain injury (Rawanduzy et al., 1997; Rami et al., 2001; Frantseva et al., 2002). In contrast, mice lacking connexin 43, and thus lacking functional gap junctions, had increased infarct size following a permanent ischaemic lesion (Siushansian et al., 2001). The discrepancy of the studies indicates the complexity of the function of the gap junctions and that the time of intervention and nature of the injury may be important for the outcome. As the gap junction channels control the spreading of different molecules between cells, this could be a pathway involved both in toxicity and protection (Perez Velazquez et al., 2003; Farahani et al., 2005). More work needs to be done in order to determine the exact role for gap junction channels and the communication through them in the propagation of injury as well as in the development of reactive gliosis.

## Oxidative stress

During normal living we are constantly exposed to free radicals. The generation of reactive oxygene species (ROS) and reactive nitrogen species (RNS) are physiological phenomenons that occur during essential metabolic processes like mitochondrial energy production, oxidation of toxins and protective cytotoxic processes of the immune response. Toxic compounds from food, exercise, cigarette smoke and fasting also increase the generation of free radicals in the body. During physiological conditions the amount of free radical production is relatively small and can be scavenged by endogenous antioxidant mechanisms and the damage can be prohibited and

repaired. However, disturbance in the redox-state by increased production of peroxides and free radicals can cause mutations and cell damage by modification of lipids, protein and DNA that can result in tissue degeneration, apoptosis or necrosis. When the production of ROS exceeds ability of the normal endogenous antioxidant systems or when the detoxification fails, it leads to an oxidative stress situation.

The brain represents about 2% of the total body weight but demands 20% of the total oxygen consumption in the body. Consequently, high levels of ROS are continuously generated during oxidative phosphorylation (Dringen, 2000). Due to high consumption of oxygen and the high content of lipids, the CNS is especially vulnerable to lipid peroxidation and oxidative stress compared to other organs (Floyd, 1999). As astrocytes represent the primary cell-source of antioxidants in the brain, and have the ability to eliminate free radicals, they play an important role for neuronal viability (Dringen et al., 2000). However, during pathological conditions or conditions where a substantial amount of oxidants are generated, these neuroprotective mechanisms become compromised which may have devastating consequences for cell survival.

Oxidative stress is implicated in many pathological conditions in the brain. For instance, oxidative stress is one of the main causes of tissue damage following ischaemic insults in the brain (Kuroda and Siesjo, 1997; Sugawara and Chan, 2003). Increased levels of oxidants during ischaemia can cause a depletion of ATP levels and result in uncontrolled cell death (Endres et al., 1997; Ying et al., 2005). Oxidative stress is also implicated in several neurodegenerative disorders such as Parkinson´s disease (Wood-Kaczmar et al., 2006), Alzheimer´s disease (Nunomura et al., 2006), amyotrophic lateral sclerosis (Goodall and Morrison, 2006) and Huntington´s disease (Browne and Beal, 2006).

## The transcription factor Nrf2

The ability to detoxify ROS/RNS is crucial for cell survival and is accomplished by complex endogenous detoxification and antioxidant mechanisms. To detoxify artificial compounds, such as toxins from the environment, food components and pharmaceuticals, cells utilize enzyme systems in two steps called phase I and phase II. Neural cells protect themselves using mainly phase II detoxifying and antioxidant enzymes, including glutathione (GSH), superoxide dismutase, catalase, glutathione reductase, glutathione transferase, glutathione peroxidase and, NAD(P)H: quinone oxidoreductase 1 (Nqo1). The transcriptions of these genes, is

regulated by the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2).

Nrf2 is a key element for the cellular redox-state and is an essential component of endogenous cellular defence. During basal conditions, most Nrf2 is kept in an inactive state sequestered in the cytoplasm by its repressor Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al., 1999; Kobayashi et al., 2002) (fig. 1). Keap1 physically entraps Nrf2 in actin filaments and targets Nrf2 for ubiquitinylation and proteasome-mediated degradation (Cullinan et al., 2004). Oxidants and other reactive chemicals induce conformational changes that release and activate Nrf2 (Eggler et al., 2005; Kobayashi and Yamamoto, 2006; Tong et al., 2006). The liberation of Nrf2 from Keap1 is suggested to be due to phosphorylation of Nrf2 by protein kinases (Huang et al., 2002; Kobayashi and Yamamoto, 2006) or modification of thiols groups in Keap1 (Dinkova-Kostova et al., 2001; Zhang, 2001). Activated Nrf2 is transported to the nucleus where it, together with small Maf proteins, bind to promoters containing the antioxidant response element (ARE) motif (Itoh et al., 1997). Binding of Nrf2 to the ARE leads to transcription of numerous cytoprotective enzymes that are, for example, involved in GSH synthesis and degradation of free radicals and aldehydes, (Ishii et al., 2000)(fig 3). The potential of Nrf2 to induce the transcription of a wide range of antioxidants, that may lead to cell protection, has lead to an increasing interest in activators of the Nrf2 system. Nrf2 activation thus represents a key step in endogenous cellular protection (Copple et al., 2008).

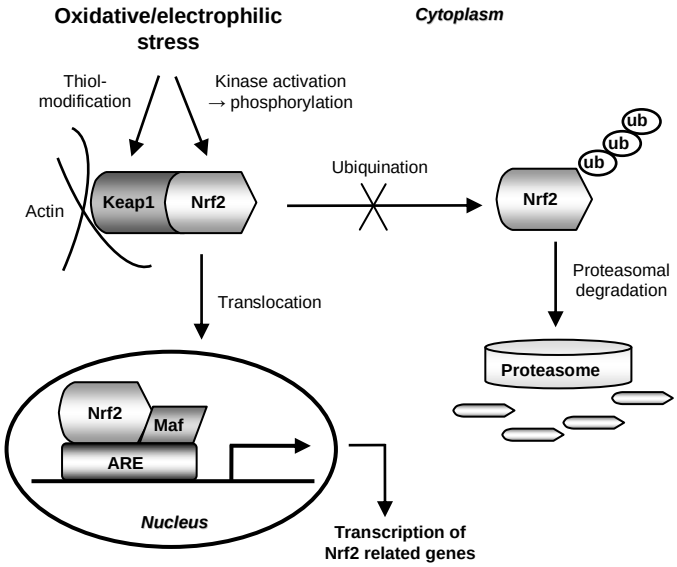


**Figure 3.** Nrf2 is sequestered in the cytoplasm and is regulated by Keap1 which under basal conditions targets Nrf2 for ubiquitinylation and proteasome-mediated degradation. Following cellular stress, Nrf2 can dissociates from Keap1 due to thiol modifications on Keap1 or Nrf2 phosphorylation by kinases. Nrf2 then translocates to the nucleus where it, together with small Maf proteins, binds to the ARE region and induces the transcription of detoxification and antioxidant enzymes (Zhang and Gordon, 2004).

## The importance of Nrf2-activation

Nrf2 is expressed in a variety of tissues (Moi et al., 1994) and is especially abundant where the main detoxification reactions occur such as in the kidney, intestine and lung (Itoh et al., 1997). Activation of Nrf2 is suggested to be the most important pathway coordinating the regulation of cell protection against oxidative stress (Dhakshinamoorthy et al., 2000). Substances that activate Nrf2 protect many different organs and tissues from several injuries and diseases (Lee et al., 2005). For instance, the Nrf2 system plays a critical role in protecting tissues from a variety of toxic insults such as carcinogens, reactive oxygen species, diesel exhaust, inflammation, calcium disturbance, UV light, and cigarette smoke (Lee et al., 2005). Conversely, mice lacking Nrf2 are much more sensitive to exposure to free radicals than their wildtype counterparts, and develop diseases from sunlight and even from minor exposure to cigarette smoke (Rangasamy et al., 2004; Hirota et al., 2005).

Nrf2 has been referred to as the multi-organ protector (Lee et al., 2005) and in comparison to many other antioxidants which act more specifically, Nrf2 regulates the transcription of a whole battery of genes encoding for proteins involved in detoxification, inflammation and free radical scavenging (Itoh et al., 1997; Ishii et al., 2000; Copple et al., 2008). These include the neuroprotective enzymes heme oxygenase-1 (Hmox1) (Alam et al., 1999), Nqo1 (Venugopal and Jaiswal, 1996) and enzymes involved in GSH synthesis and utilization, such as glutathione-S-transferase and glutamate cysteine ligase (Ikeda et al., 2002). The Nrf2-system has long been investigated as a therapeutic target for the prevention of cancer (Zhang and Gordon, 2004), while the investigations of the potential cell protective role for Nrf2 in the neuroscience field has recently dramatically increased.

In neural cells over 200 genes are regulated directly or indirectly by Nrf2 (Lee et al., 2003b; Lee et al., 2003a; Shih et al., 2003) and many of them have neuroprotective effects after cerebral ischaemia (Panahian et al., 1999; Crack et al., 2003; Hoen and Kessler, 2003; Arthur et al., 2004; Hattori et al., 2004). Mice lacking Nrf2 have a larger infarct volume following middle cerebral artery occlusion than their wildtype counterparts (Lee et al., 2003b; Kraft et al., 2004; Shih et al., 2005). The mice are also more prone to developing Parkinson's disease (Burton et al., 2006) while mice over-expressing Nrf2 are protected against Parkinson's disease or amyotrophic lateral sclerosis (Vargas et al., 2008; Chen et al., 2009). Nrf2-deficient mice also display an increased occurrence of activated microglia and astrocytes in different neurodegenerative models (Parkinson's disease, Huntington´s disease, multiple sclerosis and amyotrophic lateral sclerosis) compared to wild type controls (Kraft et al., 2004; Calkins et al., 2005; Kraft et al., 2006; Jakel et al., 2007; Vargas et al., 2008; Chen et al., 2009; Johnson et al., 2010; Rojo et al., 2010). A recent study demonstrated that variation in the human Nrf2 gene can affect the risk and the process of Parkinson´s disease (von Otter et al., 2010). In addition, astrocytic and neuronal cultures derived from mice lacking Nrf2 are more vulnerable to oxidative stress and inflammation (Lee et al., 2003b; Lee et al., 2003a).

Although Nrf2 is active in neurons, recent results indicate that astrocytes constitute the most important target for Nrf2-stimulating therapy in the brain (Vargas and Johnson, 2009). In response to Nrf2 activation (by *tert*-butylhydroquinone or sulforaphane incubation) or over-expression of Nrf2, astrocytes exhibit greater Nrf2 activation than neurons. The Nrf2 over-expression in astrocytes protects neurons from different oxidative insults (Shih et al., 2003; Kraft et al., 2004; Chen et al., 2009; Vargas and Johnson, 2009). Moreover, transplanted astrocytes over-expressing Nrf2 reduced brain

injury induced by oxidative stress (Calkins et al., 2005; Jakel et al., 2007). Studies in cultured astrocytes have shown that sulforaphane preconditioning for 48 h upregulates Nqo1 and protects cells against oxidative stress and death after oxygen and glucose deprivation in an Nrf2-dependent manner (Kraft et al., 2004; Danilov et al., 2009). Exactly how Nrf2-activated astrocytes contribute to neuroprotection is still unclear. However, genes regulated by Nrf2 encode for proteins that control key steps in, for example, heme metabolism (Alam et al., 2000), reduction of quinones (Itoh et al., 1997) and glutathione synthesis (Shih et al., 2003), mechanisms that are all involved in cell protection.

## Sulforaphane - an activator of Nrf2

Diets rich in vegetables and fruits are associated with reduced risk of several major diseases, including stroke (Gillman et al., 1995). Several fruits and vegetables contain phytochemicals which are compounds that allow the plant to protect itself from different threats, external as well as internal, including diseases. Several phytochemicals have been demonstrated to be strong activators of the Nrf2 signalling system (Mattson and Cheng, 2006). One of these phytochemicals is sulforaphane which is found in high amounts in cruciferous vegetables such as cauliflower, cabbage and broccoli. The highest concentration of sulforaphane is found in broccoli sprouts which contain 30 to 50 times more sulforaphane than mature broccoli (Fahey et al., 1997).

Sulforaphane is released from the sugar molecule glucosinolate, during chewing. Among naturally occurring substances, sulforaphane is the most potent inducer of Nrf2-regulated phase II enzymes that has been identified so far (Zhang et al., 1992; Talalay, 2000). Sulforaphane is suggested to act by modifying critical cysteine residues of Keap1 which leads to liberation of Nrf2 and the binding to the ARE region of phase II enzymes (Dinkova-Kostova et al., 2001). The anticarinogenic action of sulforaphane has long been known (Zhang et al., 1992; Zhang et al., 2006). More recently the neuroscience field has also become increasingly interested in sulforaphane due to its cytoprotective and anti-inflammatory properties (Fahey et al., 1997; Azarenko et al., 2008; Innamorato et al., 2008).

The Nrf2-mediated anti-inflammatory properties of sulforaphane have been demonstrated in both *in vivo* and *in vitro* models of inflammation (Innamorato et al., 2009). In a mouse model of inflammation, sulforaphane administration interfered with the inflammatory response by decreasing macrophage/microglia activation (Innamorato et al., 2008). In rats with high blood pressure and with high risk for stroke, sulforaphane decreased

inflammation and improved the function of heart, artery and kidney (Wu et al., 2004). Sulforaphane treatment inhibits tumor development in a number of rodent models (Talalay et al., 1978; Pearson et al., 1983; Zhang et al., 1994; Fahey et al., 2002), while mice lacking the Nrf2 gene do not acquire cancer protection from broccoli or sulforaphane (Xu et al., 2006), indicating that the positive effect of sulforaphane requires a functional Nrf2 response.

Activation of the Nrf2 pathway by sulforaphane reduces brain damage in models of transient middle cerebral artery occlusion and intracerebral hemorrhage (Zhao et al., 2006; Zhao et al., 2007). In cultured astrocytes, sulforaphane preconditioning protects the cells against oxidative stress and death after oxygen and glucose deprivation (Kraft et al., 2004; Danilov et al., 2009).

## Genes regulated by Nrf2 activation

Activation of Nrf2 increases transcription of a whole army of genes encoding for enzymes involved in detoxification, defence against ROS and other free radicals, the synthesis of glutathione, as well as inflammation inhibition. Of the many Nrf2 induced genes, Nqo1 is a known multi-protective enzyme (van Muiswinkel et al., 2000; Dinkova-Kostova and Talalay, 2010) and in the CNS the heme oxygenase and the GSH systems are particularly important for the neural defence against oxidative damage (Vargas and Johnson, 2009). However, the cell protection observed is most likely not due to one single gene but a combined effect of several upregulated proteins induced by Nrf2 activation.

### *NAD(P)H: quinone oxidoreductase 1 (Nqo1)*

Nqo1 is a known multi-protective enzyme (van Muiswinkel et al., 2000; Dinkova-Kostova and Talalay, 2010) and a well documented antioxidant that is under direct transcriptional control by Nrf2/ARE (Jaiswal, 2000). Among the inducible cytoprotective proteins, Nqo1 is one of the most consistent and robust genes (Benson et al., 1980). Nqo1 is classified as a phase II enzyme and is functionally found in the cytoplasm in all cell types, but at various concentrations (Talalay et al., 1995). Nqo1 is considered to protect cells against oxidative damage through the reduction of quinones. This subsequently prevents the generation of ROS as quinones participate in redox cycling and damage cells by depleting cellular thiol-groups such as glutathione (Nioi and Hayes, 2004; Talalay and Dinkova-Kostova, 2004). In the CNS Nqo1 is most abundant in the glial cells (Stringer et al., 2004). Upregulation of Nqo1 protects against oxidative stress in cultured astrocytes

(van Muiswinkel et al., 2000; Danilov et al., 2009) while the lack of Nqo1 enzyme induce disturbance in the redox state and seizures (Gaikwad et al., 2001; Stringer et al., 2004).

### *Heme oxygenase-1 (Hmox1)*

Among the genes regulated by Nrf2, Hmox1 (Alam and Cook, 2003) is of particularly interest due to the generation of products that display antioxidant, anti-apoptotic and anti-inflammatory effects (Prawan et al., 2005). Heme oxygenase is an essential enzyme that catalyzes the degradation of heme into biliverdin, iron and carbon monoxide (Kikuchi et al., 2005). Biliverdin can be further converted into bilirubin by biliverdin reductase. Biliverdin and bilirubin have free radical scavenging effects, and carbon monoxide has an inhibitory effect on inflammation, cell death, proliferation, and it facilitates vasodilation (Baranano et al., 2002; Li et al., 2009b). Iron, on the other hand, is cytotoxic but enhances the expression of the antioxidant ferritin that is a molecule that stores intracellular iron. Moreover, Hmox1 regulates the intracellular iron levels and inhibits the accumulation of iron that can lead to cells death (Ferris et al., 1999).

There are two isoforms of heme oxygenase, Hmox1 and Hmox2 (Maines et al., 1986). Under basal conditions Hmox2 is the isoform most widespread in the CNS with a relatively constant expression. In contrast, Hmox1 expression can be induced by its substrate heme, or in response to cellular stress, such as oxidative stress, pro-inflammatory agents or Nrf2 activation. The induction of Hmox1 is neuroprotective against oxidative stress in neuronal cultures (Chen et al., 2000) and in animal models of ischaemia (Nimura et al., 1996; Panahian et al., 1999; Zhao et al., 2006). Hmox1 induction is also important for redox regulation and to attenuate inflammation in several neurodegenerative diseases (Cuadrado and Rojo, 2008).

### *Glutathione*

Nrf2 activation is involved in the regulation of GSH synthesis and the expression of several glutathione S-transferases. GSH plays a central role in the scavenging of ROS and is the primary antioxidant in the brain. GSH is comprised of cysteine, glutamate and glycine and is synthesised in two steps by the enzymes glutamate cysteine ligase, that is the rate limiting enzyme, and by glutathione synthetase (Dringen and Hirrlinger, 2003). Glutamate cysteine ligase activity can be modulated by feedback inhibition of GSH. GSH exists in a reduced state (GSH), and in an oxidized state (glutathione disulfide, GSSG). In the brain, glutathione is mainly found in its reduced

form where approximately 90% of the intracellular GSH pool exists in the cytoplasm.

GSH is a central component of the cellular antioxidant defence and protects the cell against various ROS (Dringen, 2000; Anderson et al., 2003). GSH is able to directly detoxify ROS, by a non enzymatic process (Dringen, 2000), or can be used as a substrate (electron donor) (Dringen et al., 2005). During the reaction, two reactive GSH molecules form the oxidized state, GSSG. The GSSG will subsequently be reduced by glutathione reductase to GSH.

A compromised GSH synthesis is often observed in conditions of oxidative stress occurring during neurological diseases and stroke (Dringen and Hirrlinger, 2003). The excess of glutamate in the extracellular space that may follow a brain injury can block the cysteine uptake leading to a decreased production of GSH, as cysteine is an important building block in the synthesis of GSH. This decrease of GSH levels may be deleterious to tissues with a high metabolic activity, such as the brain and may lead to a increased oxidative stress resulting in more extensive cell injury (Pereira and Oliveira, 2000).

Among neural cells, astrocytes exhibit the highest concentration of GSH (Raps et al., 1989; Rice and Russo-Menna, 1998; Dringen et al., 2000). Astrocytic GSH acts either directly as an antioxidant or is used to increase the levels of glutathione in neurons (Dringen et al., 1999; Dringen et al., 2000; Dringen et al., 2001). The secreted glutathione from astrocytes into the extracellular space has been demonstrated to protect neurons against oxidative damage (Dringen et al., 2000; Shih et al., 2003; Kraft et al., 2004) whereas under conditions of GSH depletion this protection is lost (Drukarch et al., 1997; Gegg et al., 2005). Experimentally, the levels of glutathione are known to be difficult to increase. However, Nrf2 activation induces the astrocytes to secrete GSH through the hemichannels into the extracellular space (Stridh et al., 2010). The astrocytic secretion of GSH has demonstrated to be the most important factor for observed neuroprotection (Vargas et al., 2008).

## Summary and hypotheses

Neurons are a very well studied cell type and have for long been the main focus when investigating pathological brain conditions. However, accumulating evidence indicates a crucial role for astrocytes and other glial cells in neuronal signal processing both during normal and pathological conditions (Hamby and Sofroniew, 2010). Still, the roles of glial cells in the pathological brain are only just beginning to be defined.

Brain injury initiates an extensive glial cell response. Although reactive gliosis and scar formation exhibit protective and reparative functions, the scar is also a major obstacle for the recovery process (Fawcett and Asher, 1999). Reactive gliosis progresses over time and can be observed at great distances from a brain lesion (Moumdjian et al., 1991; Setkowicz et al., 2004) suggesting that long-distance signaling mechanisms are involved in the transformation of glial cells into their reactive states. Our hypothesis was that blocking gap junction communication would modulate reactive gliosis.

The importance of a functional Nrf2 system in the endogenous defence against oxidative damage has been described in several different models of oxidative stress and diseases (Lee et al., 2005). Sulforaphane, a substance highly enriched in cruciferous vegetables such as broccoli, is a potent activator of Nrf2. A functional Nrf2 response is required for the protective effects observed after intake of broccoli or sulforaphane (Iida et al., 2004; Xu et al., 2006). Even if sulforaphane is cleared from the body within a few hours (Ye et al., 2002) it still offers long-term protection from oxidative stress (van Poppel et al., 1999). Our hypothesis was that brief sulforaphane stimulation would be sufficient to induce prolonged Nrf2-induced gene expression.

A previous study in astrocytes has demonstrated an upregulation of Nqo1 expression and protection against oxygen and glucose deprivation for up to 48 h after constant sulforaphane-stimulation (Danilov et al., 2009). However, sulforaphane could possibly be degraded during the constant 48 h of stimulation, which could limit the Nrf2 activation at late stages of the experiment. Our hypothesis was that daily transient sulforaphane-stimulation would result in accumulation of Nrf2-mediated mRNA and protein expression and increased protection against oxidative damage.

Oxidative stress and reactive gliosis are pathological features of various brain injuries, including stroke, and contribute to the subsequent tissue damage that follows the injury. Although the neuroprotective properties of sulforaphane

are well documented, its effect on reactive gliosis after a stroke is less well investigated. Our hypothesis was that sulforaphane-induced Nrf2 stimulation would modify stroke outcome and reactive gliosis when given after permanent focal ischaemia.

# AIMS OF THE STUDIES

The general aim of this thesis was to investigate the role of intercellular communication and the Nrf2-induced endogenous antioxidant system on reactive gliosis and cellular protection after brain injury.

**The specific aims were:**

- To investigate whether the gap junction blockers octanol and carbenoxolone reduce the expression of markers of reactive gliosis after a minor traumatic brain injury in rats.

- To examine the kinetic response of two Nrf2-regulated genes, Nqo1 and Hmox1, after exposing cultured astrocytes to sulforaphane.

- To investigate whether repeated transient sulforaphane exposure results in accumulation of Nrf2-mediated mRNA and protein expression and protection against oxidative damage in cultured astrocytes

- To investigate whether stimulation of Nrf2 with sulforaphane affects stroke outcome and reactive gliosis after permanent focal ischaemia in mice.

# METHODS

## Astrocyte cell cultures (Papers I, II)

Primary astroglial cultures were prepared from 1-2 day old inbred Sprague-Dawley rats, as described in Paper I and II. Cells were used after 14-17 days in culture when a confluent monolayer had formed. For fluorescent detection of peroxide production, glutathione levels and propidium iodide exclusion, the confluent cultured cells were harvested and replated in white 96-well microtiter plates, or in 0.8 $cm^2$ chambered coverglasses pre-coated with collagen solution type 1 for confocal imaging.

### Comments:

Primary enriched astroglial cultures have been extensively used for studying astrocytic properties and function for more than 30 years (Booher and Sensenbrenner, 1972; Kimelberg, 1983). In the primary cultures, there is a mix of brain cells. Following two weeks in the petridish with medium that support the growth of astrocytes, a confluent monolayer is formed where all astrocytes are in contact with each other and can function as a network. Cell cultures enable studies of specific cellular properties and response to different stimuli, where specific mechanisms can be independently investigated without influences from other cell types. However, the *in vitro* conditions are artificial in comparison to the normal brain environment, which includes the influence from the extracellular milieu and all other cell types. Therefore one should be cautious in drawing general conclusions from studies performed in *in vitro* models. In addition, these cultures originate from the immature animal brain and therefore may have different properties to cells derived from the adult mature brain. This, must also be considered when interpreting results. To obtain a complete picture of the situation one should combine the *in vitro* experiments with the use of more complex models.

## Scrape loading/Dye transfer (Paper I)

Gap junction communication was assessed by using the scrape loading/dye transfer technique (Blomstrand et al., 1999a; Nodin et al., 2005). In brief, confluent astrocyte cultures were incubated in HEPES buffered salt solution (HBSS) with or without carbenoxolone or octanol. Lucifer yellow in calcium-free buffer was added, and two parallel scrapes were performed with a scalpel. The Lucifer yellow was allowed to diffuse into the cell layer before it was removed, and calcium containing HBSS was added. Images of each culture were captured with a Nikon Optishot 2 equipped with a Hamamatsu

C5810 chilled three-chip colour charge-coupled device camera. The extent of dye spreading from the scrapes is measured and compared to control treated cells.

### Comments:

Scrape loading/dye transfer was used to identify the permeability through the gap junction channels in cultured astrocytes following exposure to the commonly used gap junction modulators octanol and carbenoxolone. The technique is widely used (el-Fouly et al., 1987; Blomstrand et al., 1999b) and is based on the permeability through the channels of a cell membrane impermeable fluorescent substance such as Lucifer yellow (Paper I). With a single cut through the confluent cell layer Lucifer yellow can enter the cells in the cut area and then diffuse to neighbouring cells via the gap junction channels. Lucifer yellow was introduced to cells in calcium-free medium as high concentrations of calcium are known to inhibit the permeability of the gap junction channel. Lucifer yellow was allowed to enter the cell layer for just one minute to avoid toxicity from the calcium free medium. Then the substance was allowed to diffuse for another 8 min before images were taken.

## Nrf2 stimulation by sulforaphane *in vitro* (Paper II)

To stimulate Nrf2, the isothiocyanate sulforaphane was used. Sulforaphane was dissolved in dimethyl sulfoxide (DMSO) to a 10 mM stock solution and was stored at -70°C. To obtain final concentrations, sulforaphane was diluted in culture medium just before addition to the cultures.

### Comments:

The phytochemical sulforaphane is derived from glucosinolate found in cruciferous vegetables and especially broccoli sprouts. Sulforaphane is a potent inducer of genes involved in detoxifying oxidants through the activation of Nrf2 (Thimmulappa et al., 2002; McWalter et al., 2004). Many phytochemicals have a hormetic effect, where low concentrations result in beneficial effects while high concentration can lead to cell toxicity (Mattson and Cheng, 2006). However, the concentrations of sulforaphane used in the present *in vitro* study, did not induce significant cytotoxicity and the cultures stayed viable after exposure.

## Peroxide measurements (Paper II)

Changes in peroxide production in the astrocyte cultures were analysed in white 96-well microtiter plates with transparent bottoms using the non-

fluorescent probe, carboxy-$H_2$DCFDA (5-(and-6)-carboxy-2-7-dichloro-dihydrofluorescein diacetate) as described earlier (Petersen et al., 2008). Astrocytes were incubated with carboxy-$H_2$DCF-DA for a few hours and the levels of peroxides were measured after 24 h. The fluorescence was measured at an excitation wavelength of 493 nm and an emission wavelength of 522 nm in a SPECTRAmax GEMINI spectrofluorimeter.

### Comments:

Excess ROS are generated during a variety of cellular stresses including trauma and ischaemic injury. The production of hydrogen peroxide was measured in order to investigate the astrocytes ability to clear peroxides following sulforaphane stimulation. Carboxy-$H_2$DCFDA is a well established method to detect and quantify hydrogen peroxide in cell cultures (Cathcart et al., 1983). In the cell, Carboxy-$H_2$DCFDA is cleaved by esterases, yielding polarized non-fluorescent dichlorofluorescein carboxy-DCFH. Carboxy-DCFH is oxidized by peroxides to fluorescent carboxy-DCF that can be analysed using a spectrophotometer. The amount of fluorescence is correlated to the amount of peroxide there is in the media.

## GSH measurements (Paper II)

Levels of GSH were analysed in white 96-well microtiter plates with a transparent bottom as described previously (Petersen et al., 2008). The cells were incubated with monochlorobimane (MCB) that forms a fluorescent conjugate with the reduced form of GSH. Changes in GSH levels were measured after 2-3 h (excitation wavelength 380 nm, emission wavelength 460 nm). Buthionine sulfoximine is a specific inhibitor of glutamate cysteine ligase, the rate-limiting enzyme for glutathione synthesis (Anderson, 1998) and was used as a negative control.

### Comments:

MCB is a commonly used probe for measuring intracellular levels of GSH (Cook et al., 1991; Sun et al., 2005). MCB is added to the culture medium and forms a fluorescent MCB-GSH conjugate catalyzed by intracellular glutathione S-transferase. The fluorescence is a measure of the changes in GSH levels (Chatterjee et al., 1999). The advantage of using MCB is that the method is simple and can be used on living cells. It is less time consuming than, for example, high-performance liquid chromatography (Komuro et al., 1985).

## Oxidative stress generated by xanthine/xanthine oxidase (Paper II)

The effect on free radicals following activation of the Nrf2 system was explored by exposing astrocyte cultures to the superoxide radical-generating system xanthine/xanthine oxidase. The experiments were initiated by replacing the normal medium with a mixture of 0.5 mM xanthine and 5-44 mU/ml xanthine oxidase dissolved in normal medium, which was added to the cultures for 1 h. As a measure of cell viability, the ATP levels were analysed 23 h later.

### Comments:

The free radical generating xanthine/xanthine oxidase system is a classical system that results in the generation of ROS. Superoxide, hydrogen peroxide and hydroxyl radicals are cytotoxic products that contribute to oxidative stress and is formed by xanthine/xanthine oxidase (Link and Riley, 1988).

## ATP measurements (Paper II)

ATP levels, as a measure of cell viability, were measured 23 h after a 1 h superoxide challenge. To extract ATP, the cell cultures were rapidly rinsed with ice-cold phosphate buffered saline, thereafter ice-cold trichloroacetic acid was added to the cultures (Nodin et al., 2005). ATP analysis was completed using an ATP Bioluminescence Assay CLS II kit according to the manufacturer's instructions. Samples were loaded into white, flat-bottomed 96-well plates and the luminescence was determined using a Victor II plate reader (Wallac). The ATP levels were calculated as fold-change of untreated control for each independent experiment.

### Comments:

Free radicals are toxic for the cells in high concentrations and can cause cell death. ATP is a way to measure the cell's viability as it is present in all metabolically active cells and the concentration rapidly drops during necrosis or apoptosis. Therefore, the amount of ATP detected using the luminescence reaction can be correlated with cell viability.

## Propidium iodide exclusion (II)

Propidium iodide exclusion was used as a measure of late stage of cell death. Propidium iodide was added to the astrocyte cultures to a final concentration of 10 μM. The cells were stressed by the addition of $H_2O_2$ and changes in

fluorescence were measured (emission at 620 nm and excitation at 540 nm). Finally, the cells were treated with detergent and frozen at -20°C. After thawing, fluorescence measurement gave an estimate of total cellular nucleic acids in the permeabilized (dead) cells.

### Comments:

Propidium iodide exclusion measures the number of cells unable to sustain plasma membrane integrity and is used as a marker of late stages of cell death. Here it was used as an unbiased marker of cell death, after $H_2O_2$ stress, detecting both necrotic and apoptotic cells. Cellular damage and death leads to leakage of propidium iodide into the cells. Propidium iodide then combines with nucleic acids and the changes in fluorescence can be measured. The disadvantage of this method is that membrane leakage is a late marker of apoptosis and necrosis. To complement the propidium iodide exclusion assay, we also measured resistance to free radical challenge by change in ATP content which is an earlier marker of cell death

## Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) (Papers II, III)

Analyses of mRNA in cultured cells (paper II) and in tissue samples (paper III) were performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The samples were lysed and total mRNA was extracted and purified by using a MagAttract Direct mRNA M48 Kit with oligo (dT) covered magnetic beads on a GenoM-48 Robotic Workstation (Geno Vision). Standard settings for mRNA extraction were used. cDNA was synthesized from the mRNA extraction. cDNA was quantified in 96-well optical microtiter plates on a 7900HT Fast QPCR System in TaqMan® Fast Universal PCR Master Mix according to the manufacturer's protocol with minor modifications. Primers and probes used for amplification of the genes of interest are listed in papers II and III. PCR results were analyzed with SDS 2.3 software and relative quantity was determined using the $\Delta\Delta C_T$ method with untreated samples as the calibrator and Polr2a as an endogenous control.

### Comments:

RT-qPCR is a very common technique used to amplify and relative quantify specific mRNA transcripts within a sample. In comparison to normal PCR where the result is given at a set time, q-PCR detects the kinetics of the reaction during each cycle and collects data in the linear phase of the PCR reaction. By comparing the target gene expression to a gene that is

constitutively expressed (an endogenous control) the variability in mRNA can be relatively quantified.

## siRNA transfection (Paper II)

The small interfering RNA (siRNA) technique was used for down-regulation of Nrf2 expression. Astrocytes were reseeded in 24-well plates to reach 30–50% confluence at the time of transfection. The cells were incubated for 5 h with the siRNA against Nrf2 or control according to the manufacturer's instructions. After 24 h, Nrf2 was stimulated with sulforaphane. The mRNA levels of Nrf2 and its response genes Hmox1 and Nqo1 were measured with qPCR after 6 h.

### Comments:

Small interfering RNA (siRNA), also called silencing RNA, is used to down-regulate the expression of a gene product. The siRNA targets a specific RNA resulting in decreased expression of the protein of interest, in this case Nrf2. This technique was used in the present study to confirm that the effect of sulforaphane was mediated by Nrf2 activation and was not a direct effect of sulforaphane per se. The knockdown of the expression of Nrf2 was confirmed by RT-qPCR.

## Immunoblotting (Papers I, II)

Electrophoresis and western blot technique were used to determine the increase or decrease of a particular protein in the homogenate. Cell cultures or tissue samples were lysed and the protein concentration was measured. Lysates containing equal amounts of protein were introduced into each lane of the gel. Electrophoresis was conducted to separate the proteins according to the manufacturer's instructions. The separated proteins were then transferred into a membrane by western blot. Unspecific binding of antibodies to the membrane was prevented by first incubating the membrane in blocking buffer. Primary antibodies against the protein of interest were detected with horseradish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminiscence. The observed protein bands were then related to a housekeeping gene quantified by densitometry.

### Comments:

Western blot is a technique used for doing semi-quantitative measures of the amount of protein in a sample. The samples are usually homogenised and detergents are used to lyse the cells to solubilise the proteins. A protein

concentration measurement is done so equal amounts of protein will be analysed. The proteins in the sample are then separated using gel electrophoresis where the separation is based on molecular weight. For antibody detection, the proteins are transferred from the gel onto a membrane using electrical current. To avoid unspecific binding of the antibody to the membrane, a blocking step with non-fat dry milk or bovine serum albumin is done before the primary antibody incubation. Following several washing steps the membrane is incubated with a horseradish peroxidase conjugated secondary antibody. The detection reagent is a chemiluminescent agent that is cleaved by the secondary antibody and the reaction product produces luminescence. The light is detected and captured as a digital image and then by using densitometry, the relative amount of protein can be measured. The advantage of western blot technique over immunohistochemistry is that the proteins are separated according to their size which facilitates the identification of the correct antigen. In addition, the samples are loaded with equal protein concentration and can be quantified using densitometry.

## Experimental animals (Papers I, III)

In Paper I adult male Sprague Dawley rats weighing 280-310g (B&K Universal, Sweden). In Paper III we used adult male C57Bl/6 mice weighing approximately 25 g (Charles River, Germany). Animals were housed under standard conditions with a 12 h light/12 h dark cycle, temperature (24-26°C) and humidity (50-60%) and had access to food and water *ad libitum*. All experimental protocols were approved by the Animal Ethics Committee of Göteborg University and performed according to approved NIH animal care guidelines.

### Comments:

Sprague Dawley rats were used in Paper I. They are commonly used in trauma and stroke models. In Paper III, adult mice were used to enable future comparisons with gene knockout mice. Mice are most commonly used to create gene knockout models as the procedure in rats is more difficult and was first done in 2003 (Zan et al., 2003).

## Injury models (Papers I, III)

***Needle track injury (Paper I):*** To study reactive gliosis, a needle track injury was performed. Rats were anaesthetized and placed in a stereotaxic frame. The skull was exposed and a cortical stab wound was performed. After 2 days the animals were sacrificed and the brains were extracted. The brains

were fixed and transferred to cryoprotective sucrose solution. Frozen sections were cut in the horizontal plane and stored individually in 96-well plates at -20°C in cryoprotecting buffer until they were processed for immune-histochemistry or immunofluorescence. For protein determination and western blot analysis, a 2 x 2 x 5 mm tissue piece was dissected out from the ipsilateral side including the needle track and frozen immediately in liquid nitrogen. Tissue samples were then thawed and sonicated and the protein concentration was determined using the Bicinchoinic Acid Protein Assay kit.

## Comments:

The needle track and stab wound injury are commonly models utilized for studying the glial response following injury (Norton et al., 1992). Horizontal sectioning allowed us to overview the whole injury in one plane, and the progression of reactive gliosis could be easily seen and measured after immunohistochemical staining. The needle produced a discrete and restricted trauma injury with a necrotic are in the centre of the wound and reactive glial cells bordering the area.

***Photothrombotic stroke (Paper III):*** Cortical photothrombosis was induced by the Rose Bengal technique (Watson et al., 1985; Lee et al., 2007). The mice were anaesthetised and placed in a stereotaxic frame. A small scalp incision was made and the laser was positioned as described in Paper III and in a previous study (Paxinos and Watson, 2007). The photosensitive Rose Bengal was injected into the peritoneum. The laser was turned on and the area of interest was illuminated. For histochemical analysis, the animals were sacrificed 24 h and 72 h after stroke induction by an overdose of pentobarbital and transcardially perfused with saline followed by fixative. The brains were extracted and post-fixed overnight in the same fixative prior to cryoprotection in sucrose solution. Frozen sections were cut in the coronal plane and thaw mounted and stored at -20°C. For RT-qPCR analysis, 2 mm by 2 mm blocks of lateral cortex and liver from vehicle- and sulforaphane-treated mice were snap-frozen in liquid nitrogen.

## Comments:

In the photothrombotic stroke model, a dye is injected into the animal followed by illumination, which activates the dye at the site of the illumination. This leads to free radical formation, and a cascade of coagulation and aggregation that blocks the blood vessel. The photo-thrombotic stroke model has the advantage of being highly reproducible in location and in size. In addition, the photothrombotic model is minimally invasive but has a similar cell response to more invasive models such as the middle cerebral artery occlusion (MCAO) model. The infarction gives a

relatively clear border of ischaemic and non-ischaemic tissue, thus facilitating cell analysis in the peri-infarct region. In the photothrombotic model the ischeamia is permanent, and has a much smaller penumbra in comparison to a reperfusion model where some blood supply can still enter the site of injury (Kuroda and Siesjo, 1997). In consequence, lower levels of ROS are produced in the photothrombotic stroke model in comparison to a reperfusion model.

## Administration of BrdU (Papers I, III)

To detect proliferating cells, the animals were injected with bromodeoxy-uridine (BrdU). In paper I rats were intraperitoneally (i.p.) injected with 150 mg/kg BrdU twice a day, 8 h apart, with the first injection 30 min after injury, and sacrificed two days later. In paper III, 50 mg/kg BrdU was i.p. injected to the mice once a day over 3 days with the first injection 15 min after stroke onset.

### Comments:

The injection of BrdU is a common way to detect proliferating cells. BrdU is a thymidine analogue which is incorporated into the DNA of dividing cells and can be detected immunohistochemically in the daughter cells. There are alternative markers for *in vivo* cell proliferation such as Ki-67, PCNA and doublecortin. However these markers do not identify new born cells after differentiation. A potential problem of with BrdU is that it can be incorporated into cells during DNA repair. However, genome replication will include larger amount of BrdU, than a cell that repairing its DNA (Biebl et al., 2000).

## Administration of gap junction blockers (Paper I)

The rats received i.p. injections of either gap junction blocker or vehicle 30 min before or after the needle track injury. Octanol was dissolved in DMSO and a final dose of 710 mg/kg was administered to the rats (Rawanduzy et al., 1997). As octanol has a slight anaesthetic effect (Kurata et al., 1999), the rats receiving octanol before injury were anaesthetized with 85% of the normal dose of ketalar and rompun. Control rats for the octanol group received injections of DMSO only. Carbenoxolone was dissolved in saline and a final dose of 90 mg/kg administered to the rats. Control rats for carbenoxolone received injections of saline only.

### Comments:

To investigate the effect of gap junction communication on the progression of reactive gliosis we used two commonly used gap junction blockers. Octanol and carbenoxolone are identified as potent blockers of gap junction channels (Juszczak and Swiergiel, 2009). Although both of these compounds are strong gap junction blockers, neither exhibits a pure pharmacological specificity for this mechanism of action. As selective gap junction blockers are currently lacking (Juszczak and Swiergiel, 2009), and many normally used blockers have side effects that may be neuroprotective, we used octanol and carbenoxolone, two structurally different gap junction blockers.

Octanol, like other long chain alcohols, is suggested to block gap junctions quickly and reversibly. Its mechanism of action is not clear and is probably due to multiple factors. Its anaesthetic effect is probably due to octanol's agonist-like effect on $GABA_A$ receptors (Kurata et al., 1999). Carbenoxolone acts more slowly and closes the gap junctions indirectly by activating enzymes, ATPases, G-proteins for example (Jahromi et al. 2002). The ability to enter the brain probably also differs between the two drugs as octanol is lipophilic and carbenoxolone is hydrophilic. As octanol and carbenoxolone, have other effects on the cell, in addition to their ability to block gap junctions, one should be cautious when saying that possible observed effects of octanol or carbenoxolone are only due to their effect on gap junction channels.

## Nrf2 stimulation by sulforaphane *in vivo* (Paper III)

Sulforaphane was dissolved in DMSO and further diluted in either corn oil or sterile saline. For mRNA, behavioural, and histological analyses, animals were i.p. injected with 5 mg/kg or 50 mg/kg sulforaphane, 15 min after the ischaemic injury, and sacrificed 24 h later. For further histological and behavioural analyses, an additional set of animals were injected with 5 or 50 mg/kg sulforaphane 15 min, 24 h and 48 h after the ischaemic injury and sacrificed 72 h later.

### Comments:

Sulforaphane is an inducer of the Nrf2 system and is described under the paragraph "Nrf2 stimulation with sulforaphane in vitro". To maximize the Nrf2 system, sulforaphane was repeatedly administered for up to three days in order to investigate its neuroprotective effect.

## Immunohistochemistry (Papers I, III)

Immunohistochemistry was used to determine the distribution and presence of the different types of glial cells, as well as proliferating cells. Endogenous peroxidase activity was blocked with $H_2O_2$ and for detection of BrdU-labeled nuclei, the DNA was denatured. Unspecific binding was blocked and the sections were permeabilized. Primary antibodies against glial cells and proliferating cells were used. Thereafter the sections were incubated with biotinylated conjugated secondary antibodies followed by avidin-biotin-peroxidase complex. The peroxidase was detected by 3, 3´-diamino-benzidine tetrahydrochloride (DAB) solution in the presence of $H_2O_2$ and nickel ammonium sulphate. Negative controls were performed by omitting the primary antibodies and applying the secondary antibodies alone. Rinsing in water stopped the reaction and the sections were dehydrated and mounted. The sections were viewed by bright field microscopy and images were captured with a Nikon Optishot 2 and microscope equipped with a Hamamatsu C5810 colour chilled 3CCD camera.

### Comments:

Immunohistochemistry is a sensitive method and an important tool for determining cell distribution and morphology. The method is based on the specific binding of the primary antibody to an antigen on the tissue/cell. The outcome and quality of the binding is influenced by factors such as fixation and the specificity of the antibody for example. Non-specific binding of secondary antibodies can be detected by incubating some sections without adding primary antibodies.

In paper I the staining was performed on free-floating sections. The advantage with free-floating staining is that it allows the antibody to penetrate throughout the whole section. In paper III, frozen sections were put onto glass directly at sectioning. This facilitated the staining of sections from a stroked area, which is relatively fragile and could easily have been broken during a free-floating staining.

## Immunofluorescence (Paper I)

Immunofluorescence was used to determine the phenotype of the proliferating cells. Free-floating sections were treated for DNA denaturation as described above. Unspecific binding was blocked and the sections were permeabilized. Primary antibodies against glial cells and proliferating cells were used, and fluorescin conjugated secondary antibodies were subsequently

added to visualize the primary antibodies. Negative controls were performed by omitting the primary antibodies and applying the secondary antibodies alone. The sections were mounting on glass slides. The specific proteins were detected using a confocal laser-scanning microscope. Images were captured with Leica imaging software.

### Comments:

Immunofluorescence was used to detect the phenotype of proliferating cells using specific antibodies recognizing different cell types and proliferating cells. Immunofluorescence is based on the same principal as traditional immunohistochemistry with specific binding of antibodies to an antigen.

## Immunohistochemical Analysis (Paper I, III)

In both Paper I, and III the quantification was performed by an observer blinded to treatment group. In Paper I, the assessment was conducted by two observers blinded to treatment. For each brain, 2-3 sections (100 μm apart) from equivalent locations were selected. The immunostained needle track area on the sections was visualized by light microscopy using a Nikon Optishot 2 and images were captured with a Hamamatsu C5810 color chilled camera. The Easy image measurement program (Nikon, Tekno Optik, Sweden) was used to determine the area of the actual hole that the needle produced including the surrounding necrotic area, until the GFAP expression appeared. The extension of increased GFAP and NG2 was analyzed in one area that included the needle track injury (2800 x 2100 μm). A mean value was calculated and averaged, from eight compass point measurements from the edge of the necrotic area to the rim of the elevated protein expression on each section. The number of ED1 and BrdU positive cells was determined using the Nikon Easy image analyzing program, in two areas (900 x 950 μm) adjacent to the needle track. The mean value for each group was then calculated. To quantify the number of cells double-positive for BrdU and the respective cell specific markers, a confocal laser-scanning microscope (Leica TCS SP2) was used. On each section, eight images (350x350 μm and a stack of 30-40 sections) adjacent to the needle track were captured. Sections were scanned in z-direction at 0.65 μm intervals (total 20 μm). The number of double-labeled cells was expressed as percentage of the total number of BrdU-labeled cells analyzed, and the average of each group was calculated.

In Paper III, the infarct volume was quantified. Digital images containing a calibration standard of the haematoxylin and eosin stained sections were produced. Using Image J (NIH, version 1.41o), the observer, who was the

blind to drug treatment allocation, outlined the total area of the hemisphere, and the infarct on nine standard coronal planes from each brain (from Bregma in mm: 1.98, 1.54, 0.98, 0.5, 0.02, -0.58, -1.06, -2.06, -2.54). Three sections (25 µm, 200 µm apart) from 8-10 animals per group were viewed by bright field microscopy and images were captured with a Nikon Optishot 2 and microscope equipped with a Hamamatsu C5810 colour chilled 3CCD camera. The sections, from the middle of the infarct core, represented A0.9 to A0.5 mm from bregma. Using the program Stereo Investigator, the number of positive cells was determined in the peri-infarct, transition region and infarct core, each 300 µm wide, with the transition zone classified as 150 µm either side of the infarct boundary. The number of cells was divided by the area and is presented as a density.

## Comments:

In Paper I, the horizontal sections provided a clear view of the injury site and the surrounding cell activation. As reactive gliosis was most obvious close to the needle track, areas adjacent to the injury were analysed. GFAP and NG2 expression demonstrated a gradient of increased expression with highest closest to the injury. Analysis of the expression of these proteins by compass point measurements provided a good estimation of how gap junction blockage had affected the extent of the protein expression.

To determine the phenotype of proliferating cells in Paper I, confocal laser scanning microscopy was used. The confocal microscopes laser beams excite specific fluorophores conjugated to the secondary antibodies. The emitted fluorescence is obtained with high spatial resolution in the z-axis. Several fluorophores can thus be examined in single cells and a three-dimensional image of cells and structures can be performed with special software. To determine the presence of co-localization of two or more antigens in the same cell, consecutive z-series scans from different focal levels with a step size of 0.65 µm were used. In order to avoid bleed-through, which can be a problem due to partially overlapping emission spectra of fluorophores, sequential scanning was performed.

In Paper III, volumetric measurement of the infarct volume was performed on the cross-sectional areas of the neocortex, and expressed as the percentage of the contralateral hemisphere. This was to avoid overestimation of the infarct volume by including structures that have undergone secondary tissue loss.

In Paper I and III, cell quantification was performed in regions of interest bordering the injury where high glial reactivity was observed. In Paper III,

the penumbral region was divided in peri-infarct, transition region and infarct core in order to easier determine the extent of cell reactivity.

## Evaluation of neurological deficits – Behavioural testing (III)

The ability of the mice to control fine and gross motor control was assessed using multiple functional tests. Mice were acclimatized to the behavioural tests prior to the commencement of stroke induction and drug treatment, and measurements taken 24 h prior to stroke were used as their respective baseline. The animals were then tested 24 and 72 h after stroke onset. Assessors of the animal's behaviour were blinded to the treatment group of each animal. All behavioural tests had objective outcome measures.

***Beam walking-*** The distance and time taken to walk across a 60 cm long beam of 1.2 cm square diameter and a round 1 cm diameter, suspended 60 cm over the bench was recorded for each animal.

***Cylinder test-*** The method of Schallert and colleagues (Schallert et al., 2000; Schallert, 2006) was used with minor modifications. A glass cylinder, 12 cm in diameter, was used as it allowed mice to stand comfortably on the base with only 1-2 cm in front and behind them, encouraging them to stand. The number of times the mice reared, and the front paw that first made contact with the glass wall, were recorded.

***Adhesive test-*** The test was performed in the home cage of the mice, except that the bedding had been removed. Small adhesive stickers were placed onto the front paws of the mice and then length of time taken to remove the sticker was recorded. This test was repeated twice on each occasion and the mean of both scores was used in the analysis.

### Comments:

These tests are commonly used for their ability to detect motor and sensory deficits in experimental animals shortly after injury. The photothrombotic model used in Paper III affects the brain regions that represent motor-function, especially the front paw area. The beam walking test is mainly a motor test, but the time it takes to cross the beam also depends on the degree of anxiety experienced by the animal. The cylinder test is also a test where both anxiety and motor-function can be measured. Mice like to explore their environment while anxious mice will sit still. The small size of the cylinder forces the mice to stand up and rear in order to explore. This provides

information about which paw they prefer to use to lean against the glass (if one paw is paralyzed it won't be used). The adhesive test evaluates the animals sensory deficits (whether they feel the sticker or not) and motor-function (how well they then remove the stickers).

# RESULTS AND DISCUSSION

## Modulation of gap junctions decreases cell proliferation and markers for reactive gliosis after traumatic brain injury (Paper I)

**Our hypothesis** was that blocking gap junction communication would modulate reactive gliosis.

**To evaluate** the effect of gap junction blockage on reactive gliosis, two commonly used gap junction blockers, octanol (710 mg/kg) or carbenoxolone (90 mg/kg) were injected i.p. 30 min before or after traumatic brain injury induced by a needle track in the adult rat. In order to mark dividing cells, animals were injected with BrdU (150 mg/kg i.p.) twice a day, 8 h apart, with the first injection 30 min after injury, and sacrificed two days later. The expression of reactive glial cells was investigated using immuno-histochemical techniques. To investigate the extent of reactive gliosis, we measured the distance of astrocytic GFAP expression and NG2 expression from the edge of the needle track. The numbers of proliferating cells and activated microglial cells were counted in two areas adjacent to the needle track.

**We found** that the needle track injury induced reactive gliosis located in the area surrounding the injury site in the ipsilateral hemisphere. The GFAP expression was increased in the cytoplasm of hypertrophic astrocytes adjacent to the needle track with a gradient from high to low expression radiating away from the injury site. Octanol administration prior to or post injury significantly decreased the distance of GFAP expression from the wound margin by 32% and 18% respectively (fig. 4A). Treatment with carbenoxolone also reduced the GFAP expression although the difference was not statistically significant (fig. 4A). Octanol and carbenoxolone administered prior to injury significantly decreased the number of BrdU-positive cells by 60% and 70% respectively, indicating decreased cell proliferation, while injection after injury resulted in a non-significant decrease in proliferation (fig. 4B). To further investigate the effect of octanol on reactive gliosis, we analyzed the microglial response. The number of reactive microglia was significantly decreased by about 55% following octanol administration prior to or post injury (fig.4C). As the majority (about 50%) of proliferating cells analyzed were identified as NG2-positive cells (see fig. 6, Paper I), we also analyzed the effect of octanol on NG2

expression. Octanol significantly reduced the distance of NG2 expression from the needle track by 48% when administered prior to injury (fig.4D).

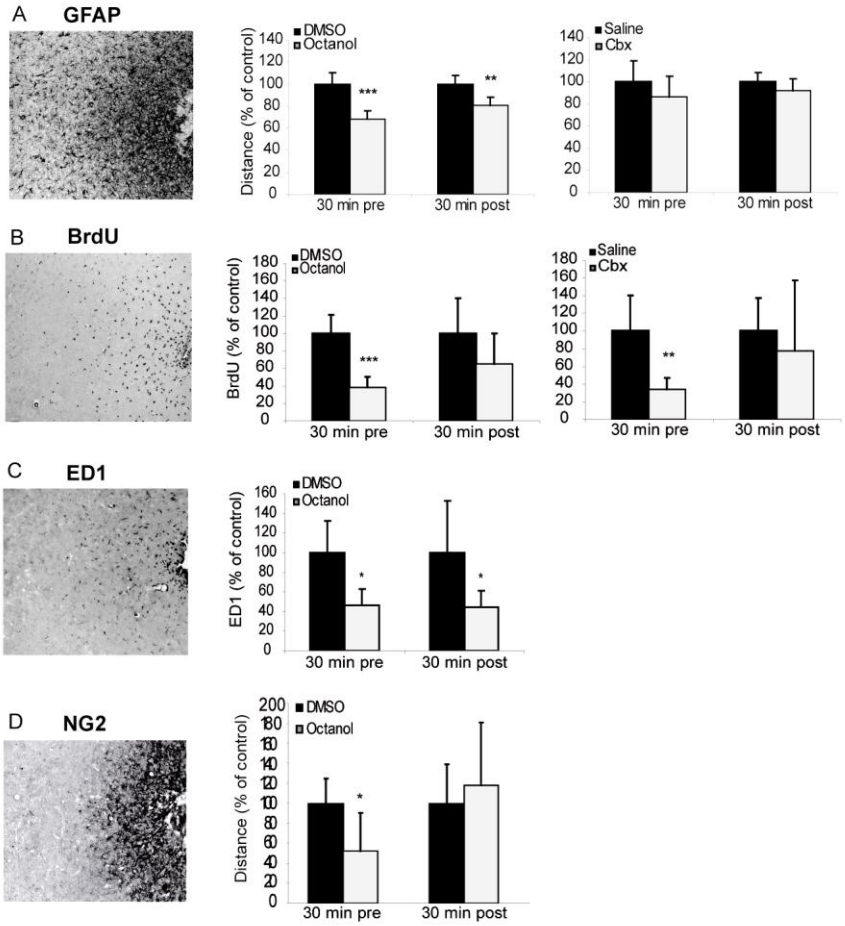


**Figure.4** The distance of GFAP expression from the wound margin was significantly decreased when octanol was administered prior to or post injury (A). Carbeonxolone decreased the distance of GFAP compared to saline, although the difference was not statistically significant (A). Octanol and carbenoxolone administered prior to injury also significantly decreased cell proliferation (B). Treatment with octanol decreased the number of reactive microglia (C) and when administered prior to injury, octanol reduced the distance of NG2 expression from the wound (D).

**In summary,** our results demonstrated that gap junction blockage with octanol and carbenoxolone decreased GFAP expression after a minor traumatic brain injury. This is line with previous studies where suppression of connexin 43 expression using an antisense oligodeoxynucleotide or mimetic peptide, which results in dysfunctional gap junctions, reduced upregulation of

GFAP expression after spinal cord injury (Cronin et al., 2008; O'Carroll et al., 2008). As it has been suggested that the gap junction channels act as pathways for triggering molecules released from dying and activated cells (Spray et al., 2006; Sofroniew, 2009), modulation of the channels with octanol or carbenoxolone, might inhibit the path for the triggering factors.

Carbenoxolone was less effective in attenuating the up-regulated GFAP expression in comparison to octanol. This could be explained by the different pharmacokinetics of the drugs, as well as their ability to pass the blood brain barrier. Octanol is lipophilic and probably it enters the brain rapidly and might therefore be able to modulate gap junctions prior to the time of injury. Carbenoxolone is hydrophilic and its possibility to cross the BBB has been questioned (Leshchenko et al., 2006). However, this is not likely to have been a problem in the present study as the traumatic injury model used results in local BBB disruption. This should provide a direct entrance of carbenoxolone into the brain parenchyma.

The attenuation of the glial response was most obvious when the drugs were given prior to injury, suggesting that octanol and carbenoxolone interfere with early cellular events following injury. For instance, both octanol and carbenoxolone decreased the number of BrdU-positive cells when given before, but not after the needle track injury. Although the mechanisms behind the attenuated cell proliferation are unclear, these results suggest that gap junction communication is involved in cell proliferation after injury.

In accordance with previous studies using the stab wound injury model (Alonso, 2005), we found that a fifth of the proliferating cells were microglia, approximately half of the population of proliferating cells were NG2 positive, and hardly any of the proliferating cells were GFAP positive. However, the ratio of glial markers co-labeled with BrdU did not differ between octanol- and control-treated animals. These results suggest that the decreased cell proliferation was not due to a specific effect on just one cell type.

As activated microglia express functional gap junction channels, which can be inhibited by gap junction blockade (Eugenin et al., 2001; Eugenin et al., 2003), the attenuated microglial response observed in our study could result from the modulating effect of octanol on gap junctions. Moreover, attenuated connexin 43 expression reduces microglial activation (Cronin et al., 2008). In addition, injury-induced morphological microglial changes involve the release of ATP via connexin channels from neighboring astrocytes (Davalos et al., 2005). Therefore, the observed attenuation of the microglial response

could result from modulation of gap junctions on both astrocytes and microglia.

NG2 cells do not express functional gap junction channels (Lin and Bergles, 2004), thus the attenuation of NG2 expression may have been mediated indirectly via other cells or by other effects of octanol. Inflammatory molecules such as cytokines, released from activated microglia and infiltrating blood cells, are known to activate both NG2 cells and astrocytes (Rhodes et al., 2006; Fitch and Silver, 2008). Hence, it is possible that the decreased inflammatory response resulted in reduced activation of NG2-positive cells and astrocytes.

Increasing evidence demonstrates the importance of the injury-induced glial response on the outcome of the degree of tissue damage and the failed neuroregeneration that follows (Fitch and Silver, 2008). The current study suggests that communication via gap junction channels is involved in the process of reactive gliosis. The results further indicate that inhibition of intercellular communication is one way to attenuate progression of reactive gliosis after traumatic brain injury.

## Brief stimulation of the Nrf2 pathway results in long-lasting antioxidative response in cultured astrocytes (Paper II)

**Our hypothesis** was that brief sulforaphane stimulation would be sufficient to induce prolonged Nrf2-induced gene expression. The aim of this study was to examine the kinetics of Nrf2-mediated gene expression, Nqo1 and Hmox1, after sulforaphane exposure in cultured astrocytes.

**To evaluate** the Nrf2 response following activation by brief sulforaphane stimulation, we examined the kinetics of two well-known Nrf2-regulated proteins, Nqo1 and Hmox1, after exposing astrocyte cultures to sulforaphane. We analyzed the induction of Nqo1 and Hmox1 mRNA and protein at various time points following transient exposure to 10 µM sulforaphane. We also analyzed the levels of GSH, the main antioxidant in the brain. In order to investigate the capacity of the astrocytes to clear peroxides, peroxide levels were measured after exposing the cells to a hydrogen peroxide challenge. To investigate whether the astrocytes exhibited alterations in the cellular defence against superoxide-mediated oxidative stress, ATP levels were analyzed as a measure of cell viability following exposure to xanthine/xanthine oxidase.

**We found** that after a 4 h sulforaphane-stimulation, Nqo1 exhibit slow induction kinetics and mRNA levels were still highly elevated at 24 h. The Nqo1 protein levels continuously accumulated for up to 48 h (fig 5A). Hmox1 mRNA accumulated during the first 6 h and then declined gradually. Hmox1 protein increased for the first 16 h. Thereafter, they started to decline, but remained elevated up to 48 h (fig 5B). In addition, the cellular GSH levels (fig 5C) and the cellular capacity to clear peroxides (fig 5D) were elevated for at least 20 h after a transient 4 h sulforaphane-stimulation. In addition, a relative preservation of cellular ATP content after a superoxide challenge was observed 20 h after sulforaphane stimulation (fig 5E).

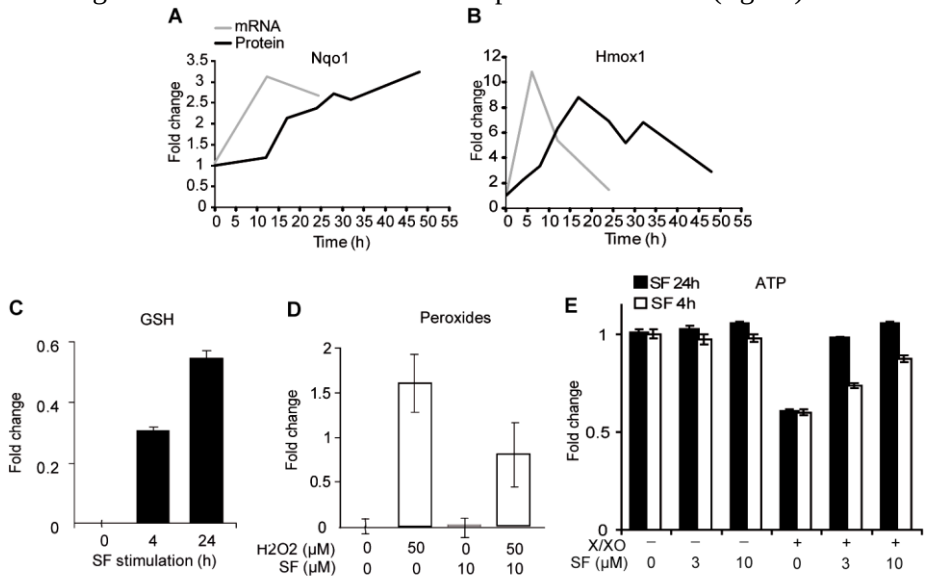


**Figure 5.** Rat astrocytes were exposed to sulforaphane stimulation (SF) for 4 h. Nqo1 and Hmox1 mRNA levels were measured by quantitative PCR and the levels of protein were analyzed using immunoblotting (A, B). The GSH levels were measured at 24 h following sulforaphane stimulation for 4 h or continuous stimulation for 24 h (C). The cells capacity to clear peroxides following a peroxide-challenge was also measured at 24 h following 4 h sulforaphane-stimulation (D). The cellular ATP content was measured at 24 h after the superoxide challenge, following continuous or 4 h sulforaphane-stimulation (3-10 μM) (E).

**In summary,** we stimulated the astrocyte cultures for 4 h with sulforaphane in order to simulate the brief sulforaphane exposure that could be expected after ingestion of broccoli. This short stimulation was sufficient to elevate levels of GSH, as well as the cells capacity to clear peroxides, for at least 20 h. In addition, a long-term increase in the expression of Nqo1 and Hmox1, two enzymes important for free radical protection in neurons and astrocytes

(Chen et al., 2000; van Muiswinkel et al., 2000), was observed following the brief sulforaphane stimulation. Furthermore, by demonstrating sustained ATP levels in sulforaphane pre-treated astrocytes after exposure to superoxide, we confirmed that the prolonged Nrf2-mediated response was protective.

The prolonged increase in Nrf2-mediated gene expression may provide an explanation for the molecular mechanisms underlying free radical-induced hormesis. Hormesis is defined as an adaptive response that can protect the cell from harmful stimuli when exposed to sub-maximal levels of a stimulus (Mattson, 2008). For example, preconditioning with a mild ischaemic lesion protects the brain against subsequent ischaemic insults (Dirnagl et al., 2003). This phenomenon is probably caused by the free radical production during the mild ischaemia that activates the Nrf2-response. Recent results indicate that astrocytes, the main source of antioxidants in the brain, are the most important target for Nrf2-stimulating therapy (Vargas and Johnson, 2009). In this study, we demonstrate that only brief stimulation of the Nrf2-pathway by sulforaphane is sufficient to induce a long-lasting elevation of endogenous antioxidants in astrocytes and results in a sustained protection against oxidative damage.

## Repeated daily stimulation of the Nrf2 pathway mediates sustained protection against radical-induced damage in cultured astrocytes (Paper II)

**Our hypothesis** was that daily transient sulforaphane-stimulations would result in accumulation of Nrf2-mediated mRNA and protein expression and increase protection against oxidative damage.

**To evaluate** the effect of intermittent sulforaphane stimulation on the Nrf2-response in cultured astrocytes, we examined Nqo1 and Hmox1 mRNA and protein expression and measured the levels of GSH and ATP, after exposing the astrocytes to 10 µM sulforaphane for 4 h per day for up to 4 days.

**We found** that repeated sulforaphane treatment resulted in accumulation of both Nqo1 mRNA and protein (fig. 6A, B). In contrast, daily 4 h sulforaphane stimulations increased Hmox1 mRNA the first day but no further increase was observed on subsequent days. Hmox1 protein also increased the first day, as expected, but remained thereafter at control levels (fig. 6C, D). Both GSH levels (fig. 7A) and the protection against superoxide-induced damage (fig. 7B) remained elevated, but no evidence of GSH accumulation or increased protection was found following daily 4 h sulforaphane stimulation.

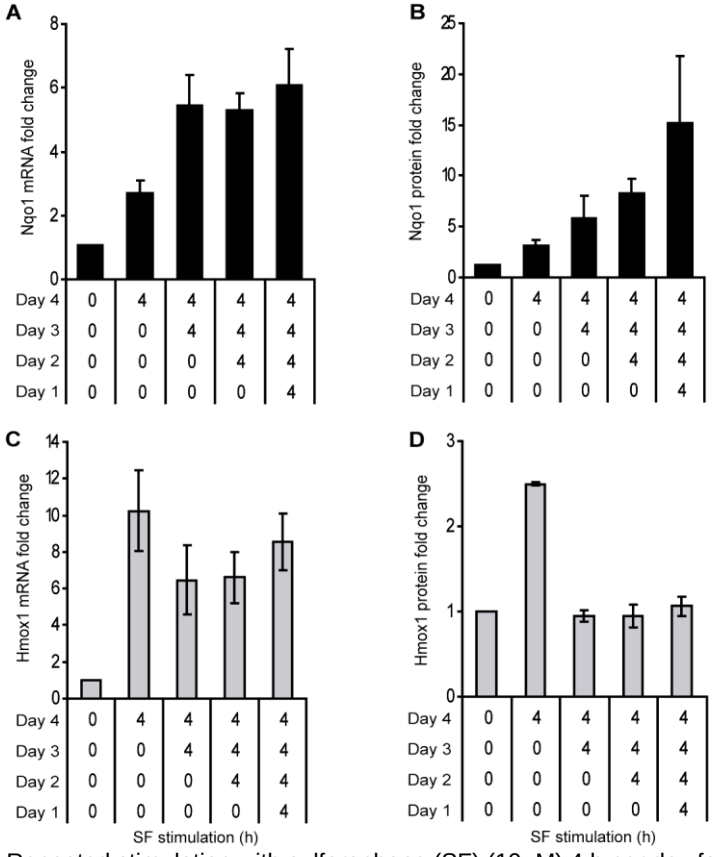


**Figure 6**. Repeated stimulation with sulforaphane (SF) (10μM) 4 h per day for up to 4 days resulted in accumulation of both Nqo1 mRNA and protein levels (A, B). In contrast, no accumulation was observed in Hmox1 mRNA or protein levels (C, D).

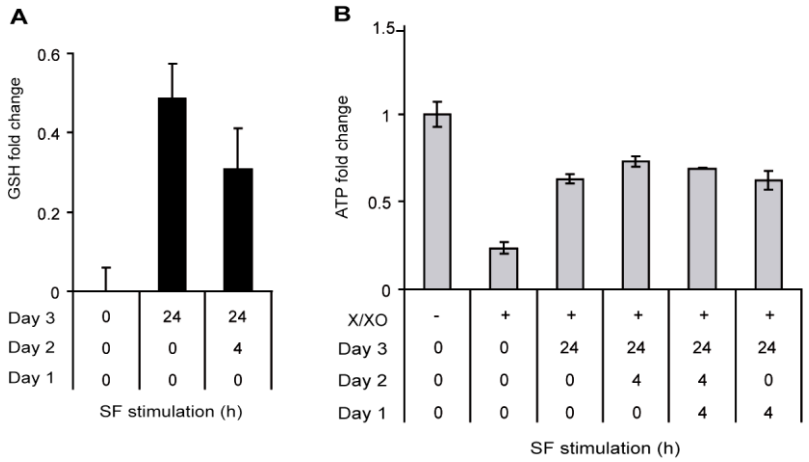


**Figure 7**. Repeated sulforaphane stimulation resulted in sustained GSH levels (A) and protection against superoxide-induced damage (B).

**In summary,** to investigate the kinetics of Nrf2-mediated mRNA and protein after repeated transient stimulation of the Nrf2-response, we exposed astrocytes to daily 4 h sulforaphane-stimulation for up to 4 days. By using repeated sulforaphane stimulations, the astrocytes were exposed to same concentration each day, which allowed, at least partly, a more efficient Nrf2-mediated response. Daily stimulation resulted in an accumulation of Nqo1 expression, continuous induction of GSH, and a persistent protection against superoxide-damage. In contrast, repeated sulforaphane stimulation did not result in an accumulation of Hmox1 mRNA or protein levels.

Our findings that Nrf2-induced prolonged gene expression following daily sulforaphane treatment, could potentially explain how consumption of vegetables and xenobiotics protect against free radical-linked disease (Primiano et al., 1995; van Poppel et al., 1999). Although Nqo1 accumulated and GSH remained increased, repeated daily sulforaphane stimulation did not result in an accumulation of Hmox1 mRNA or protein expression. These findings suggest that the Hmox1 response is subject to feedback-regulation. Thus, parts of the Nrf2-response may be attenuated by repeated sulforaphane stimulation. Even though an induced expression of Hmox1 is protective, a chronic upregulation might be toxic and cause cell death (Schipper, 2004; Stahnke et al., 2007). However, despite the fact that some parts of the Nrf2-response were attenuated, there was no attenuation of the sulforaphane-mediated superoxide protection following daily sulforaphane stimulation in our study. In conclusion, our observations demonstrated that some of the Nrf2-induced gene expression can be enhanced by repeated transient sulforaphane stimulation, which in turn could explain why intermittent intake of Nrf2-activating substances can result in long-term protection from free radical induced disease.

## The effect of sulforaphane on stroke outcome and reactive gliosis following photothrombotic stroke in mice (III)

Our hypothesis was that sulforaphane-induced Nrf2 stimulation would modify stroke outcome and reactive gliosis when given after a permanent focal ischaemia.

**To evaluate** the hypothesis that sulforaphane-induced Nrf2 activation would modify stroke outcome, reactive gliosis and cell proliferation, mice were subjected to permanent cerebral focal ischaemia induced by photothrombosis followed by sulforaphane treatment. Sulforaphane (5 or 50 mg/kg i.p.) was

administered 15 min after occlusion, either as a single dose or as a repeated daily dose for three days. To detect proliferating cells, mice were injected with BrdU (50 mg/kg) 15 min, 24 h and 48 h after the ischaemic injury. Histological investigations were performed to assess infarct volume at 24 and 72 h post ischaemic injury. To investigate the effect of sulforaphane on reactive gliosis at 72 h post-ischaemia, antibodies against GFAP (reactive astrocytes), Iba1 (activated microglia) and BrdU (proliferating cells) were used. Different behavioural tests were used to evaluate neurological functions 24 and 72 h after stroke onset. The ability of sulforaphane to activate the Nrf2-pathway was evaluated by analysing the mRNA levels of the Nrf2-regulated gene Nqo1 24 h after sulforaphane injection.

**We found** that sulforaphane administration did not alter the infarct volume (fig.1, Paper III), the number of activated glial cells or proliferating cells (fig. 2, Paper III) when analyzed 24 and 72 h after stroke. No significant effect on motor-function was observed in sulforaphane-treated animals after the photothrombotic lesion (fig. 3, Paper III). As our results showed that sulforaphane did not affect stroke outcome under these experimental conditions, an additional group of naïve mice were injected with sulforaphane to ensure that the dose of sulforaphane used was able to activate the Nrf2-pathway in mice. The mRNA levels of Nqo1 were analysed 24 h after sulforaphane injection in these naïve animals. The increased transcription of Nqo1 mRNA levels in both the liver and brain (fig.4, Paper III) after sulforaphane treatment confirmed that the Nrf2 system was activated under our experimental conditions.

**In summary,** Sulforaphane activated the Nrf2 system in naïve mice, as indicated by the upregulation of Nqo1 mRNA expression. However, glial cell markers, infarct volume and motor-function did not differ significantly between sulforaphane-treated and vehicle-treated following photothrombotic stroke.

The beneficial effects of sulforaphane are, at least partly, mediated through the Nrf2 pathway. As we did not see an effect of sulforaphane on the outcome measures in this study, we confirmed that the treatment paradigm was sufficient to upregulate Nrf2 in mice. Activation of the Nrf2-pathway after sulforaphane treatment was investigated by analysing the expression of Nqo1. Our results demonstrated that sulforaphane significantly increased the levels of Nqo1, 24 h after treatment in naïve animals. Thus, the treatment paradigm used should have been sufficient to activate Nrf2 in the experiment animals.

The photothrombotic stroke model was chosen as it has the advantage of being highly reproducible in location and in size, generating small infarcts limited to the cortex. The photothrombotic model is minimally invasive but has a similar cell response to the MCAO model (Schroeter et al., 1994; Jander et al., 1995). After photothrombosis, we observed a maximal ischaemic lesion 24 h post-ictus and at 72 h post-ictus there was no difference in infarct volume. In contrast to Zhao and colleagues (Zhao et al., 2006) where 5 mg/kg sulforaphane reduced infarct size by 30% when given 15 min after a transient middle cerebral artery occlusion in rats, we failed to observe any difference in infarct volume with 5 or 50 mg/kg sulforaphane using single or multiple doses in mice. The photothrombotic model that was used has a much smaller penumbra than a reperfusion model where the collateral blood flow is greater (Kuroda and Siesjo, 1997). This, together with the small volume of injured cortical tissue seen after photothrombosis, generates less ROS compared to middle cerebral artery occlusion. The small size of the penumbra and the lower generation of ROS may explain why we did not see neuroprotection with sulforaphane in the photothrombotic stroke model.

In this study, repeated sulforaphane treatment did not alter the number of BrdU-, GFAP- and Iba1-positive cells compared to vehicle. In line with this, dietary antioxidants can reduce oxidative stress by increasing the endogenous antioxidant defence without altering the activation of glial cells in a rat model of chronic gliosis (Bates et al., 2007). In addition, sulforaphane decreased the macrophage/microglial activation in a *in vivo* model of inflammation (Innamorato et al., 2008). However, this decrease in inflammatory response was only present when sulforaphane was administrated prior to lipolysaccharide (LPS) challenge. The fact that the number of proliferating cells, activated astrocytes and microglia were not attenuated by sulforaphane in our study suggests that, at the time points and concentrations used, sulforaphane did not interfere with the mechanisms involved in the activation of glial cells or cell proliferation after photothrombotic ischaemia.

The use of several specific tests on forepaw motor function demonstrated that photothrombotic infarction results in animals losing fine control of their paw. However, animals treated with sulforaphane had the same functional motor deficit as vehicle treated animals and recovered within the same timeframe as vehicle treated animals.

In summary, 5 or 50 mg sulforaphane was not sufficient to affect cell death or reactive gliosis when given as a single or multiple daily doses, starting 15 min after photothrombotic stroke in mice. Whether sulforaphane has an effect

on reactive gliosis in a transient stroke model, or other models of injury, is not known and needs to be investigated.

# CONCLUSIONS AND RESPONSES TO GIVEN AIMS

- The gap junction channels blockers octanol and carbenoxolone, decreased markers for reactive gliosis and cell proliferation following traumatic brain injury in the adult rat. These results suggest that cell communication through the gap junction channels are involved in the activation and progression of reactive gliosis.

- Brief sulforaphane exposure was sufficient to induce prolonged expression of the Nrf2-mediated genes Nqo1 and Hmox1 and provided a long-lasting protection against peroxide- and superoxide-induced oxidative damage. This may explain why brief sulforaphane exposure results in long-term protection against free radical-induced damage, although absorbed sulforaphane is cleared from the body within a few hours.

- Repeated transient Nrf2-activation by sulforaphane resulted in partial accumulation of the studied Nrf2-induced antioxidants and persistent cell protection against oxidative damage. This may explain why intermittent intake of Nrf2-activating drugs or vegetables can result in long-term protection from free radical-induced disease.

- Sulforaphane was not sufficient to affect cell death or reactive gliosis when given as a single dose or multiple daily doses, starting 15 min after photothrombotic permanent stroke in mice.

# CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Stroke or traumatic brain injury are major causes of death and disability. To date there is very little that can be done to treat these patients in the early stages. It is becoming increasingly clear, although the processes are still far from understood, that glial cells, and especially astrocytes, contribute to the evolving tissue damage and subsequent attempts to repair the damaged or injured areas (Fitch and Silver, 2008; Allen and Barres, 2009).

Reactive gliosis is a double edge sword when it comes to the recovery process after a brain injury. The molecular mechanisms behind reactive gliosis are not well understood. The results presented in this thesis demonstrate that modulation of gap junction channels attenuates markers for reactive gliosis, as well as the injury-induced increased cell proliferation surrounding the injury. This indicates an important role for cell communication through the gap junctions on the activation and progression of reactive gliosis. It is tempting to believe that attenuating reactive gliosis by blocking the gap junction channels may lead to modification of the glial scar and subsequently improved regeneration. This is still not known and needs to be investigated. Important aspects to further explore include when and what triggers the glial response to go from being beneficial to becoming detrimental for the outcome after brain injury.

Oxidative stress is one of the main causes of tissue damage following ischaemic insults in the brain (Kuroda and Siesjo, 1997). Astrocytes are highly involved in the defence against oxidative stress in the brain, and in many important aspects for neuronal function. Their close interactions with surrounding cells indicate that alterations in astrocytic function would also have an effect on other cells. The essential role of astrocytes in protecting neurons against oxidative insults via Nrf2 activation has been confirmed in several studies, suggesting that astrocytes constitute a primary target for future Nrf2-stimulating therapy (reviewed in (Vargas and Johnson, 2009). Here we demonstrate that Nrf2 activation in cultured astrocytes results in a long-lasting elevation of cytoprotective proteins that provide protection against free radical-induced cell damage. This provides an insight into how the Nrf2-system might be stimulated to optimize neuroprotection. In addition, we also demonstrated that part of the Nrf2-mediated response accumulates after transient repeated Nrf2 activation. These observations may provide a puzzle piece in the understanding of how short-term exposure to Nrf2-activating drugs can provide prolonged protection against free radical-linked

disease. These results also support the use of astrocyte cultures for studying Nrf2 activation, the kinetics of the response and the potency of new neuroprotective substances.

Most experimental studies demonstrate neuroprotection against oxidative stress-induced related disease when the Nrf2-pathway has been activated prior to the onset of free radical production. This suggests that Nrf2 activating drugs could be useful as prophylactic treatment in patients with a high stroke risk, which could help to protect against a potential stroke. Support for this view can be found in human studies, for example in studies where the relationship between consumption of fruit and vegetables and ischeamic stroke was investigated (Joshipura et al., 1999). In the exemplified study, cruciferous and green leafy vegetables were included in the fruit and vegetables group that was in particular associated with a reduced risk of ischeamic stroke. It is possible that the protective effects of this particular group of fruit and vegetables are mediated, at least partly, through the activation of Nrf2. In our study, activation of the Nrf2 response shortly after a permanent stroke induced by photothrombosis did not provide any neuroprotection even when sulforaphane was given as a once daily dose for three days. Due to the time it takes from gene transcription to form a cytoprotective enzyme, and the fact that free radical production is highest in the acute phase after a permanent stroke, it is conceivable that the Nrf2-system needs to be activated prior to injury in order to provide neuroprotection. However, as the extensive injury-induced cell responses, including inflammation and reactive gliosis, continue for up to weeks after an ischaemic lesion, it is motivating to find out whether activation of the Nrf2-system with repeated dosing for longer a longer time period would improve the functional outcome and facilitate the regeneration process after stroke.

# ACKNOWLEDGEMENTS

**I would like to express my sincere gratitude everybody who in different ways have contributed to this thesis. I am very grateful to you all, and especially;**

**Michael Nilsson,** for your never ending enthusiasm and encouragement, for thinking big and always seeing everything from the bright side. For being there to support and encourage me and not to forget - your contagious sense of humour!

**Michelle Anderson** for good friendship, constant encouragement and for brilliant scientific support. Thank you also for valuable proof-reading of all my works.

**Ola Hammarsten,** for your enormous passion for science, great support and scientific guidance**.**

**Michelle Porritt** for great supervision, collaboration and friendship.

**Agneta Holmäng,** Head of the Institute of Neuroscience and Physiology, and **Lars Rönnbäck**, Head of the Department of Clinical Neuroscience and Rehabilitation, for providing an inspiring scientific environment.

To the Principal Investigators at CBR, **Milos Pekny, Marcela Pekna, Klas Blomgren, Georg Kuhn and Maurice Curtis** for creating a great and interesting scientific environment!

**My present and former colleagues at CBR** for all the great times working together throughout the years and for making CBR such a interesting and positive environment for scientific research**; there is no way that I would have enjoyed this experience as much if it weren't for the colleagues at CBR.**

Special thanks to **Ina Nodin** who has always supported me since day one of my PhD, for good friendship and for being a great room-mate to discuss not only science with. **Petra Bergström** for great collaboration**,** support and friendship. **Maurice Curtis** for your

scientific guidance and inspiring discussions. **Mathilda Zetterström Axell** for being the "don't worry - be happy" girl in the lab. **Marie Kalm and Niklas Karlsson** for friendly chats and great support! **Nina Hellström and Charlotta Lindwall** for good discussions and for your confocal support and skills in Photoshop. **Carolina Roughton**, for the fun and unforgettable trip to Nebraska. **Jonas Faijerson Säljö,** for encouragement and good discussions. **Lizhen Li** for fun memories during our adventure in the states together. **Changlian Zhu**, for your valuable advice regarding my western blots experiments. **Fredrik Blomstrand** for helpful advice and support and good discussions.

**Ann-Marie Alborn** for all invaluable help throughout out my PhD period. **Birgit Linder** for laboratory help and for many friendly chats and advices. **Sheila Engdahl**, for great technical support and encouragement - I miss you! **Rita Grandér** for laboratory support and many fun conversations.

Thanks also to **Lina Bunketorp Käll, Anna Thorén, Karina Apricó, Håkan Muyderman, Karin Hultman, Henrik Landgren**, **Malin Blomstrand, Karin Gustavsson, Olle Lindberg, Anke Brederlau, Christi Cooper Kuhn, Linda Paulsson, Åsa Persson, Jenny Zhang, Andrew Naylor, Martina Hermansson, Jenny Nyberg, Cecilia Bull, Mila Komitova, Anke Brederlau, Axel Jansson, Malin Palmér, Åsa Widestrand, Yalda Rahpeymai Bogestål, Pete Smith, Daniel Andersson and Ulrika Wilhelmsson** for being good friends and excellent co-workers.

**Anki Nyberg, Ingrid Worth, Kirsten Toftered, Mari Klaesson, Oscar Bergström, Patrik Johansson och Gunnel Nordström** for being excellent help for the whole of CBR - what would we have done without you!

To all CBR-collaborators at the Högsbo Rehabcenter, especially thanks to **Thomas Lindén, Patrik Säterö, Thorleif Thorlin, Trandur Ulfarsson, Helen Davidsson, Linda Hou** for fun conversations and support, not only in science!

**Mats Sandberg** for interesting and valuable discussions. **Malin Stridh** for support and good discussions.

Former and present members of the **Astroglial group,** for good discussions and especially for many good laughs. Thanks to **Elisabeth Hansson** for good discussions and support especially during my time at Histology. **Anna Andersson** and **Mikael Persson,** thanks for all fun we have shared and the unforgettable journey in the states! **Ulrika Björklund** for the fun person you are and for scientific support. **Mikael Ängehagen, Louise Adermark** and **Anna Westerlund** for times of laughter and all entertaining coffee-breaks and discussions.

**Jan Olof Karlsson and Åsa Nilsson,** for a good collaboration and interesting discussions.

**"Syjuntan"**, my wonderful friends, for all fun times together, with and without the knitting, and for always encouraging me and believing in me.

**Barbro and Arne, Tina and Micke, Sune and the rest of the Segersäll family,** thanks for all support and encouragement.

**My family:** My parents, **Ove and Carin,** thanks for your love and for believing in me and your never ending attempts to understand my research. My big brother **Thomas** thanks for always being there for me.

**Ulf,** my partner, for all your endless love, understanding and encouragement, and for your interest in my work. Thanks for convincing me to apply to the Research School of Biomedicine where my interest in science began ♥.

To my son **Hugo** for spreading joy and happiness and for giving me a new perspective in life.

# REFERENCES

Abbott NJ (2005) Dynamics of CNS barriers: evolution, differentiation, and modulation. Cell Mol Neurobiol 25:5-23.

Alam J, Cook JL (2003) Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 9:2499-2511.

Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL (1999) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 274:26071-26078.

Alam J, Wicks C, Stewart D, Gong P, Touchard C, Otterbein S, Choi AM, Burow ME, Tou J (2000) Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. J Biol Chem 275:27694-27702.

Albright TD, Jessell TM, Kandel ER, Posner MI (2000) Neural science: a century of progress and the mysteries that remain. Cell 100 Suppl:S1-55.

Aldskogius H (2001) Microglia in neuroregeneration. Microsc Res Tech 54:40-46.

Allen NJ, Barres BA (2009) Neuroscience: Glia - more than just brain glue. Nature 457:675-677.

Alonso G (2005) NG2 proteoglycan-expressing cells of the adult rat brain: possible involvement in the formation of glial scar astrocytes following stab wound. Glia 49:318-338.

Anderson CM, Swanson RA (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1-14.

Anderson ME (1998) Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111-112:1-14.

Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS, Nilsson M (2003) Astrocytes and stroke: networking for survival? Neurochem Res 28:293-305.

Andersson M, Hanse E (2010) Astrocytes impose postburst depression of release probability at hippocampal glutamate synapses. J Neurosci 30:5776-5780.

Andersson M, Blomstrand F, Hanse E (2007) Astrocytes play a critical role in transient heterosynaptic depression in the rat hippocampal CA1 region. J Physiol 585:843-852.

Araque A, Carmignoto G, Haydon PG (2001) Dynamic signaling between astrocytes and neurons. Annu Rev Physiol 63:795-813.

Arthur PG, Lim SC, Meloni BP, Munns SE, Chan A, Knuckey NW (2004) The protective effect of hypoxic preconditioning on cortical neuronal cultures is associated with increases in the activity of several antioxidant enzymes. Brain Res 1017:146-154.

Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA (2010) Glial and neuronal control of brain blood flow. Nature 468:232-243.

Ayata C, Ropper AH (2002) Ischaemic brain oedema. J Clin Neurosci 9:113-124.

Azarenko O, Okouneva T, Singletary KW, Jordan MA, Wilson L (2008) Suppression of microtubule dynamic instability and turnover in MCF7 breast cancer cells by sulforaphane. Carcinogenesis 29:2360-2368.

Back T, Schuler OG (2004) The natural course of lesion development in brain ischemia. Acta Neurochir Suppl 89:55-61.

Baranano DE, Rao M, Ferris CD, Snyder SH (2002) Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A 99:16093-16098.

Barres BA (2008) The mystery and magic of glia: a perspective on their roles in health and disease. Neuron 60:430-440.

Bates KA, Martins RN, Harvey AR (2007) Oxidative stress in a rat model of chronic gliosis. Neurobiol Aging 28:995-1008.

Belachew S, Chittajallu R, Aguirre AA, Yuan X, Kirby M, Anderson S, Gallo V (2003) Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 161:169-186.

Bennett MV, Contreras JE, Bukauskas FF, Saez JC (2003) New roles for astrocytes: gap junction hemichannels have something to communicate. Trends Neurosci 26:610-617.

Bennett MV, Barrio LC, Bargiello TA, Spray DC, Hertzberg E, Saez JC (1991) Gap junctions: new tools, new answers, new questions. Neuron 6:305-320.

Benson AM, Hunkeler MJ, Talalay P (1980) Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. Proc Natl Acad Sci U S A 77:5216-5220.

Bergles DE, Jabs R, Steinhauser C (2010) Neuron-glia synapses in the brain. Brain Res Rev 63:130-137.

Biebl M, Cooper CM, Winkler J, Kuhn HG (2000) Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. Neurosci Lett 291:17-20.

Block ML, Hong JS (2005) Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Prog Neurobiol 76:77-98.

Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8:57-69.

Blomstrand F, Giaume C, Hansson E, Ronnback L (1999a) Distinct pharmacological properties of ET-1 and ET-3 on astroglial gap junctions and Ca(2+) signaling. Am J Physiol 277:C616-627.

Blomstrand F, Aberg ND, Eriksson PS, Hansson E, Ronnback L (1999b) Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions. Neuroscience 92:255-265.

Blomstrand F, Khatibi S, Muyderman H, Hansson E, Olsson T, Ronnback L (1999c) 5-Hydroxytryptamine and glutamate modulate velocity and extent of intercellular calcium signalling in hippocampal astroglial cells in primary cultures. Neuroscience 88:1241-1253.

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 92:7162-7166.

Booher J, Sensenbrenner M (1972) Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. Neurobiology 2:97-105.

Brambilla R, Bracchi-Ricard V, Hu WH, Frydel B, Bramwell A, Karmally S, Green EJ, Bethea JR (2005) Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. J Exp Med 202:145-156.

Brambilla R, Persaud T, Hu X, Karmally S, Shestopalov VI, Dvoriantchikova G, Ivanov D, Nathanson L, Barnum SR, Bethea JR (2009) Transgenic inhibition of astroglial NF-kappa B improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation. J Immunol 182:2628-2640.

Broer S, Brookes N (2001) Transfer of glutamine between astrocytes and neurons. J Neurochem 77:705-719.

Browne SE, Beal MF (2006) Oxidative damage in Huntington's disease pathogenesis. Antioxid Redox Signal 8:2061-2073.

Budd SL, Lipton SA (1998) Calcium tsunamis: do astrocytes transmit cell death messages via gap junctions during ischemia? Nat Neurosci 1:431-432.

Buffo A, Rolando C, Ceruti S (2009) Astrocytes in the damaged brain: molecular and cellular insights into their reactive response and healing potential. Biochem Pharmacol.

Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, Mori T, Gotz M (2008) Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. Proc Natl Acad Sci U S A 105:3581-3586.

Bukauskas FF, Jordan K, Bukauskiene A, Bennett MV, Lampe PD, Laird DW, Verselis VK (2000) Clustering of connexin 43-enhanced green fluorescent protein gap junction channels and functional coupling in living cells. Proc Natl Acad Sci U S A 97:2556-2561.

Burton NC, Kensler TW, Guilarte TR (2006) In vivo modulation of the Parkinsonian phenotype by Nrf2. Neurotoxicology 27:1094-1100.

Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, Mucke L, Johnson MH, Sofroniew MV (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. Neuron 23:297-308.

Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22:183-192.

Cafferty WB, Yang SH, Duffy PJ, Li S, Strittmatter SM (2007) Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans. J Neurosci 27:2176-2185.

Calkins MJ, Jakel RJ, Johnson DA, Chan K, Kan YW, Johnson JA (2005) Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. Proc Natl Acad Sci U S A 102:244-249.

Cathcart R, Schwiers E, Ames BN (1983) Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Anal Biochem 134:111-116.

Charles A (1998) Intercellular calcium waves in glia. Glia 24:39-49.

Chatterjee S, Noack H, Possel H, Keilhoff G, Wolf G (1999) Glutathione levels in primary glial cultures: monochlorobimane provides evidence of cell type-specific distribution. Glia 27:152-161.

Chaudhry FA, Schmitz D, Reimer RJ, Larsson P, Gray AT, Nicoll R, Kavanaugh M, Edwards RH (2002) Glutamine uptake by neurons: interaction of protons with system a transporters. J Neurosci 22:62-72.

Chen K, Gunter K, Maines MD (2000) Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J Neurochem 75:304-313.

Chen PC, Vargas MR, Pani AK, Smeyne RJ, Johnson DA, Kan YW, Johnson JA (2009) Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. Proc Natl Acad Sci U S A 106:2933-2938.

Chen ZJ, Ughrin Y, Levine JM (2002a) Inhibition of axon growth by oligodendrocyte precursor cells. Mol Cell Neurosci 20:125-139.

Chen ZJ, Negra M, Levine A, Ughrin Y, Levine JM (2002b) Oligodendrocyte precursor cells: reactive cells that inhibit axon growth and regeneration. J Neurocytol 31:481-495.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421-433.

Contreras JE, Sanchez HA, Veliz LP, Bukauskas FF, Bennett MV, Saez JC (2004) Role of connexin-based gap junction channels and hemichannels in ischemia-induced cell death in nervous tissue. Brain Res Brain Res Rev 47:290-303.

Cook JA, Iype SN, Mitchell JB (1991) Differential specificity of monochlorobimane for isozymes of human and rodent glutathione S-transferases. Cancer Res 51:1606-1612.

Copple IM, Goldring CE, Kitteringham NR, Park BK (2008) The Nrf2-Keap1 defence pathway: role in protection against drug-induced toxicity. Toxicology 246:24-33.

Cotrina ML, Kang J, Lin JH, Bueno E, Hansen TW, He L, Liu Y, Nedergaard M (1998a) Astrocytic gap junctions remain open during ischemic conditions. J Neurosci 18:2520-2537.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998b) Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci U S A 95:15735-15740.

Crack PJ, Taylor JM, de Haan JB, Kola I, Hertzog P, Iannello RC (2003) Glutathione peroxidase-1 contributes to the neuroprotection seen in the superoxide dismutase-1 transgenic mouse in response to ischemia/reperfusion injury. J Cereb Blood Flow Metab 23:19-22.

Cronin M, Anderson PN, Cook JE, Green CR, Becker DL (2008) Blocking connexin43 expression reduces inflammation and improves functional recovery after spinal cord injury. Mol Cell Neurosci 39:152-160.

Cuadrado A, Rojo AI (2008) Heme oxygenase-1 as a therapeutic target in neurodegenerative diseases and brain infections. Curr Pharm Des 14:429-442.

Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA (2004) The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol 24:8477-8486.

Danilov CA, Chandrasekaran K, Racz J, Soane L, Zielke C, Fiskum G (2009) Sulforaphane protects astrocytes against oxidative stress and delayed death caused by oxygen and glucose deprivation. Glia 57:645-656.

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB (2005) ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 8:752-758.

Davidson JS, Baumgarten IM, Harley EH (1986) Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. Biochem Biophys Res Commun 134:29-36.

De Maio A, Vega VL, Contreras JE (2002) Gap junctions, homeostasis, and injury. J Cell Physiol 191:269-282.

De Vuyst E, Decrock E, De Bock M, Yamasaki H, Naus CC, Evans WH, Leybaert L (2007) Connexin hemichannels and gap junction channels are differentially influenced by lipopolysaccharide and basic fibroblast growth factor. Mol Biol Cell 18:34-46.

Dermietzel R (1998) Diversification of gap junction proteins (connexins) in the central nervous system and the concept of functional compartments. Cell Biol Int 22:719-730.

Dermietzel R, Spray DC (1993) Gap junctions in the brain: where, what type, how many and why? Trends Neurosci 16:186-192.

Dhakshinamoorthy S, Long DJ, 2nd, Jaiswal AK (2000) Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens. Curr Top Cell Regul 36:201-216.

Di Giovanni S, Movsesyan V, Ahmed F, Cernak I, Schinelli S, Stoica B, Faden AI (2005) Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. Proc Natl Acad Sci U S A 102:8333-8338.

Dinkova-Kostova AT, Talalay P (2010) NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch Biochem Biophys.

Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proc Natl Acad Sci U S A 98:3404-3409.

Dirnagl U, Simon RP, Hallenbeck JM (2003) Ischemic tolerance and endogenous neuroprotection. Trends Neurosci 26:248-254.

Dobrenis K, Chang HY, Pina-Benabou MH, Woodroffe A, Lee SC, Rozental R, Spray DC, Scemes E (2005) Human and mouse microglia express connexin36, and functional gap junctions are formed between rodent microglia and neurons. J Neurosci Res 82:306-315.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703-716.

Dringen R (2000) Metabolism and functions of glutathione in brain. Prog Neurobiol 62:649-671.

Dringen R (2005) Oxidative and antioxidative potential of brain microglial cells. Antioxid Redox Signal 7:1223-1233.

Dringen R, Hirrlinger J (2003) Glutathione pathways in the brain. Biol Chem 384:505-516.

Dringen R, Pfeiffer B, Hamprecht B (1999) Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. J Neurosci 19:562-569.

Dringen R, Gutterer JM, Hirrlinger J (2000) Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. Eur J Biochem 267:4912-4916.

Dringen R, Pawlowski PG, Hirrlinger J (2005) Peroxide detoxification by brain cells. J Neurosci Res 79:157-165.

Dringen R, Gutterer JM, Gros C, Hirrlinger J (2001) Aminopeptidase N mediates the utilization of the GSH precursor CysGly by cultured neurons. J Neurosci Res 66:1003-1008.

Drukarch B, Schepens E, Jongenelen CA, Stoof JC, Langeveld CH (1997) Astrocyte-mediated enhancement of neuronal survival is abolished by glutathione deficiency. Brain Res 770:123-130.

Eggler AL, Liu G, Pezzuto JM, van Breemen RB, Mesecar AD (2005) Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. Proc Natl Acad Sci U S A 102:10070-10075.

el-Fouly MH, Trosko JE, Chang CC (1987) Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. Exp Cell Res 168:422-430.

Eliasson C, Sahlgren C, Berthold CH, Stakeberg J, Celis JE, Betsholtz C, Eriksson JE, Pekny M (1999) Intermediate filament protein partnership in astrocytes. J Biol Chem 274:23996-24006.

Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. J Cereb Blood Flow Metab 17:1143-1151.

Eng LF, Ghirnikar RS, Lee YL (2000) Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem Res 25:1439-1451.

Enkvist MO, McCarthy KD (1994) Astroglial gap junction communication is increased by treatment with either glutamate or high K+ concentration. J Neurochem 62:489-495.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313-1317.

Eugenin EA, Branes MC, Berman JW, Saez JC (2003) TNF-alpha plus IFN-gamma induce connexin43 expression and formation of gap junctions between human monocytes/macrophages that enhance physiological responses. J Immunol 170:1320-1328.

Eugenin EA, Eckardt D, Theis M, Willecke K, Bennett MV, Saez JC (2001) Microglia at brain stab wounds express connexin 43 and in vitro form functional gap junctions after treatment with interferon-gamma and tumor necrosis factor-alpha. Proc Natl Acad Sci U S A 98:4190-4195.

Evans WH, Boitano S (2001) Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. Biochem Soc Trans 29:606-612.

Fahey JW, Zhang Y, Talalay P (1997) Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci U S A 94:10367-10372.

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002) Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 99:7610-7615.

Farahani R, Pina-Benabou MH, Kyrozis A, Siddiq A, Barradas PC, Chiu FC, Cavalcante LA, Lai JC, Stanton PK, Rozental R (2005) Alterations in metabolism and gap junction expression may determine the role of astrocytes as "good samaritans" or executioners. Glia 50:351-361.

Farber K, Kettenmann H (2005) Physiology of microglial cells. Brain Res Brain Res Rev 48:133-143.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends Immunol 28:138-145.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 24:2143-2155.

Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377-391.

Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, Snyder SH (1999) Haem oxygenase-1 prevents cell death by regulating cellular iron. Nat Cell Biol 1:152-157.

Fields RD, Stevens-Graham B (2002) New insights into neuron-glia communication. Science 298:556-562.

Fitch MT, Silver J (1997) Activated macrophages and the blood-brain barrier: inflammation after CNS injury leads to increases in putative inhibitory molecules. Exp Neurol 148:587-603.

Fitch MT, Silver J (2008) CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp Neurol 209:294-301.

Fitch MT, Doller C, Combs CK, Landreth GE, Silver J (1999) Cellular and molecular mechanisms of glial scarring and progressive cavitation: in vivo and in vitro analysis of inflammation-induced secondary injury after CNS trauma. J Neurosci 19:8182-8198.

Floyd RA (1999) Antioxidants, oxidative stress, and degenerative neurological disorders. Proc Soc Exp Biol Med 222:236-245.

Frantseva MV, Kokarovtseva L, Naus CG, Carlen PL, MacFabe D, Perez Velazquez JL (2002) Specific gap junctions enhance the neuronal vulnerability to brain traumatic injury. J Neurosci 22:644-653.

Gaikwad A, Long DJ, 2nd, Stringer JL, Jaiswal AK (2001) In vivo role of NAD(P)H:quinone oxidoreductase 1 (NQO1) in the regulation of intracellular redox state and accumulation of abdominal adipose tissue. J Biol Chem 276:22559-22564.

Gebicke-Haerter PJ, Van Calker D, Norenberg W, Illes P (1996) Molecular mechanisms of microglial activation. A. Implications for regeneration and neurodegenerative diseases. Neurochem Int 29:1-12.

Gegg ME, Clark JB, Heales SJ (2005) Co-culture of neurones with glutathione deficient astrocytes leads to increased neuronal susceptibility to nitric oxide and increased glutamate-cysteine ligase activity. Brain Res 1036:1-6.

Gehrmann J, Schoen SW, Kreutzberg GW (1991) Lesion of the rat entorhinal cortex leads to a rapid microglial reaction in the dentate gyrus. A light and electron microscopical study. Acta Neuropathol 82:442-455.

Gentleman SM, Roberts GW, Gennarelli TA, Maxwell WL, Adams JH, Kerr S, Graham DI (1995) Axonal injury: a universal consequence of fatal closed head injury? Acta Neuropathol 89:537-543.

Ghajar J (2000) Traumatic brain injury. Lancet 356:923-929.

Giaume C, McCarthy KD (1996) Control of gap-junctional communication in astrocytic networks. Trends Neurosci 19:319-325.

Giaume C, Venance L (1998) Intercellular calcium signaling and gap junctional communication in astrocytes. Glia 24:50-64.

Giaume C, Tabernero A, Medina JM (1997) Metabolic trafficking through astrocytic gap junctions. Glia 21:114-123.

Giaume C, Kirchhoff F, Matute C, Reichenbach A, Verkhratsky A (2007) Glia: the fulcrum of brain diseases. Cell Death Differ 14:1324-1335.

Gillman MW, Cupples LA, Gagnon D, Posner BM, Ellison RC, Castelli WP, Wolf PA (1995) Protective effect of fruits and vegetables on development of stroke in men. Jama 273:1113-1117.

Giovannini MG, Scali C, Prosperi C, Bellucci A, Vannucchi MG, Rosi S, Pepeu G, Casamenti F (2002) Beta-amyloid-induced inflammation and cholinergic hypofunction in the rat brain in vivo: involvement of the p38MAPK pathway. Neurobiol Dis 11:257-274.

Giulian D (1993) Reactive glia as rivals in regulating neuronal survival. Glia 7:102-110.

Goodall EF, Morrison KE (2006) Amyotrophic lateral sclerosis (motor neuron disease): proposed mechanisms and pathways to treatment. Expert Rev Mol Med 8:1-22.

Goodenough DA, Paul DL (2009) Gap junctions. Cold Spring Harb Perspect Biol 1:a002576.

Gordon GR, Mulligan SJ, MacVicar BA (2007) Astrocyte control of the cerebrovasculature. Glia 55:1214-1221.

Graeber MB (2010) Changing face of microglia. Science 330:783-788.

Halassa MM, Fellin T, Haydon PG (2007) The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 13:54-63.

Hamby ME, Sofroniew MV (2010) Reactive astrocytes as therapeutic targets for CNS disorders. Neurotherapeutics 7:494-506.

Hampton DW, Rhodes KE, Zhao C, Franklin RJ, Fawcett JW (2004) The responses of oligodendrocyte precursor cells, astrocytes and microglia to a cortical stab injury, in the brain. Neuroscience 127:813-820.

Hanisch UK, Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10:1387-1394.

Hansson E, Muyderman H, Leonova J, Allansson L, Sinclair J, Blomstrand F, Thorlin T, Nilsson M, Ronnback L (2000) Astroglia and glutamate in physiology and pathology: aspects on glutamate transport, glutamate-induced cell swelling and gap-junction communication. Neurochem Int 37:317-329.

Hattori I, Takagi Y, Nakamura H, Nozaki K, Bai J, Kondo N, Sugino T, Nishimura M, Hashimoto N, Yodoi J (2004) Intravenous administration of thioredoxin decreases brain damage following transient focal cerebral ischemia in mice. Antioxid Redox Signal 6:81-87.

Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185-193.

Hinkerohe D, Smikalla D, Haghikia A, Heupel K, Haase CG, Dermietzel R, Faustmann PM (2005) Effects of cytokines on microglial phenotypes and astroglial coupling in an inflammatory coculture model. Glia 52:85-97.

Hirota A, Kawachi Y, Itoh K, Nakamura Y, Xu X, Banno T, Takahashi T, Yamamoto M, Otsuka F (2005) Ultraviolet A irradiation induces NF-E2-related factor 2 activation in dermal fibroblasts: protective role in UVA-induced apoptosis. J Invest Dermatol 124:825-832.

Hoen B, Kessler M (2003) [Iron, oxidative stress and infection in hemodialysis]. Nephrologie 24:7-8.

Houades V, Rouach N, Ezan P, Kirchhoff F, Koulakoff A, Giaume C (2006) Shapes of astrocyte networks in the juvenile brain. Neuron Glia Biol 2:3-14.

Hu X, Zhang D, Pang H, Caudle WM, Li Y, Gao H, Liu Y, Qian L, Wilson B, Di Monte DA, Ali SF, Zhang J, Block ML, Hong JS (2008) Macrophage antigen complex-1 mediates reactive microgliosis and progressive dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. J Immunol 181:7194-7204.

Huang HC, Nguyen T, Pickett CB (2002) Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem 277:42769-42774.

Iadecola C, Nedergaard M (2007) Glial regulation of the cerebral microvasculature. Nat Neurosci 10:1369-1376.

Iida K, Itoh K, Kumagai Y, Oyasu R, Hattori K, Kawai K, Shimazui T, Akaza H, Yamamoto M (2004) Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. Cancer Res 64:6424-6431.

Ikeda H, Serria MS, Kakizaki I, Hatayama I, Satoh K, Tsuchida S, Muramatsu M, Nishi S, Sakai M (2002) Activation of mouse Pi-class glutathione S-transferase gene by Nrf2(NF-E2-related factor 2) and androgen. Biochem J 364:563-570.

Innamorato NG, Lastres-Becker I, Cuadrado A (2009) Role of microglial redox balance in modulation of neuroinflammation. Curr Opin Neurol 22:308-314.

Innamorato NG, Rojo AI, Garcia-Yague AJ, Yamamoto M, de Ceballos ML, Cuadrado A (2008) The Transcription Factor Nrf2 Is a Therapeutic Target against Brain Inflammation. J Immunol 181:680-689.

Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 275:16023-16029.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76-86.

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236:313-322.

Jaiswal AK (2000) Regulation of genes encoding NAD(P)H:quinone oxidoreductases. Free Radic Biol Med 29:254-262.

Jakel RJ, Townsend JA, Kraft AD, Johnson JA (2007) Nrf2-mediated protection against 6-hydroxydopamine. Brain Res 1144C:192-201.

Jander S, Kraemer M, Schroeter M, Witte OW, Stoll G (1995) Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex. J Cereb Blood Flow Metab 15:42-51.

Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA (2010) The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. Toxicol Sci 114:237-246.

Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH (2002) NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. J Neurosci 22:2792-2803.

Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC (1999) Fruit and vegetable intake in relation to risk of ischemic stroke. Jama 282:1233-1239.

Juszczak GR, Swiergiel AH (2009) Properties of gap junction blockers and their behavioural, cognitive and electrophysiological effects: animal and human studies. Prog Neuropsychopharmacol Biol Psychiatry 33:181-198.

Kikuchi G, Yoshida T, Noguchi M (2005) Heme oxygenase and heme degradation. Biochem Biophys Res Commun 338:558-567.

Kimelberg HK (1983) Primary astrocyte cultures--a key to astrocyte function. Cell Mol Neurobiol 3:1-16.

Kirchhoff F, Dringen R, Giaume C (2001) Pathways of neuron-astrocyte interactions and their possible role in neuroprotection. Eur Arch Psychiatry Clin Neurosci 251:159-169.

Kobayashi M, Yamamoto M (2006) Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. Adv Enzyme Regul 46:113-140.

Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, Takagi Y, Yamamoto M (2002) Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. Genes Cells 7:807-820.

Kochanek PM, Clark RS, Ruppel RA, Adelson PD, Bell MJ, Whalen MJ, Robertson CL, Satchell MA, Seidberg NA, Marion DW, Jenkins LW (2000) Biochemical, cellular, and molecular mechanisms in the evolution of secondary damage after severe traumatic brain injury in infants and children: Lessons learned from the bedside. Pediatr Crit Care Med 1:4-19.

Komuro C, Ono K, Shibamoto Y, Nishidai T, Takahashi M, Abe M (1985) Rapid and simple method for quantitative determination of non-protein sulphydryls in mouse liver by reversed-phase high-performance liquid chromatography. J Chromatogr 338:209-212.

Kraft AD, Johnson DA, Johnson JA (2004) Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. J Neurosci 24:1101-1112.

Kraft AD, Lee JM, Johnson DA, Kan YW, Johnson JA (2006) Neuronal sensitivity to kainic acid is dependent on the Nrf2-mediated actions of the antioxidant response element. J Neurochem 98:1852-1865.

Kreutzberg GW (1995) Microglia, the first line of defence in brain pathologies. Arzneimittelforschung 45:357-360.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312-318.

Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027-2033.

Kumar NM, Gilula NB (1996) The gap junction communication channel. Cell 84:381-388.

Kurata Y, Marszalec W, Yeh JZ, Narahashi T (1999) Agonist and potentiation actions of n-octanol on gamma-aminobutyric acid type A receptors. Mol Pharmacol 55:1011-1019.

Kuroda S, Siesjo BK (1997) Reperfusion damage following focal ischemia: pathophysiology and therapeutic windows. Clin Neurosci 4:199-212.

Laird DW (2006) Life cycle of connexins in health and disease. Biochem J 394:527-543.

Lawson LJ, Perry VH, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39:151-170.

Lee JK, Park MS, Kim YS, Moon KS, Joo SP, Kim TS, Kim JH, Kim SH (2007) Photochemically induced cerebral ischemia in a mouse model. Surg Neurol 67:620-625; discussion 625.

Lee JM, Shih AY, Murphy TH, Johnson JA (2003a) NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. J Biol Chem 278:37948-37956.

Lee JM, Calkins MJ, Chan K, Kan YW, Johnson JA (2003b) Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J Biol Chem 278:12029-12038.

Lee JM, Li J, Johnson DA, Stein TD, Kraft AD, Calkins MJ, Jakel RJ, Johnson JA (2005) Nrf2, a multi-organ protector? FASEB J 19:1061-1066.

Leoni G, Rattray M, Butt AM (2009) NG2 cells differentiate into astrocytes in cerebellar slices. Mol Cell Neurosci 42:208-218.

Leshchenko Y, Likhodii S, Yue W, Burnham WM, Perez Velazquez JL (2006) Carbenoxolone does not cross the blood brain barrier: an HPLC study. BMC Neurosci 7:3.

Levine JM (1994) Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. J Neurosci 14:4716-4730.

Levine JM, Enquist LW, Card JP (1998) Reactions of oligodendrocyte precursor cells to alpha herpesvirus infection of the central nervous system. Glia 23:316-328.

Levine JM, Reynolds R, Fawcett JW (2001) The oligodendrocyte precursor cell in health and disease. Trends Neurosci 24:39-47.

Li B, Mahmood A, Lu D, Wu H, Xiong Y, Qu C, Chopp M (2009a) Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1beta level after traumatic brain injury. Neurosurgery 65:179-185; discussion 185-176.

Li L, Hsu A, Moore PK (2009b) Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation--a tale of three gases! Pharmacol Ther 123:386-400.

Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, Nodin C, Stahlberg A, Aprico K, Larsson K, Yabe T, Moons L, Fotheringham A, Davies I, Carmeliet P, Schwartz JP, Pekna M, Kubista M, Blomstrand F, Maragakis N, Nilsson M, Pekny M (2007) Protective role of reactive astrocytes in brain ischemia. J Cereb Blood Flow Metab.

Li Y, Chopp M, Jiang N, Zaloga C (1995a) In situ detection of DNA fragmentation after focal cerebral ischemia in mice. Brain Res Mol Brain Res 28:164-168.

Li Y, Chopp M, Jiang N, Yao F, Zaloga C (1995b) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 15:389-397.

Li Y, Chopp M, Jiang N, Zhang ZG, Zaloga C (1995c) Induction of DNA fragmentation after 10 to 120 minutes of focal cerebral ischemia in rats. Stroke 26:1252-1257; discussion 1257-1258.

Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergaard M (1998) Gap-junction-mediated propagation and amplification of cell injury. Nat Neurosci 1:494-500.

Lin SC, Bergles DE (2004) Synaptic signaling between neurons and glia. Glia 47:290-298.

Link EM, Riley PA (1988) Role of hydrogen peroxide in the cytotoxicity of the xanthine/xanthine oxidase system. Biochem J 249:391-399.

Little AR, O'Callagha JP (2001) Astrogliosis in the adult and developing CNS: is there a role for proinflammatory cytokines? Neurotoxicology 22:607-618.

Maines MD, Trakshel GM, Kutty RK (1986) Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. J Biol Chem 261:411-419.

Martinez AD, Saez JC (2000) Regulation of astrocyte gap junctions by hypoxia-reoxygenation. Brain Res Brain Res Rev 32:250-258.

Mattson MP (2008) Hormesis defined. Ageing Res Rev 7:1-7.

Mattson MP, Cheng A (2006) Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses. Trends Neurosci 29:632-639.

McGraw J, Hiebert GW, Steeves JD (2001) Modulating astrogliosis after neurotrauma. J Neurosci Res 63:109-115.

McWalter GK, Higgins LG, McLellan LI, Henderson CJ, Song L, Thornalley PJ, Itoh K, Yamamoto M, Hayes JD (2004) Transcription factor Nrf2 is essential for induction of NAD(P)H:quinone oxidoreductase 1, glutathione S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates. J Nutr 134:3499S-3506S.

Moi P, Chan K, Asunis I, Cao A, Kan YW (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A 91:9926-9930.

Morioka T, Kalehua AN, Streit WJ (1991) The microglial reaction in the rat dorsal hippocampus following transient forebrain ischemia. J Cereb Blood Flow Metab 11:966-973.

Moumdjian RA, Antel JP, Yong VW (1991) Origin of contralateral reactive gliosis in surgically injured rat cerebral cortex. Brain Res 547:223-228.

Nagy JI, Rash JE (2000) Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS. Brain Res Brain Res Rev 32:29-44.

Nave KA (2010) Myelination and support of axonal integrity by glia. Nature 468:244-252.

Nedergaard M, Ransom B, Goldman SA (2003) New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 26:523-530.

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318.

Nimura T, Weinstein PR, Massa SM, Panter S, Sharp FR (1996) Heme oxygenase-1 (HO-1) protein induction in rat brain following focal ischemia. Brain Res Mol Brain Res 37:201-208.

Nioi P, Hayes JD (2004) Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. Mutat Res 555:149-171.

Nishiyama A, Komitova M, Suzuki R, Zhu X (2009) Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat Rev Neurosci 10:9-22.

Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB (1996) Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. J Neurosci Res 43:299-314.

Nodin C, Nilsson M, Blomstrand F (2005) Gap junction blockage limits intercellular spreading of astrocytic apoptosis induced by metabolic depression. J Neurochem 94:1111-1123.

Norton WT (1999) Cell reactions following acute brain injury: a review. Neurochem Res 24:213-218.

Norton WT, Aquino DA, Hozumi I, Chiu FC, Brosnan CF (1992) Quantitative aspects of reactive gliosis: a review. Neurochem Res 17:877-885.

Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA (2006) Involvement of oxidative stress in Alzheimer disease. J Neuropathol Exp Neurol 65:631-641.

O'Carroll SJ, Alkadhi M, Nicholson LF, Green CR (2008) Connexin 43 mimetic peptides reduce swelling, astrogliosis, and neuronal cell death after spinal cord injury. Cell Commun Adhes 15:27-42.

Oberheim NA, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M (2009) Uniquely hominid features of adult human astrocytes. J Neurosci 29:3276-3287.

Panahian N, Yoshiura M, Maines MD (1999) Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. J Neurochem 72:1187-1203.

Park E, Bell JD, Baker AJ (2008) Traumatic brain injury: can the consequences be stopped? Cmaj 178:1163-1170.

Parri R, Crunelli V (2003) An astrocyte bridge from synapse to blood flow. Nat Neurosci 6:5-6.

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, Sixth Edition. London: Elsevier Inc.

Pearson WR, Windle JJ, Morrow JF, Benson AM, Talalay P (1983) Increased synthesis of glutathione S-transferases in response to anticarcinogenic antioxidants. Cloning and measurement of messenger RNA. J Biol Chem 258:2052-2062.

Pekny M, Pekna M (2004) Astrocyte intermediate filaments in CNS pathologies and regeneration. J Pathol 204:428-437.

Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. Glia 50:427-434.

Pekny M, Leveen P, Pekna M, Eliasson C, Berthold CH, Westermark B, Betsholtz C (1995) Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. Embo J 14:1590-1598.

Pekny M, Johansson CB, Eliasson C, Stakeberg J, Wallen A, Perlmann T, Lendahl U, Betsholtz C, Berthold CH, Frisen J (1999) Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. J Cell Biol 145:503-514.

Pekovic S, Filipovic R, Subasic S, Lavrnja I, Stojkov D, Nedeljkovic N, Rakic L, Stojiljkovic M (2005) Downregulation of glial scarring after brain injury: the effect of purine nucleoside analogue ribavirin. Ann N Y Acad Sci 1048:296-310.

Perea G, Araque A (2010) GLIA modulates synaptic transmission. Brain Res Rev 63:93-102.

Perea G, Navarrete M, Araque A (2009) Tripartite synapses: astrocytes process and control synaptic information. Trends Neurosci 32:421-431.

Pereira CF, Oliveira CR (2000) Oxidative glutamate toxicity involves mitochondrial dysfunction and perturbation of intracellular Ca2+ homeostasis. Neurosci Res 37:227-236.

Perez Velazquez JL, Frantseva MV, Naus CC (2003) Gap junctions and neuronal injury: protectants or executioners? Neuroscientist 9:5-9.

Peters A (2004) A fourth type of neuroglial cell in the adult central nervous system. J Neurocytol 33:345-357.

Petersen A, Carlsson T, Karlsson JO, Jonhede S, Zetterberg M (2008) Effects of dexamethasone on human lens epithelial cells in culture. Mol Vis 14:1344-1352.

Powell EM, Geller HM (1999) Dissection of astrocyte-mediated cues in neuronal guidance and process extension. Glia 26:73-83.

Povlishock JT, Christman CW (1995) The pathobiology of traumatically induced axonal injury in animals and humans: a review of current thoughts. J Neurotrauma 12:555-564.

Prawan A, Kundu JK, Surh YJ (2005) Molecular basis of heme oxygenase-1 induction: implications for chemoprevention and chemoprotection. Antioxid Redox Signal 7:1688-1703.

Primiano T, Egner PA, Sutter TR, Kelloff GJ, Roebuck BD, Kensler TW (1995) Intermittent dosing with oltipraz: relationship between chemoprevention of aflatoxin-induced tumorigenesis and induction of glutathione S-transferases. Cancer Res 55:4319-4324.

Privat A, Gimenez y Ribotta M, Ridet JL (1995) Morphology of astrocytes. In: Neuroglia (Ransom B, Kettenmann H, eds), pp 3-22: Oxford University Press, New York.

Raivich G (2005) Like cops on the beat: the active role of resting microglia. Trends Neurosci 28:571-573.

Rami A, Volkmann T, Winckler J (2001) Effective reduction of neuronal death by inhibiting gap junctional intercellular communication in a rodent model of global transient cerebral ischemia. Exp Neurol 170:297-304.

Rana S, Dringen R (2007) Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes. Neurosci Lett 415:45-48.

Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tuder RM, Biswal S (2004) Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. J Clin Invest 114:1248-1259.

Ransom B, Behar T, Nedergaard M (2003) New roles for astrocytes (stars at last). Trends Neurosci 26:520-522.

Rao VL, Baskaya MK, Dogan A, Rothstein JD, Dempsey RJ (1998) Traumatic brain injury down-regulates glial glutamate transporter (GLT-1 and GLAST) proteins in rat brain. J Neurochem 70:2020-2027.

Raps SP, Lai JC, Hertz L, Cooper AJ (1989) Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. Brain Res 493:398-401.

Rawanduzy A, Hansen A, Hansen TW, Nedergaard M (1997) Effective reduction of infarct volume by gap junction blockade in a rodent model of stroke. J Neurosurg 87:916-920.

Retamal MA, Froger N, Palacios-Prado N, Ezan P, Saez PJ, Saez JC, Giaume C (2007) Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia. J Neurosci 27:13781-13792.

Rhodes KE, Moon LD, Fawcett JW (2003) Inhibiting cell proliferation during formation of the glial scar: effects on axon regeneration in the CNS. Neuroscience 120:41-56.

Rhodes KE, Raivich G, Fawcett JW (2006) The injury response of oligodendrocyte precursor cells is induced by platelets, macrophages and inflammation-associated cytokines. Neuroscience 140:87-100.

Rice ME, Russo-Menna I (1998) Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. Neuroscience 82:1213-1223.

Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20:570-577.

Rohl C, Lucius R, Sievers J (2007) The effect of activated microglia on astrogliosis parameters in astrocyte cultures. Brain Res 1129:43-52.

Rojo AI, Innamorato NG, Martin-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A (2010) Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. Glia 58:588-598.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675-686.

Rouach N, Glowinski J, Giaume C (2000) Activity-dependent neuronal control of gap-junctional communication in astrocytes. J Cell Biol 149:1513-1526.

Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, Giaume C (2002) Gap junctions and connexin expression in the normal and pathological central nervous system. Biol Cell 94:457-475.

Rozental R, Srinivas M, Spray DC (2001) How to close a gap junction channel. Efficacies and potencies of uncoupling agents. Methods Mol Biol 154:447-476.

Ryu JK, Franciosi S, Sattayaprasert P, Kim SU, McLarnon JG (2004) Minocycline inhibits neuronal death and glial activation induced by beta-amyloid peptide in rat hippocampus. Glia 48:85-90.

Sandvig A, Berry M, Barrett LB, Butt A, Logan A (2004) Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. Glia 46:225-251.

Scali C, Giovannini MG, Prosperi C, Bellucci A, Pepeu G, Casamenti F (2003) The selective cyclooxygenase-2 inhibitor rofecoxib suppresses brain inflammation and protects cholinergic neurons from excitotoxic degeneration in vivo. Neuroscience 117:909-919.

Schallert T (2006) Behavioral tests for preclinical intervention assessment. NeuroRx 3:497-504.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke,

cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39:777-787.

Schipper HM (2004) Heme oxygenase expression in human central nervous system disorders. Free Radic Biol Med 37:1995-2011.

Schroeter M, Jander S, Witte OW, Stoll G (1994) Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. J Neuroimmunol 55:195-203.

Seifert G, Schilling K, Steinhauser C (2006) Astrocyte dysfunction in neurological disorders: a molecular perspective. Nat Rev Neurosci 7:194-206.

Seri B, Garcia-Verdugo JM, Collado-Morente L, McEwen BS, Alvarez-Buylla A (2004) Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J Comp Neurol 478:359-378.

Setkowicz Z, Bien E, Janeczko K (2004) Contralateral response of macrophages and astrocytes to injury in the cerebral hemisphere of 6-day-old rat following prenatal gamma irradiation. Int J Dev Neurosci 22:1-9.

Shih AY, Li P, Murphy TH (2005) A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. J Neurosci 25:10321-10335.

Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, Murphy TH (2003) Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 23:3394-3406.

Silver J, Miller JH (2004) Regeneration beyond the glial scar. Nat Rev Neurosci 5:146-156.

Siushansian R, Bechberger JF, Cechetto DF, Hachinski VC, Naus CC (2001) Connexin43 null mutation increases infarct size after stroke. J Comp Neurol 440:387-394.

Sofroniew MV (2005) Reactive astrocytes in neural repair and protection. Neuroscientist 11:400-407.

Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci.

Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathol 119:7-35.

Sohl G, Willecke K (2004) Gap junctions and the connexin protein family. Cardiovasc Res 62:228-232.

Spray DC, Ye ZC, Ransom BR (2006) Functional connexin "hemichannels": a critical appraisal. Glia 54:758-773.

Stahnke T, Stadelmann C, Netzler A, Bruck W, Richter-Landsberg C (2007) Differential upregulation of heme oxygenase-1 (HSP32) in glial cells after oxidative stress and in demyelinating disorders. J Mol Neurosci 32:25-37.

Staugaitis SM, Trapp BD (2009) NG2-positive glia in the human central nervous system. Neuron Glia Biol 5:35-44.

Streit WJ (2002) Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40:133-139.

Streit WJ, Walter SA, Pennell NA (1999) Reactive microgliosis. Prog Neurobiol 57:563-581.

Stridh MH, Correa F, Nodin C, Weber SG, Blomstrand F, Nilsson M, Sandberg M (2010) Enhanced glutathione efflux from astrocytes in culture by low extracellular Ca2+ and curcumin. Neurochem Res 35:1231-1238.

Stringer JL, Gaikwad A, Gonzales BN, Long DJ, Jr., Marks LM, Jaiswal AK (2004) Presence and induction of the enzyme NAD(P)H: quinone oxidoreductase 1 in the central nervous system. J Comp Neurol 471:289-297.

Sugawara T, Chan PH (2003) Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. Antioxid Redox Signal 5:597-607.

Sun X, Erb H, Murphy TH (2005) Coordinate regulation of glutathione metabolism in astrocytes by Nrf2. Biochem Biophys Res Commun 326:371-377.

Swanson RA, Ying W, Kauppinen TM (2004) Astrocyte influences on ischemic neuronal death. Curr Mol Med 4:193-205.

Sykova E (2001) Glial diffusion barriers during aging and pathological states. Prog Brain Res 132:339-363.

Tabernero A, Medina JM, Giaume C (2006) Glucose metabolism and proliferation in glia: role of astrocytic gap junctions. J Neurochem 99:1049-1061.

Takano T, Kang J, Jaiswal JK, Simon SM, Lin JH, Yu Y, Li Y, Yang J, Dienel G, Zielke HR, Nedergaard M (2005) Receptor-mediated glutamate release from volume sensitive channels in astrocytes. Proc Natl Acad Sci U S A 102:16466-16471.

Talalay P (2000) Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors 12:5-11.

Talalay P, Dinkova-Kostova AT (2004) Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates. Methods Enzymol 382:355-364.

Talalay P, Batzinger RP, Benson AM, Bueding E, Cha YN (1978) Biochemical studies on the mechanisms by which dietary antioxidants suppress mutagenic activity. Adv Enzyme Regul 17:23-36.

Talalay P, Fahey JW, Holtzclaw WD, Prestera T, Zhang Y (1995) Chemoprotection against cancer by phase 2 enzyme induction. Toxicol Lett 82-83:173-179.

Tan AM, Zhang W, Levine JM (2005) NG2: a component of the glial scar that inhibits axon growth. J Anat 207:717-725.

Tan AM, Colletti M, Rorai AT, Skene JH, Levine JM (2006) Antibodies against the NG2 proteoglycan promote the regeneration of sensory axons within the dorsal columns of the spinal cord. J Neurosci 26:4729-4739.

Tanaka K, Nogawa S, Ito D, Suzuki S, Dembo T, Kosakai A, Fukuuchi Y (2001) Activation of NG2-positive oligodendrocyte progenitor cells during post-ischemic reperfusion in the rat brain. Neuroreport 12:2169-2174.

Tatsumi K, Haga S, Matsuyoshi H, Inoue M, Manabe T, Makinodan M, Wanaka A (2005) Characterization of cells with proliferative activity after a brain injury. Neurochem Int 46:381-389.

Theis M, Sohl G, Eiberger J, Willecke K (2005) Emerging complexities in identity and function of glial connexins. Trends Neurosci 28:188-195.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res 62:5196-5203.

Tian DS, Dong Q, Pan DJ, He Y, Yu ZY, Xie MJ, Wang W (2007) Attenuation of astrogliosis by suppressing of microglial proliferation with the cell cycle inhibitor olomoucine in rat spinal cord injury model. Brain Res 1154:206-214.

Tong KI, Kobayashi A, Katsuoka F, Yamamoto M (2006) Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. Biol Chem 387:1311-1320.

Trotter J, Karram K, Nishiyama A (2010) NG2 cells: Properties, progeny and origin. Brain Res Rev 63:72-82.

Walter L, Neumann H (2009) Role of microglia in neuronal degeneration and regeneration. Semin Immunopathol 31:513-525.

van Muiswinkel FL, Riemers FM, Peters GJ, LaFleur MV, Siegel D, Jongenelen CA, Drukarch B (2000) L-Dopa stimulates expression of the antioxidant enzyme NAD(P)H:quinone oxidoreductase (NQO) in cultured astroglial cells. Free Radic Biol Med 29:442-453.

van Poppel G, Verhoeven DT, Verhagen H, Goldbohm RA (1999) Brassica vegetables and cancer prevention. Epidemiology and mechanisms. Adv Exp Med Biol 472:159-168.

Wang J, Ma M, Locovei S, Keane RW, Dahl G (2007) Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. Am J Physiol Cell Physiol 293:C1112-1119.

Vargas MR, Johnson JA (2009) The Nrf2-ARE cytoprotective pathway in astrocytes. Expert Rev Mol Med 11:e17.

Vargas MR, Johnson DA, Sirkis DW, Messing A, Johnson JA (2008) Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. J Neurosci 28:13574-13581.

Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD (1985) Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol 17:497-504.

Ventura R, Harris KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci 19:6897-6906.

Venugopal R, Jaiswal AK (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 93:14960-14965.

Wigley R, Butt AM (2009) Integration of NG2-glia (synantocytes) into the neuroglial network. Neuron Glia Biol 5:21-28.

Wilhelmsson U, Li L, Pekna M, Berthold CH, Blom S, Eliasson C, Renner O, Bushong E, Ellisman M, Morgan TE, Pekny M (2004) Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. J Neurosci 24:5016-5021.

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G (2002) Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383:725-737.

Wilson JX (1997) Antioxidant defense of the brain: a role for astrocytes. Can J Physiol Pharmacol 75:1149-1163.

Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6:626-640.

von Otter M, Landgren S, Nilsson S, Celojevic D, Bergstrom P, Hakansson A, Nissbrandt H, Drozdzik M, Bialecka M, Kurzawski M, Blennow K, Nilsson M, Hammarsten O, Zetterberg H (2010) Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease. BMC Med Genet 11:36.

Wood-Kaczmar A, Gandhi S, Wood NW (2006) Understanding the molecular causes of Parkinson's disease. Trends Mol Med 12:521-528.

Wu H, Mahmood A, Lu D, Jiang H, Xiong Y, Zhou D, Chopp M (2010) Attenuation of astrogliosis and modulation of endothelial growth factor receptor in lipid rafts by simvastatin after traumatic brain injury. J Neurosurg 113:591-597.

Wu L, Noyan Ashraf MH, Facci M, Wang R, Paterson PG, Ferrie A, Juurlink BH (2004) Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. Proc Natl Acad Sci U S A 101:7094-7099.

Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, Conney AH, Kong AN (2006) Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer Res 66:8293-8296.

Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P (2002) Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta 316:43-53.

Ye ZC, Wyeth MS, Baltan-Tekkok S, Ransom BR (2003) Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J Neurosci 23:3588-3596.

Ying W, Alano CC, Garnier P, Swanson RA (2005) NAD+ as a metabolic link between DNA damage and cell death. J Neurosci Res 79:216-223.

Zador Z, Stiver S, Wang V, Manley GT (2009) Role of aquaporin-4 in cerebral edema and stroke. Handb Exp Pharmacol:159-170.

Zan Y, Haag JD, Chen KS, Shepel LA, Wigington D, Wang YR, Hu R, Lopez-Guajardo CC, Brose HL, Porter KI, Leonard RA, Hitt AA, Schommer SL, Elegbede AF, Gould MN (2003) Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. Nat Biotechnol 21:645-651.

Zhang D, Hu X, Qian L, O'Callaghan JP, Hong JS (2010) Astrogliosis in CNS Pathologies: Is There A Role for Microglia? Mol Neurobiol.

Zhang Y (2001) Molecular mechanism of rapid cellular accumulation of anticarcinogenic isothiocyanates. Carcinogenesis 22:425-431.

Zhang Y, Gordon GB (2004) A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. Mol Cancer Ther 3:885-893.

Zhang Y, Yao S, Li J (2006) Vegetable-derived isothiocyanates: anti-proliferative activity and mechanism of action. Proc Nutr Soc 65:68-75.

Zhang Y, Talalay P, Cho CG, Posner GH (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A 89:2399-2403.

Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P (1994) Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci U S A 91:3147-3150.

Zhao J, Kobori N, Aronowski J, Dash PK (2006) Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. Neurosci Lett 393:108-112.

Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, Grotta JC, Aronowski J (2007) Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. Stroke 38:3280-3286.

Zhu X, Bergles DE, Nishiyama A (2008) NG2 cells generate both oligodendrocytes and gray matter astrocytes. Development 135:145-157.

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 6:43-50.