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Novel Players in Neuroprotection and Tissue Reorganisation after Hippocampal Lesion

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1. Zusammenfassung

Im Rahmen der vorliegenden Doktorarbeit sollten Gene identifiziert und funktionell charakterisiert werden, die bei der Neuroprotektion und der postläsionalen Restrukturierung eine Rolle spielen. Dabei waren einerseits Gene von Interesse, die intrazellulär neuroprotektive Mechanismen vermitteln. Andererseits interessierten uns auch Gene, die für Wachstumsfaktoren kodieren, die neuroprotektiv wirken oder die auf andere Weise die Reparatur des geschädigten Zentralnervensystems beeinflussen. Aufgrund der Vorergebnisse unserer Gruppe an einem Läsionsmodell der Haut wurden drei Kandidatengene ausgewählt, nämlich die Nrf Transkriptionsfaktoren, sowie die Wachstumsfaktoren Activin und *Connective Tissue Growth Factor* (CTGF). Die im Rahmen dieser Arbeit erzielten Ergebnisse sind untenstehend zusammengefasst.

1.1 Nrf-1 und Nrf-2

Die Transkriptionsfaktoren Nrf-1 und Nrf-2 regulieren die Expression verschiedener zytoprotektiver Proteine, wie z.B. von Enzymen, die reaktive Sauerstoffspezies detoxifizieren. Letztere spielen eine zentrale Rolle beim neuronalen Tod nach traumatischen oder ischämischen Hirnverletzungen. Es ist nicht bekannt, ob Nrf-1 oder Nrf-2 eine neuroprotektive Funktion erfüllen. In der vorliegenden Arbeit untersuchten wir daher die zeitlichen und örtlichen Expressionsmuster von Nrf-1 und Nrf-2 nach unilateraler, excitotoxischer Schädigung des Hippokampus der Maus. In deutlichem Gegensatz zu früheren *in vitro* Studien, bei denen nie eine Regulation dieser Transkriptionsfaktoren auf mRNA-Ebene gezeigt werden konnte, konnten wir eine starke Hochregulation von Nrf-1 mRNA nachweisen, begleitet von einer schwächeren Induktion von Nrf-2 mRNA. Die folgenden Ergebnisse sprechen für eine wichtige Funktion von Nrf-1 beim Überleben von Neuronen: (1) neu gebildetes Nrf-1 Protein ist im verletzten Hippokampus im Zellkern der Neuronen lokalisiert, und die Expression von Hämoxygenase, einem der wichtigsten Zielproteine von Nrf-1 und Nrf-2, wurde zusammen mit der von Nrf-1 und Nrf-2 in den hippokampalen

Neuronen induziert. Da Nrf-1 und Nrf-2 auch andere cytoprotektive Proteine induzieren, legen unsere Daten nahe, dass die Induktion dieser Transkriptionsfaktoren einen wichtigen Schritt für das Überleben von Neuronen nach akuter Hirnschädigung darstellt.

1.2 Activin

Die exogene Verabreichung von neurotrophen Wachstumsfaktoren hat sich als eine neue und vielversprechende Methode erwiesen, nicht nur die funktionelle Wiederherstellung des Zentralnervensystems zu unterstützen, sondern auch Neuronen vor den unmittelbaren Folgen der Schädigung zu schützen. Unter den vielen bisher untersuchten Wachstumsfaktoren und Zytokinen ist die neuroprotektive und neurotrophe Wirkung des Fibroblastenwachstumsfaktors 2 (bFGF or FGF2) am genauesten untersucht. Anhand eines Tiermodells, bei dem eine lokale excitotoxische Hirnläsion induziert wird, konnten wir zeigen, dass die neuroprotektive Wirkung von bFGF, welches bereits an Schlaganfallpatienten klinisch getestet wurde, abhängig ist von der Induktion von Activin A, einem Mitglied der *Transforming growth factor* β Familie. Ein erster Hinweis für diesen bisher unbekannten Mechanismus war für uns die starke läsionsbedingte Hochregulation von Activin A nach bFGF-Gabe. In Gegenwart des Activin-bindenden Proteins Follistatin war bFGF hingegen nicht in der Lage, seine neuroprotektive Wirkung zu entfalten. Rekombinantes Activin A war seinerseits in der Lage, diesen Effekt auch alleine zu vermitteln. Unsere Daten sprechen dafür, dass man durch die Entwicklung von Substanzen, die die Activin-Expression oder dessen Rezeptorbindung beeinflussen, ein wirksames Mittel für die akute Therapie des Schlaganfalls finden könnte.

1.3 CTGF

Vor kurzem veröffentlichte Untersuchungen zeigten, dass CTGF bei Reparaturprozessen sowie bei Fibrosierung der Haut und verschiedener anderer Organe eine wichtige Rolle spielt. Dagegen konnte eine Beteiligung dieses Wachstumsfaktors an Reparaturvorgängen im Zentralnervensystem

noch nie gezeigt werden. Im Rahmen meiner Doktorarbeit untersuchte ich daher die zeitliche und räumliche Verteilung von CTGF nach unilateraler Kainatläsion der CA3 Region des Hippokampus der Maus. Wir konnten eine starke Induktion von CTGF mRNA und Protein in Neuronen und Gliazellen des geschädigten Hippokampus nachweisen. Interessanterweise ging die verstärkte Expression von CTGF mit einer Induktion der Fibronektinexpression einher, welches als Zielprotein von CTGF bekannt ist. Unsere Daten sprechen daher für eine neue Funktion von CTGF bei der posttraumatischen Reorganisation des Hippokampus, wobei es möglicherweise an der Glianarbenbildung beteiligt ist.

2. Summary

The goal of my thesis was to identify and functionally characterize new players involved in neuroprotection and CNS repair after hippocampal lesion. For this purpose we used an animal model of acute excitotoxic brain injury, where the excitotoxin kainate was unilaterally injected into the ventricle, leading to a severe lesion in the CA3 region of the hippocampus. On the one hand we were interested in the intracellular signalling mechanisms which are involved in the injury response, and on the other hand we attempted to identify growth factors involved in neuroprotection and/or post-lesional restructuring of the injured brain. Based on previous results from our group that were obtained in a skin injury model, we selected candidate genes that are involved in cutaneous wound repair: the Nrf-1 and Nrf-2 transcription factors as well as the growth factors activin and connective tissue growth factor. The results presented in this thesis reveal novel roles of these genes in CNS repair. Whereas the Nrf transcription factors and activin appear to be predominantly involved in neuroprotection, connective tissue growth factor seems to play a role in glial scarring. The detailed results obtained with all three genes are listed below:

2.1 Nrf-1 and Nrf-2

The Nrf-1 and Nrf-2 transcription factors play a pivotal role in the cellular defence against the toxic effects of reactive oxygen species (ROS). Although ROS are key effectors of neuronal death after ischemic and traumatic brain injury, it is not known whether Nrf-1 and Nrf-2 are involved in neuroprotective signalling. Here, we analyzed the temporal and spatial expression pattern of Nrf-1 and Nrf-2 after unilateral excitotoxic lesion of mouse hippocampus. In marked contrast to previous *in vitro* studies, where up-regulation of these transcription factors on the mRNA level was never detected, we found a strong induction of Nrf-1 mRNA and protein expression in neurons of the lesioned hippocampus, accompanied by a weak elevation of Nrf-2 mRNA levels. The following observations underscore the functional significance of Nrf-1 in surviving neurons: Firstly, the newly formed Nrf-1 predominantly localized to the

nucleus in the injured hippocampus. Secondly, expression of the cytoprotective enzyme, heme oxygenase-1, a major target of Nrf-1 and Nrf-2 action, was co-regulated with Nrf-1 in the same hippocampal neurons. Since Nrf-1 and Nrf-2 are potent inducers of various other cytoprotective proteins, our data implicate the induction of these factors as a critical step in neuronal survival after acute brain injury.

2.2 Activin

Exogenous application of neurotrophic growth factors has emerged as a new and particularly promising approach not only to promote functional recovery following acute brain injury, but also to protect neurons against the immediate impact of the injury. Among the various growth factors and cytokines studied so far, the neuroprotective and neurotrophic profile of basic fibroblast growth factor (bFGF) is best documented. Using an animal model of acute excitotoxic brain injury, we report here that the neuroprotective action of basic fibroblast growth factor (bFGF), which is currently tested in stroke patients, critically depends on the induction of activin A, a member of the transforming growth factor-β (TGF-β) superfamily. Our evidence for this novel mechanism of action of bFGF is the following: (1) bFGF strongly enhances lesion-associated activin A induction, (2) in the presence of the activin-neutralizing protein, follistatin, bFGF is no longer capable of rescuing neurons from excitotoxic death, and (3) recombinant activin A exerts a neuroprotective effect by itself. Development of substances that influence activin expression or receptor binding should offer new ways to fight neuronal loss in ischemic and traumatic brain injury.

2.3 CTGF

Recent studies have suggested a role of connective tissue growth factor (CTGF) in repair processes of the skin as well as in various types of fibrotic disease. However, a function of this molecule in CNS repair has not been

demonstrated yet. In this study we analyzed the temporal and spatial expression pattern of CTGF after unilateral kainic acid lesions of the hippocampal CA3 region in mice. We found a strong induction of CTGF mRNA and protein expression in neurons and glial cells of the lesioned hippocampus. Interestingly, increased expression of this mitogen was accompanied by elevated levels of the extracellular matrix molecule fibronectin which is a known target of CTGF action. Therefore, our data indicate a novel function of CTGF in postlesional restructuring of the hippocampus, where it possibly participates in glial scar formation.

3. Introduction

For a long time, it was one of the dogmas in neurobiology that the adult CNS is endowed with very limited capacity for regeneration and repair after injury. Neuronal damage often inflicts long-term impairments upon the affected individuals, associated with enormous costs for the health care system. In order to promote survival of neurons and to restrict the extent of secondary brain damage, it is a prerequisite to elucidate basic mechanisms involved in protection and recovery of the CNS.

An acute lesion of the brain initiates a complex pattern of responses that is orchestrated by a variety of cytokines, growth- and differentiation factors. In the present work I used a circumscribed excitotoxic lesion of the rodent hippocampus to identify new players in the neuronal response to acute injury and to characterise their roles in neuroprotection and glial scarring.

3.1. The hippocampus

The hippocampus is part of the archicortex and represents an important part of the limbic system. Its topography in rodent the brain is illustrated in Fig. 1A. In man, the hippocampus plays an essential role in learning and transient storage of declarative memory contents. In rodents, the hippocampus is especially important for spatial learning and orientation. It is generally believed that synaptic plasticity, in particular long-term potentiation (LTP) and long-term depression (LTD), are the neurophysiological correlates of learning on the cellular level. Owing to its highly regular organisation, the hippocampal circuitry and its synaptic physiology are easily accessible to investigations both *in vivo* and *in vitro*, making the hippocampus the model brain structure to study the physiological and pathophysiological aspects (excitotoxicity, see below) of excitatory synaptic transmission. In terms of basic connectivity, the hippocampal formation represents a trisynaptic excitatory synaptic circuit, with glutamate as the principal transmitter. Signals coming from the entorhinal cortex (EC) via the perforant path (PP) are sequentially processed at three glutamatergic synapses before leaving the hippocampus again. The PP excites granule cells of the dentate

gyrus (DG), whose axons, the so-called mossy fibres, transmit the signals to the pyramidal neurons of the CA3 region. The axons of the CA3 pyramidal cells form the so-called Schaffer-collaterals, which excite the pyramidal cells of the CA1 region, from where the signals are then projected to various cortical and subcortical areas. Throughout the trisynaptic circuit, excitatory synaptic transmission is effectively controlled by different types of interneurons.

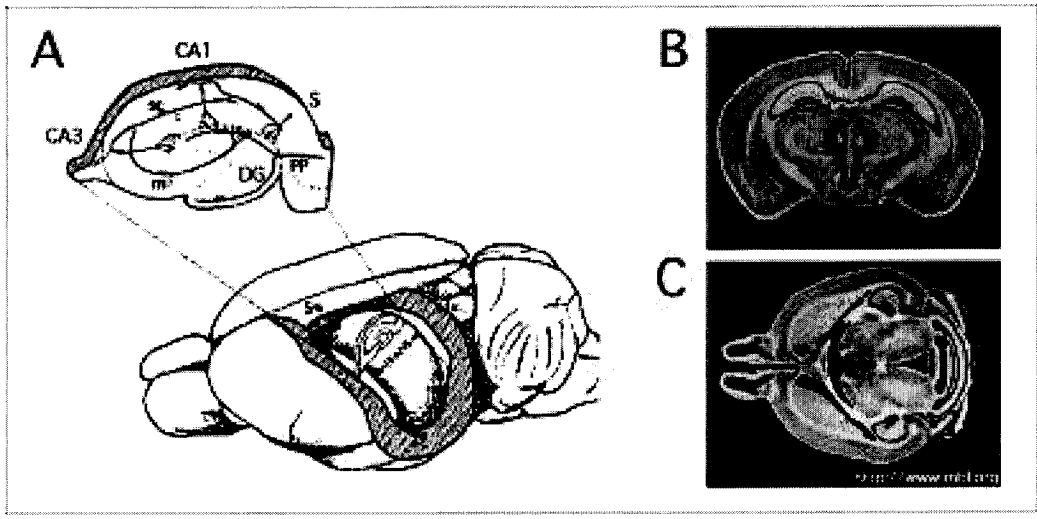


Fig.1 ***A)*** *shows a schematic drawing of the mouse brain (lower part) and a magnification of the hippocampal circuits in the slice preparation (upper part)* ***PP=*** *perforant path,* ***GD=*** *dentate gyrus,* ***m=*** *mossy fibres,* ***sc=*** *Schaffer collaterals (figure from Amaral and Witter, 1989)*
B) *coronal section through the mouse brain showing the hippocampus near the site of injection for the lesion described later* ***C)*** *horizontal section through the mouse brain at the level of the hippocampus*

3.2. The excitotoxic effect

In the CNS, the amino acids glutamate and presumably aspartate are the predominant excitatory neurotransmitters. Abnormally high concentrations of glutamate have been shown to exert a toxic effect on neurons. To reflect their double role as excitatory transmitters and toxins, the term "excitotoxicity" was coined. An excitotoxic insult leads to a loss of ion homeostasis followed by osmotic swelling and death. Under normal conditions L-glutamate rises during synaptic transmission for a short time. Lucas and Newhouse showed 1957 for

the first time that a longer lasting exposition to glutamate destroyed neurons of the retina. Later it was shown that pathologically high levels of L-glutamate have a similar effect on neurons of other brain regions, including the hippocampus. Based on these findings Olney and Shape (Olney and Shape, 1969; Olney *et al.*,1971) developed a more general concept of excitotoxicity, which is widely accepted today. This implies that a variety of clinical situations like ischemia, trauma, hypoxic or hypoglycemic conditions lead via different mechanisms to a dramatic increase in extracellular glutamate. A schematic diagram of these events and the underlying mechanisms is given in Fig.2 which was taken from a review by Choi (1988)

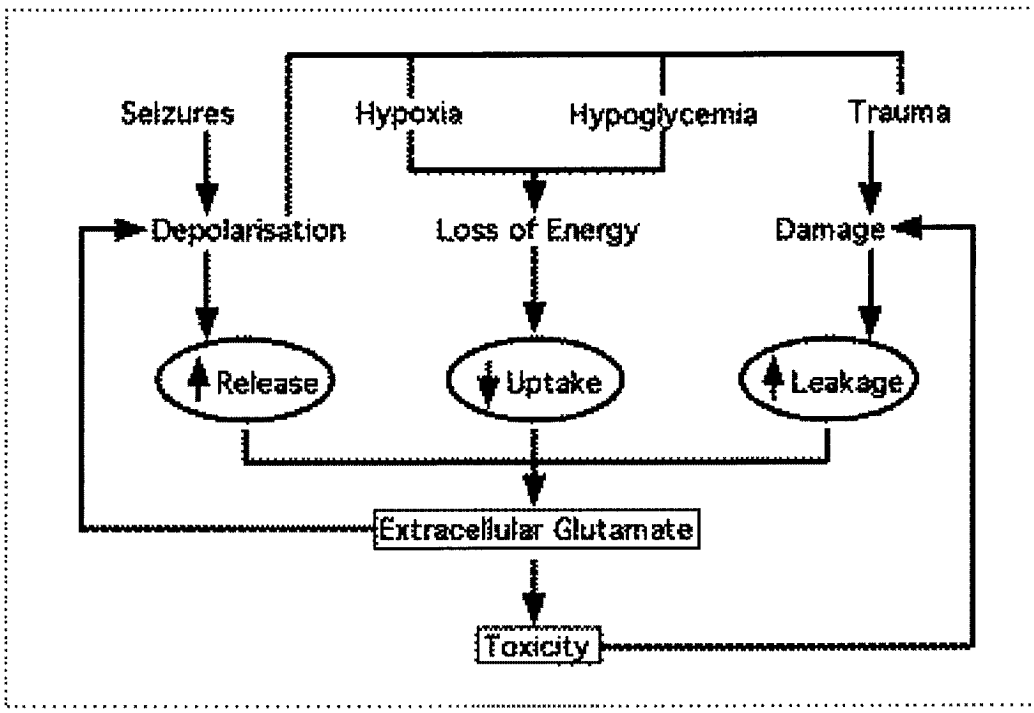


Fig. 2: Rise in extracellular glutamate as the common pathway leading to neuronal death in several acute brain disorders (Choi, 1988)

Under ischemic conditions, glutamate is released into the extracellular space where it reaches near millimolar concentrations. Such a massive flooding leads to an over-stimulation of glutamate receptors, promoting Na^+ influx through glutamate receptors. Stimulation of one type of glutamate receptors, the N-methyl-D-aspartate (NMDA)-receptors (see 1.5) induces the opening of the receptor cation channel. Since this channel is highly permeable to Ca^{2+}

(Choi,1995) the intracellular Ca^{2+}-concentration rises significantly. The entry of Na^{+} and Ca^{2+} ions is accompanied by an influx of Cl^{-} and water into the cytoplasm of the neurons, leading to swelling of cell bodies and dendrites which might result in necrotic cell death. Furthermore, high levels of intracellular Ca^{2+} in neurons exposed to glutamate trigger a cascade of molecular events resulting in apoptotic neuronal death. The dramatically enhanced Ca^{2+} -levels activate kinases and proteases and promote formation of reactive oxygen species (ROS) which play a central role in neuronal death (see below).

3.3. Kainic acid (KA)

KA is an excitatory amino acid extracted from a red algea. Although its basic structure is a heterocycle, KA shows structural similarities to glutamic acid (Fig.3) and binds to glutamatergic receptors, in particular to the kainate receptor subtype. In animal experiments examining excitotoxicity, kainate is widely used instead of glutamate because KA is not taken up by glutamate transporters. In addition to its direct excitotoxic effect, kainate promotes neuronal death through induction of long lasting seizure (Schwob *et al.*, 1980; Ben-Ari, 1985; Okazaki and Nadler, 1988)

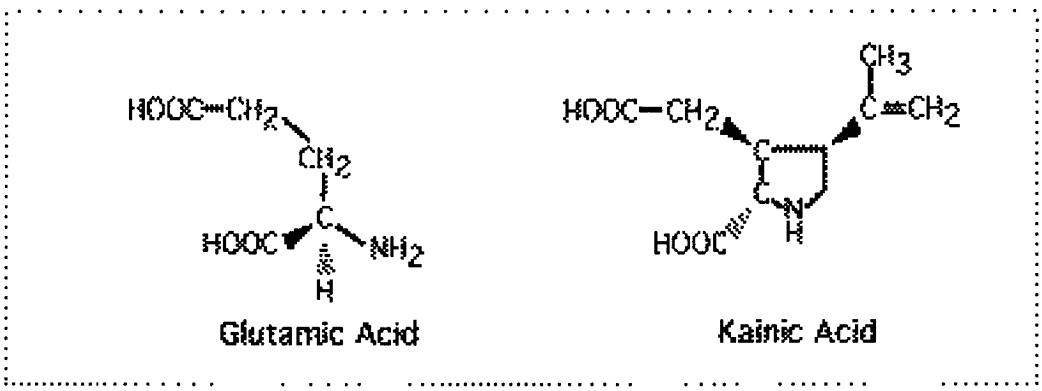


Fig.3: The structure formulas of glutamic acid and kainic acid

3.4. Necrosis and apoptosis

In general, two different types of cell death can be distinguished. One is the necrotic cell death which occurs in a highly unordered fashion. During the necrotic

process the cell swells, accompanied by disintegration of all cellular membranes and organelles and release of the cellular contents into the intercellular space. These released intracellular components usually trigger an inflammatory reaction.

A more controlled or programmed form of cell death is the regulated disassembly of a cell first described by Kerr and Wyllie *et al.*(1972) nearly 30 years ago. A series of morphological changes are shared by dying cells in various model systems. These alterations were largely detected by electron microscopy and included cell shrinkage, plasma and nuclear membrane blebbing, organelle delocalisation and compaction, chromatin condensation, DNA laddering, and production of membrane-enclosed vesicles containing intracellular material. These typical "apoptotic bodies" are removed from the tissue by phagocytosis, a process avoiding inflammatory processes in the tissue.

In the KA lesion model both, apoptotic and necrotic cell death occurs. The first wave of neuronal death has been attributed to necrosis, whereas delayed neuronal loss seems to result from apoptotic processes (Fujikawa *et al.*, 2000)

3.5. Glutamate receptors

Glutamate receptors (GluR) are divided into two major classes based on their molecular, pharmacological and electrophysiological properties. One class are ionotropic receptors which are divided into three subtypes according to their pharmacological affinities: NMDA-(N-Methyl-D-Aspartate), AMPA (α-Amino-3-Hydroxy-5-Methyl-isoxazole-4-Propionicacid) receptors and kainic acid receptors (Monagahan *et al.*, 1989; Hollmann and Heinemann , 1994; Bettler and Mulle, 1995). The second class are the G protein-coupled glutamate receptors (Sladeczek *et al.*, 1985), which come so far in eight subtypes. These metabotropic glutamate receptors are classified into three groups based on similarities in their primary structure, their coupling to second messenger systems and their pharmacology (Keele at al., 2000)

3.6. The kainic acid lesion

The KA lesion is a widely used model to investigate the consequences of excitotoxic brain damage (reviewed in Ben-Ari, 1985; Ben-Ari and Cossart, 2000). Following *intraperitoneal* injection, KA gives rise to epileptic seizures and to relatively wide-spread lesions in susceptible brain regions. By contrast, *intracerebroventricular* (*icv*) injection or direct injection of KA into the brain tissue causes a selective neuronal loss in the CA3 region of the ipsilateral hippocampus (Nadler *et al.*, 1978 and Monahgan *et al.*,1993). This typical lesion pattern was confirmed in the present study (see second paper)

All three forms of application have been employed to investigate the mechanisms and functional consequences of the neuronal loss which results from the KA-induced seizure activity. Electrophysiological investigations have shown that repeated seizure activity leads to preferential cell loss in the CA3 region and to synaptic reorganisation in the dentate gyrus (DG) and in the CA1 region. It is not clear, whether the observed postlesional hyperexcitability of the CA1 region results primarily from sprouting of excitatory fibres, from the loss of inhibition, or from both (Meier and Dudek, 1996; Perez *et al.*, 1996)

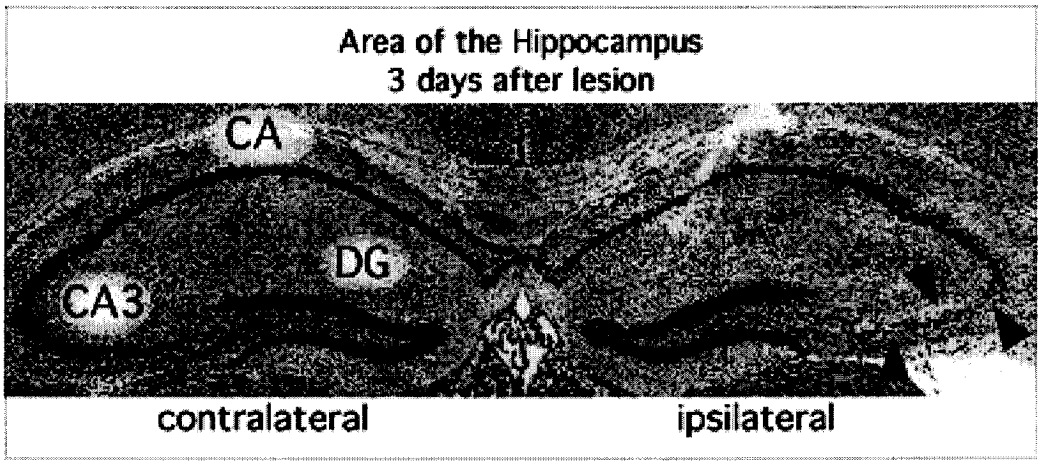


Fig. 4: Coronal section of the hippocampus showing a typical KA lesion as performed in this study. The lesion is restricted to the ipsilateral CA3 region, where the neurons *are almost completely lost (Nissl stain, 3d after lesion)*

Previous studies have shown that the *icv* injection of KA, which was also employed in the present study, has the following characteristics:

1) KA causes damage only in the CA3 region but not in the CA1 region.
2) Necrotic neurons are phagocytosed by activated glial cells.
3) Axons of damaged but surviving neurons start sprouting and generate new synapses.
4) Axonal sprouting is disrupted by the gradual formation of a glial scar.

3.7. Reactive oxygen species

Reactive oxygen species (ROS) are central effectors of neuronal death in the excitotoxic lesion (Boldyrev *et al.*, 2000, Bondy *et al.*, 1993). The term ROS comprises different molecules, which are capable of oxidising other molecules via radical formation, such as hydrogen peroxide, organic hydroperoxides and oxyradicals. ROS are frequent intermediates and (by-) products of numerous chemical reactions e.g. redox reactions and homolytic molecular cleavages. In addition, endogenous ROS are generated in all cell types in the course of a broad variety of common metabolic processes, e.g. the respiratory chain. Particularly large amounts of ROS are produced by leukocytes in inflamed tissues. Interestingly, increased detoxification of ROS appears to be a major part of neuroprotection. The neuroprotective growth factor basic fibroblast growth factor (b-FGF, FGF2) has been shown to protect neurons in hippocampal cultures by suppression of ROS accumulation, suggesting that the defence against ROS is part of the neuroprotective mechanism (Mark *et al.*,1997). Indeed, it has been shown that bFGF induces the expression and activity of enzymes that detoxify ROS in hippocampal neurons (Mattson *et al.*, 1995).

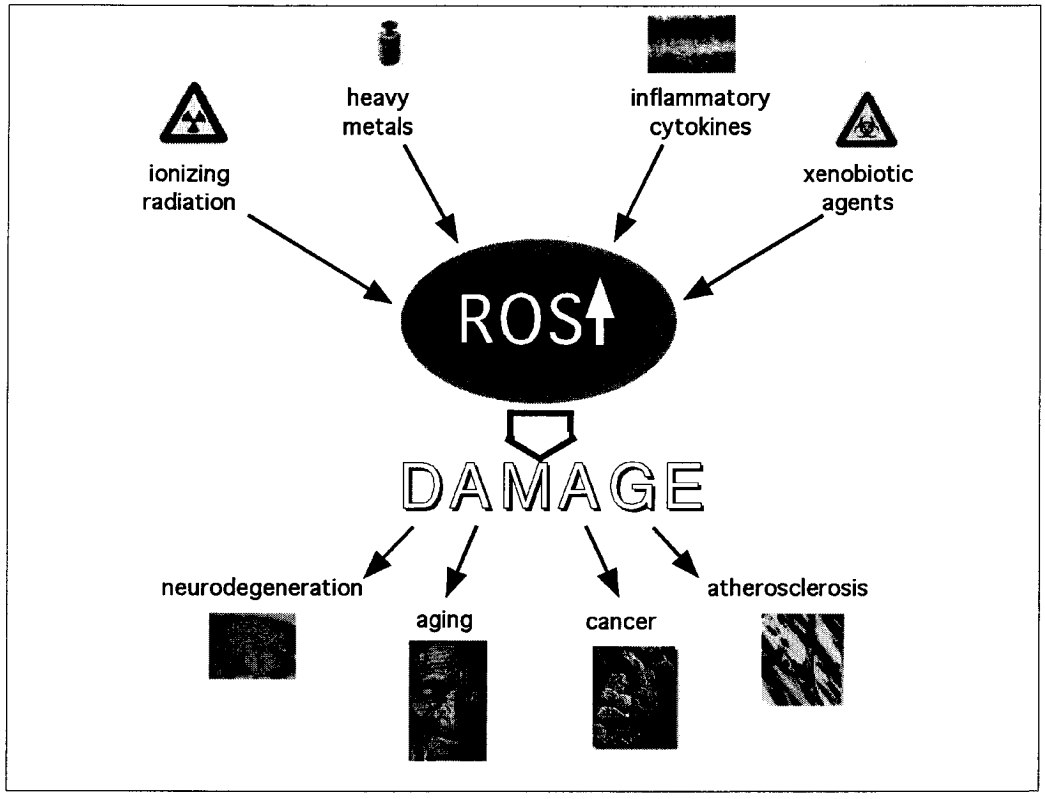


Fig.5: Scheme showing the induction and effects of ROS

3.8. Detoxifying enzymes

Interest in ROS arose in the early seventies, after biochemists had discovered superoxide dismutase, an enzyme specific for a free radical substrate (Fridovich, 1974). To date a large number of ROS detoxifying enzymes is known and the up-regulation after brain lesion is a common feature of many of these enzymes (Kim *et al.*, 2000, McIntosh *et al.*, 1998, Nakaso *et al.*, 1999)

Some of the respective genes are regulated by the Nrf-family transcription factors which bind to the antioxidant response element (ARE) (Wasserman *et al.*, 1997), in the promoters of these genes.

3.9. Nrf-1 and Nrf-2 transcription factors

Nrf-1 is a member of the "cap'n collar" family of transcription factors, which include p45-NF-E2, Nrf-1, Nrf-2 and Nrf3 as well as Bach1 and Bach2. These

proteins bind to the NF-E2 binding sites that are essential for the regulation of erythroid genes. In addition, Nrf-1 and 2 also bind to a very similar element, the antioxidant response element (ARE) or electrophile response element (EpRE). This sequence with the consensus sequence TGACNNNGC was identified in the promoter of several genes involved in the cellular stress response (Rushmore and Pickett, 1993; Jaiswal, 1994, Mulcahy *et al.*,1997). By binding to this element, Nrf-1 and Nrf-2 induce the expression of a variety of phase II detoxifying enzymes and oxidative stress inducible proteins such as NAD(P)H:quinone oxidoreductase, glutathione S-transferase A2, UDP glucuronosyl transferase, epoxide hydrolase, γ-glutamylcysteine synthetase, and also heme oxygenase-1 (Prestera *et al.*,1995; Venugopal and Jaiswal, 1996; Itoh *et al.*, 1997; Venugopal and Jaiswal 1998, Wild *et al.*, 1999, Kwong *et al.*, 1999, Alam *et al*, 1999). Nrf-1 and Nrf-2 are leucine zipper proteins which do not dimerize with each other, but need a heterologous leucine zipper binding partner for their activity (Chan *et al.*,1993: Venugopal and Jaiswal 1996/1998 and Moi *et al.*, 1994). Some of the interaction partners identified until now are Jun (c-Jun, Jun-B, Jun-D) as well as small Maf proteins (H-Maf, MafK, MafG) (Venugopal and Jaiswal, 1998; Marini *et al.*,1997; Johnson *et al.*, 1998)

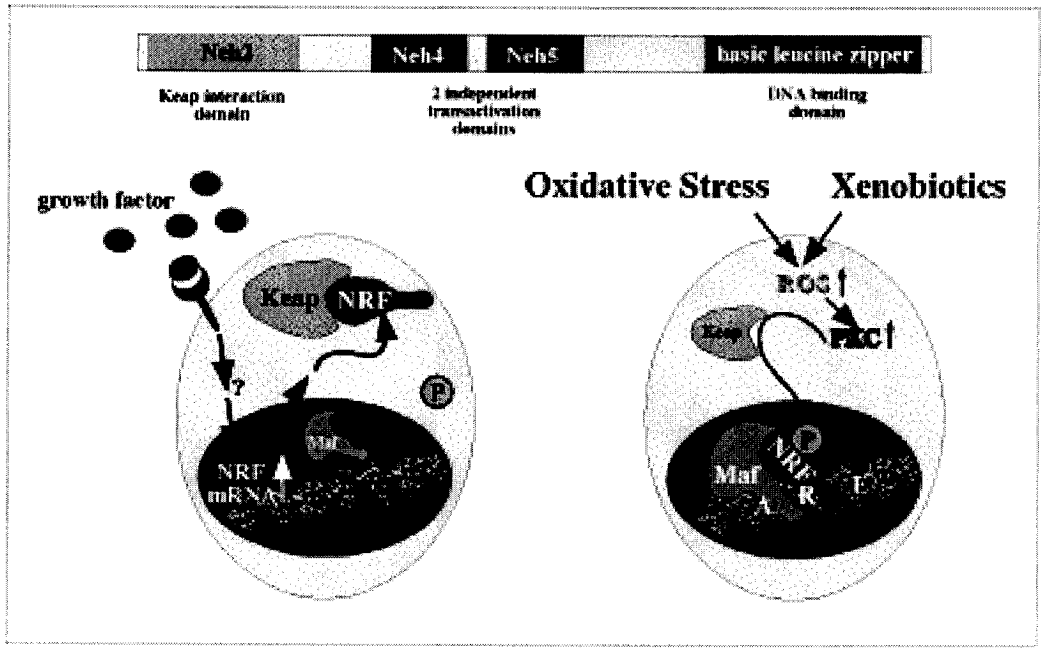


Fig.6 **Top:**The structure of the Nrf-Protein **Bottom:** Predicted induction of Nrf and its function in the cell

A multitude of xenobiotics is able to induce the binding of Nrf-1 or Nrf-2 to the ARE sequence, suggesting that signals from oxidative stress agents are transduced via these transcription factors. This effect might be mediated via a recently discovered protein, Keap-1, a homologue of the *Drosophila* actin binding protein Kelch (Itoh *et al.*, 1999). Keap-1 is ubiquitously expressed and was originally found in a yeast two-hybrid screen using Nrf-2 as a bait. Keap-1 was shown to repress Nrf-2 ARE binding by direct binding to this transcription factor. Electrophilic agents functionally liberate Nrf-2 transactivation activity from repression by Keap-1, allowing induction of the above mentioned detoxifying enzymes.

Recent knockout experiments have provided first insight into the *in vivo* function of these transcription factors. Nrf-2 knockout animals have no obvious phenotype under normal laboratory conditions (Chen *et al.*,1996), but treatment of these animals with butylated hydroxytoluene resulted in death from respiratory distress syndrome (Chan *et al.*, 1999). Nrf-1 gene disruption resulted in anemia and embryonic lethality (Chan *et al.*, 1998). Cultured fibroblasts from these animals showed lower levels of glutathione and enhanced sensitivity to the toxic effect of oxidant compounds (Kwong *et al.*; 1999).

Since these findings implicate a possible role of Nrf-1 and Nrf-2 in cytoprotection, I was interested in a possible role of these transcription factors in neuroprotection after hippocampal injury. For this purpose I analysed the temporal and spatial expression pattern of Nrf-1 and Nrf-2 mRNAs and proteins in our KA lesion model. Furthermore, we addressed the question whether Nrf-1 and Nrf-2 are functionally active in the lesioned hippocampus.

In addition to the intracellular mechanisms involved in neuroprotection, I was also interested in growth factors that mediate neuroprotection and/or glial scarring. In a first study we analysed the effects of the known neuroprotective growth factor bFGF in our lesion model, and we identified a novel player in CNS repair, namely activin A.

3.10. Basic fibroblast growth factor (bFGF)

Basic FGF, also named FGF-2, is a member of the FGF family which already includes 22 different members (rev. by Ornitz and Itoh, 2001). These factors are capable of modulating cellular functions in a wide range of cell types. Thus bFGF has been identified as a potent stimulator of angiogenesis and cutaneous wound repair (reviewed by Nagent and Rizzo, 2000). Most interestingly it is a potent neuroprotective growth factor for different types of neurons, including cultured rat hippocampal and cortical neurons (Morrison *et al.*, 1986; Walicke *et al.*, 1986) and neurons in rat hippocampal slices (Nakagami *et al.*, 1997). This neurotrophic effect was shown to be independent from bFGF's mitotic effect on glial cells (Walicke *et al.*, 1988). Since the effect could be blocked with staurosporine, it was suggested that the action is mediated by protein phosphorylation (Abe *et al.*, 1991).

In vivo, bFGF plays a important role in development and also in neuroprotection and neuronal reconstitution. bFGF is present in the telencephalon as early as E9.5 and high levels are found in the cerebral cortex throughout neurogenesis and into adulthood (Baird, 1994). bFGF knockout mice revealed abnormalities in the cytoarchitecture of the neocortex, combined with a significant reduction in neuronal density, most pronounced in layer V (Palmer *et al.*, 1999; Palmer *et al.*, 1999a). A neuroprotective function was predicted after it was shown that bFGF was up-regulated in mechanical brain lesions (e.g. Finklestein *et al.*; 1988; Logan *et al.*, 1991), ischemic insults (Takami *et al.*, 1992; Lippoldt *et al.*, 1993), and after convulsive seizures (e.g. Riva *et al.*,1992). A direct neuroprotective effect in a variety of different neuronal lesions was reported. If administered during or within hours of acute injury, systemic or intracerebroventricular (icv.) bFGF reduces infarct size after stroke (e.g. Koketsu *et al.*,1994), diminishes histopathologic damage associated with fluid percussion injury (Dietrich *et al.* 1996), affords neuroprotection against N-methyl-D-aspartate (NMDA) and kainate receptor-mediated excitotoxicity (e.g. Liu *et al.*, 1997), and prevents the death of axotomized CNS neurons (e.g. Otto *et al.*, 1989). On the other hand, treatment with neutralising anti-bFGF antibodies after a unilateral suction lesion of the motorcortex reduced the score of the treated animals in a manipulation test (Rowntree *et al.*, 1997).

Our studies revealed that bFGF is also neuroprotective in the KA lesion model and that this effect is mediated by activin A

3.11. The activin family

Activin A is a member of the TGFβ superfamily, consisting of more than 50 structurally related and evolutionary well conserved members. Members of this family are secreted proteins, which form dimers via disulfide bridges. Other members of the family are the TGFβs themselfes (TGFβ1, -β2, -β3), bone morphogenetic proteins (BMP), the *Drosophila* decapentaplegic (dpp) protein, mullerian inhibitory substance, growth differentiation factor, nodal-related proteins and others (reviewed in Mathews *et al.*, 1994; Phillips *et al.*, 2000)

Activins and their heteromeric antagonists, the inhibins, were first extracted from ovarian fluid. They are named after their ability to stimulate (activin) or inhibit (inhibin) the release of follicle stimulating hormone (FSH) from pituitary cells.

Activins are either homo- or heterodimers, composed of two activin β subunits (βA or βB) called activin A (βA,βA), B (βB,βB), or AB (βA,βB). Heterodimers between a β-chain and an α subunit are called inhibin A (α,βA) or B (α,βB), depending on which β chain is used (Fig.7)

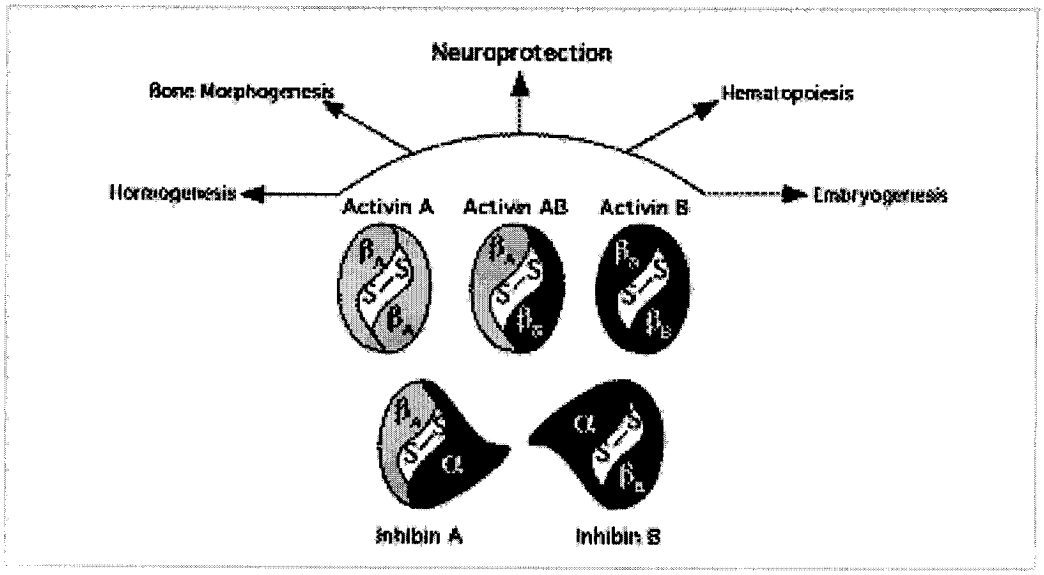


Fig.7 Different activin dimers and their targets during developmental and repair processes.

Over the last years a wide spectrum of novel activin functions have been described: Activin induces the differentiation of several cell types, e.g. osteoblasts (Ogawa *et al.*, 1992) and endocrine cells (Katayama *et al.*, 1990). A growth inhibitory effect was described for hepatocytes (Yasuda *et al.*, 1993), endothelial cells (McCarthy and Bicknell, 1993) and epithelial cells of the lung (Carcamo *et al*, 1994). Furthermore, activin is known to play important roles in development and cell survival, for example it induces mesoderm in *Xenopus laevis* (reviewed by Asashima *et al.*, 2000). Most interestingly, different roles of activin in the CNS have been reported: First evidence that activin promotes nerve cell survival came from Schubert *et al.* (1990). Fann and Patterson (1994) could show that activin is able to promote the synthesis of neuropeptides in cultured sympathetic neurons. Recent studies from our and other laboratories have shown a possible involvement of activin A in the early response to brain injury. Yvonne Tretter and co-workers from our group studied the temporal and spatial expression of activin/inhibin βA, βB and α subunits after unilateral kainic acid lesion of the hippocampal CA3 region and this studies revealed strikingly increased expression of βA mRNA in the ipsilateral hippocampus 6-24 h after injury. By contrast, the βB and α mRNAs were expressed at equally low levels in normal and injured hippocampi, suggesting that the β_A transcripts give rise to activin A, but not to activin AB or inhibin. *In situ* hybridization demonstrated the presence of βA mRNA in neurons near the site of lesion. Expression of all known types of activin receptors could be demonstrated in normal and injured hippocampi by RT-PCR. These findings suggested a role of activin A in brain injury (Tretter *et al.*, 1996). Up-regulation of activin β_A mRNA and activin A protein was described by several laboratories in different CNS injury models (reviewed in Hubner *et al.* 1999; Munz *et al.*, 1999). Most interestingly, activin A was found to promote survival of midbrain and hippocampal neurons *in vitro* (Krieglstein *et al.* 1995; Ivahory *et al.*, 1997), to reduce ischemic brain injury in infant rats (Wu *et al.*, 1999), and to protect striatal and midbrain neurons against neurotoxic damage (Kriegelstein *et al.*, 1995; Hughes *et al.*, 1999).

The biological activity of activins is controlled and mediated by several activin binding proteins. Their role in neuroprotection is largely unknown and will be a topic of further investigations by our group.

3.12. The activin binding proteins

A family of transmembrane serine/threonine kinases is acting as receptors for activins as well as for other members of the TGF β superfamily. Proteins of this receptor family are found in insects, mammals and reptiles (reviewed in Ebendal *et al.*, 1998, Mathews *et al.*, 1994; Phillips *et al.*, 2000; Piek *et al.*, 1999). The family falls into two distinct subclasses, the type I and type II receptors, distinguished by their kinase domains and other structural features. Functional receptor units are formed by a hetero-oligomeric complex of type I and II receptors.

In contrast to the type II receptors, the type I receptors have a conserved 30-amino acid glycine-serine rich sequence in the juxtamembrane region (GS domain) just upstream of the kinase domain. For the transmembrane signal transduction, a complex has to be formed between the ligand and the two receptor subtypes. The type I receptor has a weak affinity for the ligands. However, binding of activin to the type II receptor leads to recruitment of the type I receptor. The heteromeric complex binds the ligand with high affinity. Once the complex is formed, the serine/threonine residues of the GS domain of the type I receptor become transphosphorylated by the kinase of the type II receptor, leading to a signal transduction from the extracellular space through the membrane to members of the Smad family. Smads are intracellular proteins involved in signal transduction downstream of serine/threonine kinase receptors (Chen *et al.*, 1997; Ebendal *et al* 1998, Heldin *et al.*, 1997, Pieck *et al.*, 1999).

Besides these membrane-bound signal transducing receptors, there is a second type of activin binding protein named follistatin. Follistatin is a secreted glycoprotein that is expressed in many tissues and organs. The primary physiological function of follistatin is the binding and neutralisation of activin. Follistatin binds to activin with high affinity (K_d = 50-500 pM) and thereby prevents the binding to the activin receptors. It was shown that follistatin also binds some bone morphogenetic proteins (BMP2, -4, -7, -11) but with a significantly lower

affinity (Iemura *et al.*, 1998; Gamer *et al.*, 1999). Due to its activin-inhibitory function it can be used as a tool to block the biological function of activin *in vitro* and *in vivo* .

The use of this protein to block activin function in the injured hippocampus is described in the second paper of this thesis.

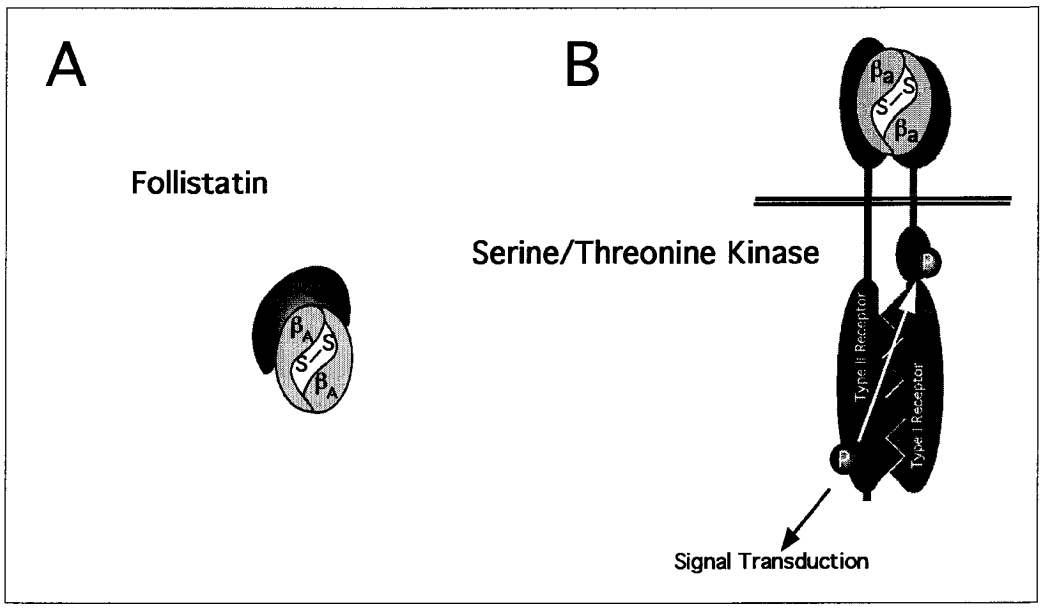


Abb.8 Structure of the different activin receptors

Finally, we were interested in the growth factors that are important for glial scarring. Since connective tissue growth factor is associated with fibrotic processes in other organs, this factor appeared to be a likely candidate

3.13. Connective tissue growth factor (CTGF)

CTGF was first described by Bradham *et al.*, (1991) as a factor secreted by human endothelial cells in culture which stimulates DNA synthesis and chemotaxis in fibroblasts. It is a member of the growing CCN (CTGF/cysteine-rich61/nephroblastoma over-expressed) gene family which also contains cyr61, nov, elm1, Cop1 and WISP-3 (reviewed by Bringstock, 1999).

Members of the CNN family are described as mosaic proteins that are composed of four structural modules. The first module is an IGF binding domain,

and module II shows a "von Willebrand" factor type C domain. This part is followed by a thrombospondin (module III) and a cysteine knot motive (module IV). Overall, 38 fully conserved cysteine residues are spread over the molecule, predicted to be involved in intramolecular disulfide bridging. Two very distinct functions were observed for CTGF, which are exerted by either the N-terminal or the C-terminal part of the protein. The C-terminal part with its IGF binding domain promotes mitosis (Brigstock *et al.*, 1997; Brigstock *et al.*, 1999; Surveyor *et al.*, 1998), whereas an extracellular matrix interacting activity is found in the N-terminal part (Kireeva *et al.*,1997; Rieser *et al.*, 2000).

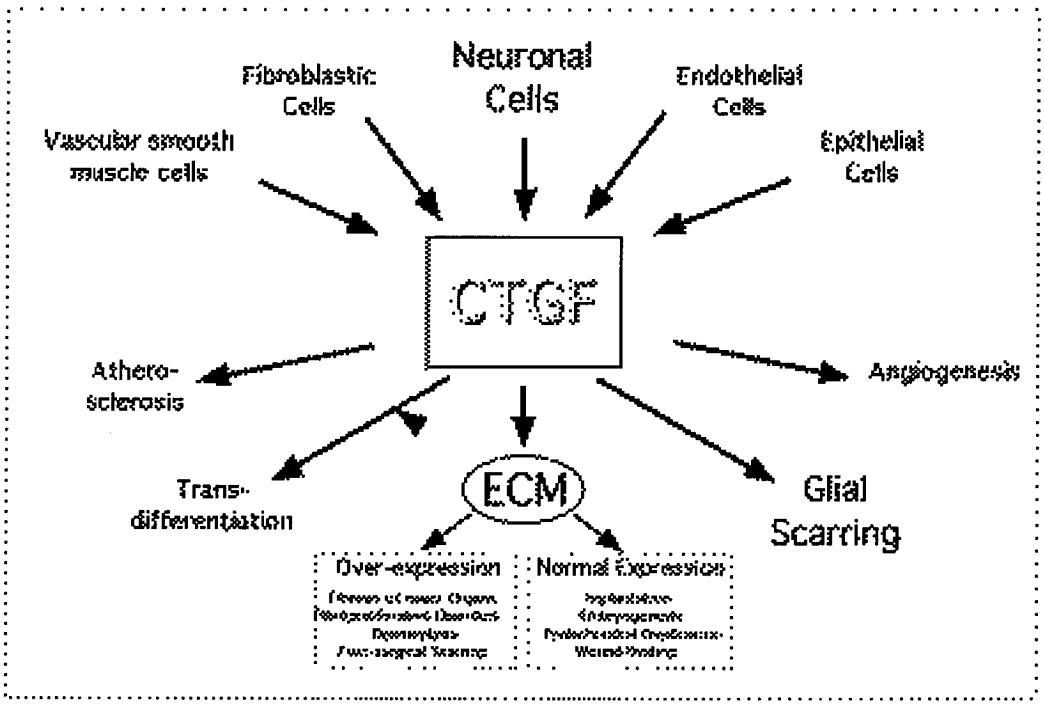


Fig.9 Effects of CTGF in different tissues as reviewed in Moussad and Brigstock (2000)

Very little is known about possible interaction partners or receptors of CTGF, and the intracellular signalling pathways have not been elucidated. The most potent stimulator of CTGF expression is TGF-β (Igarashi *et al.*, 1993), but glycocorticoids and ROS have recently also been shown to induce expression of the CTGF gene (Dammeier *et al.*, 1997; Park *et al.*, 2001)

A series of studies have provided evidence for an important role of CTGF in the development of fibrosis. Thus, CTGF is over-expressed in several fibrotic dis-

eases including lung fibrosis (Paradis *et al.*, 1999), kidney fibrosis (Goldschmeding *et al.*, 2000), liver fibrosis (Rieser *et al.*, 2000) and atherosclerosis (Oemar *et al.*, 1997). By contrast, little is known about a role of CTGF in the central nervous system. Due to its pro-fibrotic effect in other tissues, we were interested in a possible role of CTGF in glial scarring. Therefore, one goal of this thesis was to determine the temporal and spatial expression pattern of CTGF in normal and lesioned hippocampus and to compare this expression pattern with that of known extracellular matrix molecules associated with glial scars.

4. Materials and Methods

4.1. Chemicals

Most chemicals were purchased from Sigma Aldrich (Deisenhofen/Buchs) Merck (Darmstadt/Basel) or Roche (Mannheim/Basel). Chemicals from other suppliers are mentioned separately.

4.2. Growth factors

bFGF	Roche Biochemicals, Mannheim/Basel
Activin	kindly provided by CHIRON, Emeryville, CA, USA
Follistatin	kindly provided by the National Hormone and Pituitary Program, Torrance, CA, USA

4.3. Frequently used solutions

10x PBS

1.3 M	NaCl
70 mM	Na_2HPO_4
30 mM	Na_2HPO_4

TBE

10 mM	Tris
20 mM	Boric Acid
20 mM	EDTA pH 8.0

TAE

0.04 M	Tris-Acetate
0.1 mM	EDTA pH 8.0

20x SSC

3 M	NaCl
0.3 M	Na_3 Citrate

TE

10 mM	Tris/HCl pH 8.0
1 mM	EDTA

Phenol/Chloroform

250 g phenol were melted with 250 mg 8-hydroxychinoline at 65°C and frozen in 22.5 ml aliquots. Before use an aliquot was mixed with 22.5 ml chloroform and 5 ml 1M Tris/HCl pH 9.5 and stored at 4°C.

Phenol, water saturated

250 g phenol was melted with 250 mg 8-hydroxychinoline at 65°C and mixed with 100 ml DEPC-treated water, the mixture was stored in aliquots at –20°C, aliquots in use were stored at 4°C.

Ethidium bromide (EtBr)

Stock solution:

10 mg/ml EtBr in ddH2O

Solution for use: 0.5µg/ml

Methods

4.4. DNA-methods

4.4.1. Reverse transcription

The cDNA was initially generated by reverse transcription of a mixture of RNAs isolated from hippocampi, 6 hours and 2 days after lesion. We used an oligonucleotide primer consisting solely of 15 thymidines, that anneals specifically to the 3' poly-A tail of mRNAs, and M- MU LV-reverse-transcriptase to catalyse the reverse transcription.

Reaction mixture:

5 µl	*10x RT buffer*
1-2 µg	*RNA template*
5 µl	*10x dNTP (10 mM of each)*
5 µl	*10x oligo dT primer (500 µg/ml)*
50 U	*RNasin*
1 µl	*BSA (5000 µg/ml)*
4 µl	*M- Mu LV reverse transcriptase*

The reaction mixture was incubated for 1 hour at 37° C and then stored at -20° C.

4.4.2. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an easy way to amplify DNA fragments. In the presence of suitable oligonucleotide primers it is able to copy in a high number any fragment of a template DNA. The reactions were performed in a 200 µl sterile thin-walled PCR tube.

a) Amplification of cDNA

The oligonucleotide primers used were chosen by searching Genbank database sequences for regions of equal amounts of G/C and A/T, the melting temperature was calculated approximately by the following formula:
(A+T)2+(G+C)4=Tm

The reaction was performed in a 50 µl volume using a Peltier heated PCR machine from MWG Biotech.

Reaction mixture:

5.0 µl	*10x PCR buffer*
5.0 µl	*10x dNTP*
0.2 µl	*Taq polymerase (5 U/µl)*
1.0 µl	*cDNA of choice*
1.0 µl	*5' primer 100µM*
1.0 µl	*3' primer 100µM*
36.8 µl	*sterilized Millipore water*

PCR program:

Denaturation	*2 min*	*94°C*		
Denaturation	*30 sec*	*94°C*	}	
Annealing temperature	*30 sec*	*(Tm -7°C)*	}	*x35*
Polymerization	*1 min*	*72°C*	}	

The reaction mix was subsequently incubated at 72°C for 5 minutes to enable completion of the ends of the fragments and then cooled down to 4°C.

PCR product processing

b) Analysis of the amplified products

To separate the produced DNA fragments, agarose gel electrophoresis with 2% gels containing ethidium bromide (0.02 µg/l) in TBE buffer was performed. The samples were mixed with 1/5 volume of loading buffer and run at 120 V for 20-30 minutes. The bands were visualised on an UV-light box and photographed.

Loading buffer:

30% glycerol
0.025% bromophenol blue
0.025% xylene cyanol
dd H_2O

c) Purification of the PCR fragment

In the case of one clear single band (highly stringent conditions) the fragment was cleaned by using a PCR purification kit (Qiagen) as described in the manual. The fragment was dissolved in a high salt buffer, applied to a column containing a silica gel membrane, washed and eluted in the appropriate amount of low salt buffer or distilled water. Dependent on the concentration of the fragment the product was eluted in 20-50 µl ion exchanged, autoclaved water.

For a multiple band PCR (less stringent conditions) the complete amplification mix was run on a gel (as described above), visualised on an UV- screen and the desired band was excised. The fragment was eluted from the gel by using a QIAquick gel extraction kit (Qiagen) as described by the manufacturer. The gel slice was melted in the provided solution and proceeded as described above. Dependent on the concentration of the fragment, the product was eluted in 20-50µl distilled, autoclaved water.

4.4.3. Ligation

a) Blunt end ligation

Blunt end ligation is the easiest, but not very efficient way to insert a chosen fragment into a vector. Both fragment and vector have blunt ends (no overhanging oligonucleotides at their ends). The major problem with this method is the high likelihood that the vector will religate with itself.

b) Pre-treatment of the vector

300ng pBluescript SK II Vector DNA (Stratagene, La Jolla, USA) was linearized by digestion with the restriction enzyme EcoRV.

c) Pre-treatment of the insert

The insert was treated with 1µl of Klenow polymerase and incubated for 30 min at 37°C. Further, it was isolated and purified as described above.

d) Ligation

The ligation was performed in a 0.2 ml PCR tube. About 300 ng of vector was mixed with 1 µg of insert and was incubated at 16°C overnight.

Ligation mix:

11.5 µl	*insert dissolved in ion exchanged water*
1.0 µl	*purified, linearized vector*
1.0 µl	*DNA ligase*
1.5 µl	*10x ligation buffer*

e) Sticky end ligation

Using specially designed primers, two restriction enzyme sites were added to the ends of the amplified fragment. Both, the vector and amplified fragment were cut with the corresponding restriction enzymes. After this treatment there were different overhangs at each end of the digested vector which were compatible to the ends of the amplified fragments but not to each other. The advantage of this method is that there is barely any religation of the vector. Furthermore, sticky end ligation is more efficient than blunt end ligation.

4.4.4. Transformation of competent *Escherichia coli*

a) Preparation of competent *E. coli*

Competent *E. coli* are specially treated bacteria which are able to take up DNA through their membrane.

The bacteria were grown in 5ml Tetracycline treated LB medium overnight and used to inoculate 500 ml cultures. They were allowed to grow up to an OD of =0,4 at 600 nm, placed for 5 minutes on ice and afterwards spun for 10 minutes at 3500 rpm at 4°C. The pellet was re-suspended in 1 ml 0.1 M $MgCl_2$ and spun again for 10 minutes at 3500 rpm at 4°C.

To this mixture 3 ml cold, sterile, 0.1 M $CaCl_2$ was added and it was put on ice for 3-4 hours. Then 1.6 ml 0.1 M $CaCl_2$ and 87% glycerol was mixed in, the bacteria were aliquoted in 200 µl in 1.5 ml Eppendorf tubes and snap frozen in liquid nitrogen. The aliquots were stored at -80°C.

b) Transformation

The ligation was performed as described above and 10-20 µl of the mix was added to a 100 µl aliquot of competent *E. coli* on ice and incubated for 30 minutes. After the incubation, a heat shock for 2 min at 42°C was performed and the cells were put back on ice for 10 min. 800 µl of LB medium (without any antibiotics) was pipetted into the transformation mix and incubated for one hour on a shaker (225 rpm) at 37°C. The 200 µl of the bacterial culture were plated out on LB agar plates containing 50 µg/ml ampicillin. The plates were incubated at 37°C overnight.

When the pBluescript plasmid was used as the vector the plates were pretreated with 40 µl of 0.1 M IPTG and 100 µl of X-gal (2% stock in dimethyl formamide). This procedure allowed us to apply the blue/white screening method. All colonies containing a plasmid with an insert stayed white while every other colony turned blue.

c) Isolation of plasmid DNA from *E.coli*

Agar plates containing the overnight colonies were screened via blue/white screening, whereby colonies containing an insert should lack α-complementation. Therefore, the X-gal staining is not working on positive clones, so that they can be easily distinguished from the blue stained negative ones. Single colonies were picked for starting small scale (3 ml LB medium, 50 µg/ml ampicillin) or large scale (150 ml LB medium, 50 µg/ml ampicillin) cultures. They were incubated on a shaker at 225 rpm at 37°C overnight.

4.4.5. Preparation of plasmid DNA

a) Isolation of plasmid DNA

For small scale plasmid DNA preparation two different methods were used. The cleaner and more efficient way was using a Qiagen plasmid "Mini" kit as described by the manufacturer. The protocol is based on a modified alkaline lysis of bacteria, followed by adsorption of plasmid DNA onto a silica column at high salt concentrations. The DNA was washed with an ethanol based buffer and eluted in a low salt buffer or distilled water.

The second method was also based on an alkaline lysis of the bacteria, followed by a phenol/ chloroform extraction and ethanol precipitation of the DNA. The bacteria from 1.5 ml culture medium were precipitated (5 min 5000 rpm), the supernatant was discarded and the pellet re-suspended in 100 µl of solution I by vortexing. 200 µl of solution II were added and the tube was inverted several times. The mixture was incubated for exact 3 min at room temperature, afterwards 150 µl of solution III were added, the tube inverted and incubated on ice for 10 min to 1hour. After a last centrifugation of 5 min at 13000 rpm the pellet was washed with 95% ethanol and air-dried. For use, it was diluted in 70-200 µl ddH_2O or TE buffer containing 20µg/ml RNase A.

For large-scale plasmid DNA preparations a Qiagen plasmid "Midi" kit was used as described by the manufacturer. This method is also based on an alkaline lysis of bacteria. The mixture is applied to a Qiagen anion exchange resin to bind the DNA under low salt and pH conditions. RNA, proteins and low molecular weight impurities were removed by medium salt wash. Plasmid DNA was eluted in a high salt buffer and then concentrated and desalted by isopropanol precipitation.

Solution I:

5 mM	*Sucrose*
10 mM	*EDTA*
25 mM	*Tris/HCl pH 8.0*

Solution II:

0.2 N	*NaOH*
1%	*SDS*

Solution III:

3 M NaAc *pH 4.8*

b) Analysis of the eluted DNA by agarose gel electrophoresis and UV photometry

The concentration of the eluted DNA was determined with a "BioPhotometer" (Eppendorf).

To analyse the quality of the plasmid, it was digested with an appropriate restriction enzyme and loaded on an agarose gel. The gel was stained with ethidium bromide and visualised on an UV screen.

4.4.6. Sequencing of a cloned DNA fragment

a) Principle

The principle is based on a PCR reaction using a dNTP mix containing also at low concentrations fluorescence labelled ddNTP. Each of the four ddNTPs is marked with a different coloured fluorophor. If the polymerase is coincidentally using one of the labelled ddNTPs, the extension reaction is stopped as the polymerisation of the nascent DNA strand cannot proceed further. As this happens randomly, DNA fragments of all possible different sizes are produced, each having a labelled end corresponding to the last base synthesised. The PCR mix is then loaded to the sequencer and analysed by capillary electrophoresis.

b) Sequencing PCR

The DNA used for sequencing must be purified on a column (in our case the plasmid was isolated with the Qiagen "Mini" preparation kit).

PCR- mix:

3 µl	*5x sequencing buffer*
0.5- 1µl	*3.2 pmol/µl primer*
100- 500 ng	*ds DNA*
2 µl	*Terminal reaction mix*

add sterile ion exchanged water up to 20 µl

The sample was mixed by gently flicking, the mixture was spun down, and the PCR reaction was performed in an Eppendorf thermocycler (Mastercycler gradient).

PCR program:

10 sec	*96°C*
5 sec	*50°C*
4 min	*60°C*

25 cycles of this program were performed.

c) Precipitation of the PCR products

2 µl of 3 M sodium acetate (pH 5.2) and 50 µl of 95% ethanol was added to the reaction, mixed thoroughly and spun for 30 min at room temperature at 13000 rpm. The supernatant was discarded, the pellet was rinsed with 250 µl of 70% ethanol and spun for 5 min at 13000 rpm. The invisible pellet was dried completely in a speed vac (avoiding exposure to direct light) and re-suspended by vortexing in 25 µl template suppression reagent.

The sample was denatured by boiling for 2 min at 95°C, put immediately on ice and loaded onto the sequencer.

4.5. RNA-methods

4.5.1. RNase free vessels and solutions

As RNA is very susceptible to degradation by RNases, sterile plastic or baked glass vessels (180°C, > 8 hours) were used for all experiments. The solutions were pre-treated with diethylpyrocarbonate (DEPC) to destroy RNases. They were mixed with 0.05% DEPC and incubated for at least 16 hours at room temperature. Finally, they were autoclaved to destroy DEPC. Gloves were always worn while performing these experiments.

4.5.2. RNA Isolation

Hippocampi were dissected from the mouse brain and directly frozen on dry ice on a piece of aluminium foil and stored at –70 °C .

For RNA isolation, 4 ml of cold GSCN solution was added to 3-6 hippocampi and they were immediately homogenised with an "Ultra Turrax" homogeniser. Homogenised material was aliquoted to 400 µl aliquots. 400 µl of 2M sodium acetate (pH 4) was added to each tube and mixed by inversion. 400 µl of water-saturated phenol was added and the mixture was immediately vortexed. Afterwards, 400 µl of chloroform was added, tubes were shaken to mix and left on ice for 15 min. The mixture was centrifuged for 15 min at 3000 rpm at 4°C. The aqueous phase including some of the interphase was transferred into a fresh microcentrifuge tube and spun for 10 min at 13000 rpm at 4°C. The supernatant was transferred again to a fresh microcentrifuge tube, this time avoiding taking up the interphase. 2.5 volumes of 100% ethanol was added and the solution was incubated at -20°C for one hour. The samples were centrifuged again for 15 min at 4000 rpm at 4°C, the supernatant was discarded and the pellet reunified by resuspending in a total volume of 200 µl DEPC water. The suspension was extracted with 400 µl phenol/chloroform by vortexing for 30 sec and centrifuged for 5 min at 4°C and 13000rpm. The upper phase was taken again, 400 µl chloroform was added, vortexed for 30 sec and centrifuged for 5 min at 13000 rpm at 4°C. The upper phase was taken, 40 µl 3 M sodium acetate pH 5 and 1 ml 100% ethanol were added and the mixture was left for precipitation for one hour at -20°C. After spinning for 15 min at 13000 rpm at 4°C the resulting pellet was washed with 70% ethanol. The ethanol was aspirated and the pellet re-suspended in 100-200 µl DEPC water and heated for 5 min at 65°C. The concentration of RNA was determined by measuring the absorption at 260 nm in a "Biophotometer" (Eppendorf) and the quality was determined by running 1 µg of each sample on a 1.2% agarose/TBE gel for 10 min at 90 V. The ethidium bromide within the gel enabled us to see distinct bands of the 5, 18 and 28 S rRNA under an UV illuminator, a clear sign that the RNA was not degraded. The RNA was aliquoted into 10 µg aliquots in safety-lock microcentrifuge tubes and

stored in 0.1% volume of 3 M sodium acetate pH 5 and 2.6 volumes 100% ethanol at -20°C.

4.5.3. RNase protection assay

This method allows the detection of specific mRNAs and is based on hybridisation with a suitable complementary probe sequence. As it is highly sensitive and specific, it is a powerful tool to detect as little as 0.1 pg of RNA out of total cellular RNA from any tissue.

a) Preparation of DNA templates for run off transcription

The template was digested with the best suited restriction enzyme, cutting it such that no 3'prime overhang is formed, so that the antisense RNA probe could be generated by run off transcription. The digested DNA was purified by phenol/chloroform extraction and 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH5.2) were added. The DNA was precipitated at -20°C for 30 min and centrifuged for 15 min at 13000 rpm and 4°C. The resulting pellet was rinsed with 70% ethanol, air-dried for 5 min, and re-suspended in DEPC-dH_2O at a concentration of 1 µg/µl.

b) Synthesis of $\alpha[^{32}P]$ labelled riboprobe

A reaction mix was prepared as follows:

4.0 µl	*DEPC dH_2O*
1.0 µl	*10x transcription buffer*
1.0 µl	*10x nucleotide mix (5 mM ATP,CTP, GTP)*
0.5 µl	*RNasin (20 U)*
0.5 µl	*RNA polymerase (for antisense RNA, 10 U)*
2.0 µl	*$\alpha\ ^{32}P$ - UTP (20mCi/ml)*
1.0 µl	*linearised template (1 µg/µl- first denatured for 5 min at 65°C)*

The transcription reaction was mixed and incubated for 60 min at 37°C

c) Purification and isolation of the riboprobe by PAGE

The riboprobe was mixed with 10 µl FLB 100 and loaded straight onto a denaturing gel containing 5% acrylamide / 7 M urea with 1x TBE running buffer. The gel was run for 1 hour at 250 V.

The gel running device was disassembled and the gel left on one glass plate covered with "Saranwrap" to avoid any contamination. The orientation was labelled with a permanent marker pen and the gel exposed to a X-ray film for one minute. After developing the film, the band corresponding to the full-length probe was excised using a scalpel. The X-ray film was superimposed on the gel according to the marker pen lines, and the piece of gel containing the full-length probe was excised.

The gel slice was cut into 4 small pieces and transferred to a 1.5 ml safe-lock tube. 150 µl elution buffer (0.1x TBE/ 0.2% SDS) were added to the gel fragment and the probe was eluted by vortexing for 2 hours. The elution mixture was spun at 13000 rpm for 2 min at 4°C, the supernatant collected and transferred to a fresh tube. 1 µl of it was pipetted up and the tip ejected into a scintillation vial for analysis of the probe's activity in a scintillation counter (counting Cerenkow radiation without scintillation cocktail). The minimum activity of the probe needed is 10^4 cpm/µl.

FLB 100:
100% deionised formamide
0.025% (w/v) bromophenol blue
0.025% (w/v) xylene xyanol

d) Hybridisation of the probe with the RNA sample

To a 20 µg RNA sample 10^5 cpm probe (max 10 µl, because of SDS concentration) was added. The RNA sample was stored in 0.3 M volume of sodium acetate (5.2 pH) and 2.5 volumes of ethanol. The hybridisation mix was incubated at -80°C for 30 minutes and centrifuged for 15 min at 13000 rpm at 4°C. After checking the supernatant for residual radioactivity, it was discarded. The RNA pellet was air-dried and afterwards re-suspended in 30 µl of FAB by pipetting

30-50 times. The dissolved RNA was denatured for 10 min at 85°C and hybridised overnight in a 42°C water bath.

FAB

80%	*deionised formamide*
400 mM	*NaCl*
40 mM	*PIPES pH 6.4*
1 mM	*EDTA*

e) RNase digest of hybridised samples

The hybridised samples were placed on ice, 300 µl of RNase cocktail was added to each.

RNase cocktail:

297 µl	*RNase buffer*
1 µl	*RNase A (10 µg/µl)*
2 µl	*RNase T1 (100 U/µl)*

Samples were vortexed and incubated for 60min at 30°C.

RNase buffer:

300 mM	*NaAc pH 7.0*
10 mM	*Tris/HCl pH 7.5*
5 mM	*EDTA*

6.6 µl 10% SDS and 4.4 µl proteinase K (10 µg/µl) were added. The mixture was briefly vortexed, incubated for 15 min at 42°C and placed on ice. Proteins were precipitated by addition of 350 µl phenol/chloroform mixture (1:1, Tris buffered, pH 8.5), vortexed and spun for 3 min at 13000 rpm at 4°C. The upper phase was collected in a fresh tube. 1.5 µl tRNA (10 µg/µl) and 880 µl ethanol were added. The sample was precipitated for 30 min at -80°C and afterwards centrifuged for 15 min at 13000 rpm at 4°C.

f) Electrophoresis of samples

The supernatant was discarded and the pellet was suspended in 30 µl of FLB 80 buffer by pipetting up and down. The denatured sample (5 min at 95°C) was loaded onto a denaturing gel (5% acrylamide/ 7 M urea) with 1x TBE as running buffer, and run at 250 V until the bromophenol blue had left the gel. After removing the plates from the gel, it was transferred to Whatman paper, covered with "Saran" wrap and dried for 2 hours at 80°C. The dried gel was exposed to X-ray film overnight at -80°C. Alternatively, it was exposed to a phosphor-imager screen for two hours up to 3 days.

FLB 80

80%	*deionized formamide*
1x	*TBE*
1 mM	*EDTA*
0.05%	*bromophenol blue*
0.05%	*xylenecyanol*

4.1.4. *In situ* hybridisation

With *in situ* hybridisation the expression of a given mRNA could be detected within a specific tissue.

In this thesis two different methods of radioactive *in situ* hybridisation were used. In one case a radioactive, end labelled, oligonucleotide was used as a probe (activin paper); in the other case the probe was a radioactive antisense RNA probe similar to the probe used for the RNase protection assay but labelled with ^{35}S UTP instead of ^{32}P UTP (Nrf Paper).

Method 1

a) Probing with a radioactively labelled oligonucleotide
(Wisden *et al.*, 1991).

This method is in relation to the given alternative much faster and less work intensive, since only a β-max X-ray film (Kodak, Rochester NY, USA) was used for detection instead of film emulsion. With this protocol only the brain area in which expression occurred could be identified, whereas resolution at the cellular level was not possible.

A specific 40-50 mer oligodeoxyribonucleotide was used, which was labelled with [α^{35}S]-dATP using terminal deoxynucleotidyl-transferase (TdT).

b) Preparation of the specimens

The preparation of the specimens was performed as described for immunohistochemistry. Briefly, sagittal brain sections (10 μm thick) were produced with a cryostat, dried for 30 min at RT and stored at –70 °C. On the day of the experiment the sections were thawed, dried for 30 min at 56°C, and fixed in ice-cold paraformaldehyde (PFA) solution for 5 min.

PFA:

10 g of PFA were suspended in 125 ml of water and the milky suspension was heated to 60°C under permanent stirring. After a while the paraformaldehyde should decompose to formaldehyde and the solution should become clear. If not, 2-3 drops of 2 M NaOH were added to neutralise the solution. The clear solution was filtered through a folded filter on ice, and 125 ml of 2x PBS was added. The solution was prepared on the day of the experiment and was not stored for more than 10 days to avoid the formation of oxidation products which could destroy the nucleic acids.

c) Terminal transferase reaction

5x reaction buffer:

1M	*Potassium cacodylate*
125 mM	*Tris*
1.25 mg/ml	*pig serum albumin (pH6.6 at 25°C)*

TNES:

10 mM	Tris
1mM	EDTA

Antisense oligonucleotides were used which were complementary to a certain sequence of the RNA of interest. Oligonucleotides were produced by a company or by the facility of the Max-Planck Institute, and dissolved to a concentration of 10 nmol/µl in sterile water.The following oligonucleotide was used for the detection of activin β_A mRNA:

Activin βA:
5´ AAT CCA GCA ACT TGC CAA CAG AAA TCC TCT CAG CCA AAG CAA GGG CAT 3´

The corresponding sense oligonucleotide was used as a control for unspecific hybridisation.

d) Radiolabelling

The oligonucleotides were diluted to a concentration of 0.3 pmol/µl, at this dilution a 45mer would have a concentration of 5 ng/µl, calculated for an average molecular weight of each nucleotide of 330.
The radiolabeling was performed by adding [γ^{35}S]-dATP to the 3´OH end of the oligonucleotide using terminal deoxynucleotidyl transferase (TdT). This enzyme catalyses the synthesis of polydeoxyribonucleotides out of deoxyribonukleotide triphosphates under release of pyrophosphates.
In a standard reaction 0.3 pmol oligonucleotide were labelled with 10 pmol of radioactive dATP. The reaction should add 10-20 dATP to the oligonucleotide. If

the tails are longer, the unspecific background rises, whereas shorter tails prolong the exposure time.
The following components were added to the reaction in the given order:

11.3 µl	*DEPC-treated water*
1 µl	*oligonucleotide*
1.2 µl	*25 mM $CoCl_2$*
4 µl	*5x reaction buffer*
1.5 µl	*radioactive dATP (1300 Ci/mM)*
1 µl	*TdT (25 U/µl)*

Cacodylate which is present in the reaction buffer and $CoCl_2$ are cofactors for the TdT enzyme

Reaction mixes were incubated for 5-10 minutes at 37°C. The reaction was stopped by addition of 30 µl TNES and the oligonucleotide was separated from unincorporated dATP by purification on a single use size exclusion column (BioSpin 6, Biorad, Glattbrugg, Switzerland).
2 µl of the eluate were counted in the szintillation counter. If the activity was between 50 000 and 200 000 cpm the probe was used for the experiment. DTT was subsequently added to a final concentration of 100 mM to avoid oxidation, and the probe was then stored at -20°C.

e) Hybridisation

Hybridisation buffer:

20 ml	*100% deionised formamide*
10 ml	*20x SSC*
2.5 ml	*0.5 M sodium phosphate pH 7*
0.5 ml	*0.1 M sodium pyrophosphate*
5 ml	*50x Denhard's solution*
2.5 ml	*sonicated salmon sperm DNA*
1 ml	*polyadenylate*
5 g	*dextran sulfate*

The dextran sulfate was dissolved by shaking for 2 h in a 40°C waterbath. Afterwards, the mixture was filled up to 50 ml and stored at 4°C for up to one year.

Frozen sections were dried for 1 h at room temperature. The radioactive oligonucleotide was mixed with the hybridisation buffer at a 1:100 ratio. Every slide was overlaid with 100 µl of the diluted probe and covered with a "Parafilm" strip. The specimens were incubated in a wet chamber at 42°C overnight. To humidify the chambers, the bottom was coverd with a "Whatman" paper soaked with box buffer (40% formamide, 4x SSC).

On the following day the Parafilm was carefully removed with a pair of tweezers and the slides were transferred to 1xSSC. Slides were washed for 30 min in 1xSSC at 55°C, afterwards they were transferred to 1xSSC at room temperature and then to 0.1xSSC to avoid precipitation of salt. In a final step the slides were dehydrated in 70% and 95% ethanol and dried for at least 1h at room temperature. To visualize the signal, slides were exposed to a sensitive X-ray film (Hyperfilm βmax, Kodak) for 2 weeks.

Method 2

a) *In situ* hybridisation with an antisense RNA probe:

The tissue mRNAs hybridise with an antisense RNA probe which is radioactively labelled. The probes should be between 120 and 400 bp in length, usually the templates were identical to the ones used for RNase protection assay.

b) Synthesis of a [^{35}S] antisense RNA probe

Reaction mix:

100 µCi	*[α^{35}S]-rUTP*
1 µl	*transcription buffer*
1 µl	*dNTPs*
0.5 µl	*RNasin (20U)*
1 µl	*RNA polymerases T3 or T7 (20 U)*

linearised template (first denatured for 5 min at 65°C)
DEPC-H_2O was added to a total volume of 10 µl

The mix was incubated for 90 min at 37°C. After the transcription reaction 1 µl DNase Q1 (10 U) was added to digest the plasmid. After a 15 min incubation at 37°C 100 µl phenol/chloroform was added to denature protein components. At the same time 90 µl DEPC- H_2O was added to increase the extraction volume, and 1 µl tRNA (50 µg/µl) to help precipitation. The tubes were vortexed and centrifuged 4 min at room temperature. 1 µl of the upper phase was mixed with 10 µl FLB80 (see RPA protocol) for the control gel (probe 1). The rest of the upper phase was mixed with 10µl NaAc (3 M, pH 5.2) and 250 µl 100% ethanol, and the RNA was precipitated at –20°C overnight. The quality of the transcript was tested on an acrylamide/urea gel. The sample was denatured for 5 min at 95°C and loaded on the gel. It was run for 30 min at 200 V, dried for 30 min at 80°C on Whatman paper (3mm) and exposed to a phosphor imager screen for 30 min or to a X-ray film overnight.

c) Preparation of the sections

Sections were thawed and dried for 30 min at 56°C. They were fixed for 10 min in 4% PFA in PBS at 4°C and washed twice for 5 min in PBS. They were incubated for 12 min in pronase buffer to make the tissue accessible for the probe. After 5 min in PBS the sections were fixed again for 10 min in 4% PFA in PBS at 4°C and washed for 3 min in PBS.

They were incubated twice in 2.5% acetanhydride/0.1 M triethanolamine (375 µl acetanhydride in 150 ml 0.1 M triethanolamine) to acetylate the OH groups. This acetylation should reduce unspecific binding of the probe.

Sections were then dehydrated by passing through a series of solutions with increasing ethanol concentrations:

5 min	*PBS*
5 min	*0.9% NaCl*
10 sec	*30% EtOH/0.9% NaCl*
10 sec	*60% EtOH/0.9% NaCl*
10 sec	*85% EtOH/0.9% NaCl*
10 sec	*95% EtOH/0.9% NaCl*
10 sec	*100% EtOH*
10 sec	*100% EtOH*
2 min	*100% EtOH*

The sections were air dried for 2 hours.

d) Hybridisation of the cellular RNA with the probe

The RNA probe was centrifuged for 10 min at 13000 pm and 4°C, washed once with 70% ethanol and re-suspended in 50 µl 10mM DTT. The activity of 1 µl probe was measured in a scintillation counter. The probe was diluted to 10000-20000 cpm/µl with hybridisation mix. DTT was added to a final concentration of 10 mM and the probe was denatured 3 min at 80°C. The tube was then transferred to a 65°C heating block for 5 minutes.

The treated sections were overlaid with 900.000 cpm (90 µl of a 10000 cpm/µl hybridisation mix), covered with parafilm and incubated in a humid chamber covered with Whatman paper soaked in box buffer for at least 16 h at 52°C.

Hybridisation Mix:

50% (v/v)	*Formamide*
0.3 M	*NaCl*
20 mM	*Tris/HCl pH 8*
5 mM	*EDTA*
1x	*Denhardt's solution*
10 mM	*DTT*
10% (w/v)	*dextran sulfate (add last!!)*
ad	*H_2O DEPC*
Box buffer:	
4x	*SSC (DEPC)*
50% (v/v)	*formamide*

e) RNase treatment and washing steps

On the following day the sections were washed in wash solution 1 at room temperature. Unspecifically bound probe was removed by washing for 25 min at 55°C in wash solution 2. After three10 min washes at 37°C in RNase buffer the single-stranded RNA was digested by incubating the slides for 30min at 37°C in RNase buffer with RNase A (200ml RNase buffer + 4 mg RNase or 400 µl 10 mg/ml solution). They were washed for 15 min at 37°C in RNase buffer and for 25 min at 55°C in wash solution 2. To avoid salt precipitation they were washed for 15 min at 37°C in 2xSSC and for 15 min at 30°C in 0.1xSSC before dehydration.

Solution 1:

2x	*SSC*
10 mM	*β-mercaptoethanol*
1 mM	*EDTA*

Solution 2:

0.1x	*SSC*
10 mM	*β-mercaptoethanol*
1 mM	*EDTA*

1 min	*30% ethanol/3M ammonium acetate*
	60% ethanol/3M ammonium acetate
	80% ethanol/3M ammonium acetate
	95% ethanol/3M ammonium acetate
	100% ethanol/3M ammonium acetate
	100% ethanol/3M ammonium acetate

After 2 hours at 30°C the slides were dipped in warm (44°C) film emulsion (KODAK) in the dark. They were stored at 4°C in a box which contained a desiccating agent (silicagel). A few slides were developed after 2-3 weeks to determine the intensity of the signal. The rest of the slides was developed after 2-6 weeks.

f) Development of the sections

The sections were warmed for 1 hour to room temperature. The solutions were pre-warmed to 15°C and the developer diluted 1:1 with ddH_2O.

All the following steps were performed in the dark:

3 min	*developer*
20 sec	*ddH_2O*
3 min	*fixative*
20 sec	*ddH_2O*
5 min	*ddH_2O*

The sections were stained using the Nissl staining protocol.

4.2. Protein methods

4.2.1. Total cell lysates

Hippocampi were collected on dry ice and stored at –70°C until used for preparation of lysates. They were then homogenised in urea buffer at room temperature with an Ultra Turrax (IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged (15 min,13 000 rpm/RT) and the protein concentration of the supernatant was determined using the Bradford assay.

Urea buffer:

9.5 M	*Urea*
10 mM	*Tris/HCl pH8.0*
2 mM	*EDTA*
2 mM	*PMSF (add fresh before use)*

4.2.2. Nuclear lysates

a) Nuclear extracts obtained by trypsin digest

Mice were sacrificed at different time points after KA injection. Control hippocampi from non-injected mice and ipsilateral and contralateral hippocampi from KA-injected mice were removed, transferred into ice-cold serum-free Dulbecco's modified Eagle's medium (DMEM), washed twice in ice-cold PBS, and digested with PBS/0.1% trypsin for 30 min at 37°C. After two washing steps in DMEM/10% FCS, the cells were isolated by pipetting the suspension through five silanised Pasteur pipettes with decreasing diameters of the opening. Cells were precipitated (5 min 2000 rpm, 4°C) and re-suspended in 400 µl buffer A by gently pipetting up and down 5 times. Cells were then incubated for 10 minutes on ice for swelling. Afterwards they were vortexed for 10 sec and the nuclei were precipitated (15 sec 13000 rpm/ 4°C). Nuclei were re-suspended in 50 µl (for 2 hippocampi) ice-cold buffer B and incubated for 20 min on ice. Chromatin and cell debris were collected by centrifugation (2 min, 13000 rpm/ 4°C) and the supernatant was collected as the nuclear protein fraction.

Buffer A:

10 mM	*HEPES/KOH pH7.8*
1.5 mM	*$MgCl_2$*
10 mM	*KCl*
1 mM	*DTT*
1 mM	*PMSF*

Buffer B:

20 mM	*HEPES/KOH pH7.8*
25%	*Glycerol*
420 mM	*NaCl*
1.5 mM	*$MgCl_2$*
0.2 mM	*EDTA*
1 mM	*DTT*
1 mM	*PMSF*

b) Nuclear extracts obtained by homogenisation

Mice were sacrificed at different time points after KA injection. Control hippocampi from non-injected mice and ipsilateral and contralateral hippocampi from KA-injected mice were removed and transferred to 8 ml ice-cold homogenisation buffer. The complete hippocampi were homogenized at 4°C with a motor-driven Teflon glass homogeniser (10 strokes). The homogenised tissue was overlayed on a cushion of homogenisation buffer (material identical with the buffer used for homogenisation) in an ultracentrifuge tube. Nuclei were pelleted by ultracentrifugation (30 min, 80000xg/ 0°C). Nuclear pellets were resuspended in 3.5 ml nuclear lysis buffer and lysed with a all glass douncer. 1/10 volume of 4M ammonium sulfate, titrated to pH 7.6 with NaOH, was dropwise added and the mixture was shaken carefully for 30 min. The highly viscous lysate was centrifuged for 60 min in an ultracentrifuge (80 000xg/ 0°C) to get rid of the chromatin. Afterwards fine pulverised ammonium sulfate (0.3 g/ml) was added over a period of 15 min to precipitate all proteins. Proteins were collected by a final ultracentrifugation step (30 min, 80000 xg/0°C).

Homogenization Buffer:

10 mM	*HEPES/KOH pH 7.6*
25 mM	*KCl*
0.15 mM	*Spermidine*
1 mM	*EDTA*
1 mM	*EGTA*
2 M	*Sucrose*
10%	*Glycerol*

Nuclear Lysis Buffer

10 mM	*HEPES/KOH pH 7.6*
100 mM	*KCl*
3 mM	*$MgCl_2$*
0.1 mM	*EDTA*
1 mM	*DTT (add fresh)*
0.1 mM	*PMSF (add fresh)*
10%	*Glycerol*
0.1 mM	*NaF (add fresh)*
0.1 mM	*Na_3VO_4 (add fresh)*
0.5%	*SDS*

4.2.3. SDS-PAGE

Aliquots of 5-10 µg protein were boiled for 5 min in Laemmli sample buffer containing β-mercaptoethanol, and loaded to 10-15% SDS minigels (39.2%/0.8% acrylamide/bisacrylamide). Gels were run until the bromophenol blue reached the end of the gel. The separation was normally performed at 50 mA.

As a size control a pre-stained protein marker was loaded in one of the lanes.

4.2.4. Western blot

a) Blotting

The gel was soaked in blot buffer for one minute and then placed on a buffer-soaked sponge that was covered by two Whatman papers (3 mm thick). It was then covered with a nitrocellulose membrane followed by two layers of Whatman paper and a second sponge. The stack was placed into a plastic holder which was inserted into a blotting chamber in the right polarity. The proteins were transferred from the gel to the membrane at 200 mA for 2 hours. The stack was disassembled and the proteins that had been transferred to the membrane were stained with Ponceau Red for 10 minutes.

Blot buffer:

25 mM	*Tris*
192 mM	*Glycine*
20%	*Methanol*

b) Blocking of the membrane and binding of the antibody

The membrane was blocked with PVP blocking buffer for 10 min to avoid unspecific binding of the antibody and afterwards incubated overnight at 4°C with the first antibody (1:100-1:1000 diluted in PVP blocking buffer). The excess of unbound antibody was washed away by three 10 min washes in washing buffer. Secondary antibody was added in PVP blocking buffer (diluted 1:7500). After three additional washing steps with washing buffer the antibody-binding proteins were visualised using a peroxidase staining kit or the ECL detection system (Amersham Pharmacia Biotech, Dübendorf CH).

Blocking buffer:

2.2%	*Polyvinylpyrrolidone (PVP)*
150 mM	*NaCl*
50 mM	*Tris/HCl pH 7.0*

Wash buffer:

0.18%	*PVP*
0.1%	*Tween 20*
150 mM	*NaCl*
50 mM	*Tris/HCl pH 7.0*

PVP stock solution:

39 g 10000 Mol Wt PVP
39 g 40000 Mol Wt PVP
10 g 360000 Mol Wt PVP
to produce a 8.8% w/v solution

4.2.5. Coomassie staining

Gels were stained in a Coomassie solution for approximately one hour, afterwards excess of stain was washed away overnight with de-staining solution. To preserve the gel, it was dried in a plastic frame between two celluloid film sheets overnight.

4.2.6. EMSA

a) Preparation of the double-strand oligonucleotide

Equal amounts of synthesised single-stranded oligonucleotide, e.g. 5 or 10 µg, were mixed in 20 µl water. The mixture was boiled for 10 minutes in a beaker and transferred quickly to a heating block with the calculated melting temperature of the oligonucleotide. After a 30 min incubation, the hybridised dsDNA was placed on ice. For purification the dsDNA was loaded on a 12% acrylamide gel, which was subsequently stained with ethidium bromide. The higher running band representing the double-stranded oligonucleotide was excised and eluted in water at 37°C overnight.

b) Radiolabelling

5 pmol of dsDNA were mixed with 10 pmol of γ ^{32}P ATP in 20 µl of water with the suitable buffer for T4 polymerase. The labelling reaction was performed for 60 min with 20 U of enzyme. The labelled enzyme was stored for up to 3 weeks in the –20 °C freezer.

5 pmol	*dsDNA*
2 µl	*10x Buffer*
10 pmol	*γ^{32}P ATP (20 mCi/ml)*
1 µl	*T4 polymerase (10 U/µl)*
ad	*20 µl H_2O*

c) Bandshift

10 µg of nuclear proteins were mixed on ice with 1.5 µl of Oligo(d(I-T)) (1µg/µl) in water with gelshift buffer (total volume was calculated to be 30 µl after addition of the 30.000 cpm radiolabelled oligonucleotide). After a 30 min incubation on ice, 30.000 cpm radiolabeled oligonucleotide were added and a further 30 min incubation on ice was performed. In this step the antibody for a possible supershift was also added.

As a control for unspecific binding, unlabeled dsDNA with the same sequence was added in a 5-10 fold excess.

The mixture was loaded on a 4% acrylamide gel in the cold room together with pre-cooled loading buffer. The gel was run until the bromophenol blue band reached 2/3 of the gel. The gel was then mounted on a Whatman paper (3mm) and dried on a vacuum dryer. The gel was exposed to a phosphor imager plate for 3 hours to 4 days to visualise the bands.

2.5x bandshift buffer:

25 mM	*HEPES/KOH pH 7.5*
10%	*glycerol*
100 mM	*KCl*
1 mM	*EDTA*
1 mM	DTT (add fresh)
25 µM	*$ZnSO_4$*
ddH2O	

4.3. Histology and immunostaining

4.3.1. Isolation of mouse brains

Mice were sacrificed by cervical dislocation, followed by complete decapitation. With a small pair of scissors an incision was made on both sides, right under the brain, from the posterior opening of the skull towards the jaws. A third cut was made from the posterior opening along the midline of the skull towards the

bregma. The two parts of the skull, laying over the hemispheres were now moved to the lateral with a fine pair of tweezers.

The brain was removed with a spatula, washed with ACSF and placed on a piece of aluminum foil in contact with dry ice. Frozen brains were used immediately or could be stored at -70°C if protected against freeze drying.

Artificial cerebrospinal fluid (ACSF)

125 mM	*NaCl*
3 mM	*KCl*
25 mM	*$NaHCO_3$*
1.25 mM	*NaH_2PO_4*
10 mM	*D-Glucose $1xH_2O$*

Add freshly on the day of use and after saturation with carbogen (95% O_2, 5% CO_2):

2 mM	*$MgCl_2$*
2 mM	*$CaCl_2$*

4.3.2. Coating of microscope slides

For better adhesion of the specimens the microscope slides were coated with poly L-lysine hydrobromide solution. Non-coated washed microscope slides were baked at 200°C in an oven to obtain a better hydrophilic surface. A 0.01% solution of poly L-lysine hydrobromide solution was striked on the surface of the slide, the slides were dried at room temperature and stored at 4°C up to a week.

Poly-L-lysine hydrobromide solution:

25 mg poly-L-lysine hydrobromide salt was dissolved in 5 ml DEPC-treated water and stored in 250 µl aliquots at -20°C. For use, aliquots were thawed and dissolved in 12.5 ml of DEPC treated water to obtain a 0.01 % solution.

4.3.3. Nissl staining

Nissl staining allows staining of nuclei and tigroid plaques (Nissl Schollen) in nerve cells. Best results were obtained with basic tar dyes or cresyl violet. The basic dye binds by electrostatic forces to the acidic nucleic acids.

Slices were stained for 1-2 minutes in cresyl violet solution followed by fast dips in 75% and 95% EtOH and a 4 minutes incubation in 100% EtOH. Specimens were then incubated twice in xylene and sealed with a hydrophobic mounting medium. Nuclei and nigroid plaques appeared in dark violet, nerve cells appeared in lighter blue.

Cresyl violet:
0.5 g of cresyl violet dissolved in 100 ml acetate buffer (pH 3.8-4)
acetate buffer:
2.72 g sodium acetate were dissolved in 800 ml distilled water,180 ml 1M HCl was added, and the solution was adjusted to a pH of 3.8-4 with 0.1 M HCl

4.3.4. Preparation of the slides

Slices of 6 µm nominal thickness were prepared with the use of a Leica kryostat or a Microm vacutome. For this purpose the tissue was removed from the -70°C freezer and warmed to the chamber temperature of the kryostat, normally between -18 to -16°C. The brains were fixed on a specimen holder with a drop of tissue freezing medium, facing with the *bulbus olfactorius* towards the holder. The brains were cut in coronal sections, starting with the *cerebellum.* Sections through the hemispheres, showing the hippocampus, were mounted on coated microscope slides. In a typical experiment 25 slides were placed in a row. The sections were mounted on the slides such that every slide contains 6 specimens of different areas of the hippocampus. Slides were subsequently dried for at least 30 min at room temperature and then stored in boxes at -70°C.

4.3.5. Immunohistochemistry

For immunohistochemistry slides were taken from the storage boxes at –70°C and dried at room temperature for 30 min. Then the specimens were fixed in ice-cold acetone for 10 minutes, briefly dried and washed 3 times in PBS for 10 minutes. Endogenous peroxidases were blocked by incubating the slides for 10 minutes in 0.3% hydrogen peroxide in PBS, followed by three washing steps in PBS, 10 min each. Drops of about 100 µl normal serum (depending on the species the secondary antibody had been raised in) were added to the sections to

block unspecific binding of the primary antibody and incubated for 20 minutes. After removal of the serum and a brief washing step, specimens were incubated with the primary antibody for one hour at room temperature or overnight at 4°C. An excess of antibody was washed away by two rinses in PBS, 10 minutes each. The slides were incubated with secondary biotinylated antibody for 15 min at 37°C in a humid chamber. After two washes with PBS, the biotin of the secondary antibody was coupled to streptavidin-peroxidase by a 15 min incubation at 37°C. The colour reaction was performed after two further 10 minute washing steps with PBS, using the peroxidase substrate diaminobenzidine until the desired intensity of the signal was obtained. A counter-stain was performed with Nissl stain, specimens were dehydrated in a series of solutions with increasing alcohol concentrations (30%, 45%, 60%, 95%, 100%) followed by two incubations in xylene. Finally, the slices were mounted with a drop of xylene-based embedding medium and a coverslip.

4.3.6. Immunofluorescence

Slides were dried, fixed, and washed as described above, washed again 3 times in PBS, then pre-incubated for 20 minutes with antibody dilution solution containing 12% BSA. The solution was then replaced by a dilution of the primary antibody of choice and the slides were incubated for 1 h at room temperature, or overnight at 4°C in a humid chamber. After three washes with PBS for 10 min each, the secondary antibody was added in a drop of antibody dilution solution. After three final washes with PBS the slides were rinsed with 0.1 M Tris/HCl (pH 8.0) and mounted with a water-based mounting medium (Mowiol). Specimens were stored in the dark and analysed under a fluorescence microscope.

Mowiol:

24g *Mowiol (Hoechst)*

60g *glycerol (48.8 ml 87% solution)*

60ml *deionized water*

stir for 5 minutes, let swell overnight

Add 120ml *Tris/HCl pH 8.0*

Shake 10 minutes at 50°C

Centrifuge 15 minutes at 5000xg

Take supernatant and add 6.6g DABCO (1,4Diazabicyclo-(222)octane) (Sigma D-2522)

Vortex until dissolved

Store 1ml aliquots at –20 °C

4.4. Surgery

4.4.1. Mice

The inbred strain of C57/Bl6 mice (Charles River, Sulzfeld, Germany) was used for all experiments. The animals had a weight of 20-22 g and were around 6-8 weeks old. All experiments were approved by the government of Upper-Bavaria and performed according to their guidelines at the Institute of Physiology, University of Munich.

4.4.2. Stereotaxic lesion

Young adult male mice were anaesthetised by intraperitoneal injection of 7% chloralhydrate (10 µl/g body weight). The hair on the head was shaved and the head of the mouse was placed into the headholder of a stereotaxic apparatus. The skull was exposed and the following coordinates from bregma were used for injection into the right lateral ventricle: -2.0 mm anteroposterior, -3.2 mm dorsoventral and -2.9 mm lateral. The cannula (tip diameter 0.49 mm) of a Hamilton microliter syringe was lowered into the ventricle after a small hole (0.7

mm in diameter) had been drilled into the skull and 0.25-0.3 µl of kainic acid in PBS (1 µg/µl pH 7.0) were slowly injected within one minute.

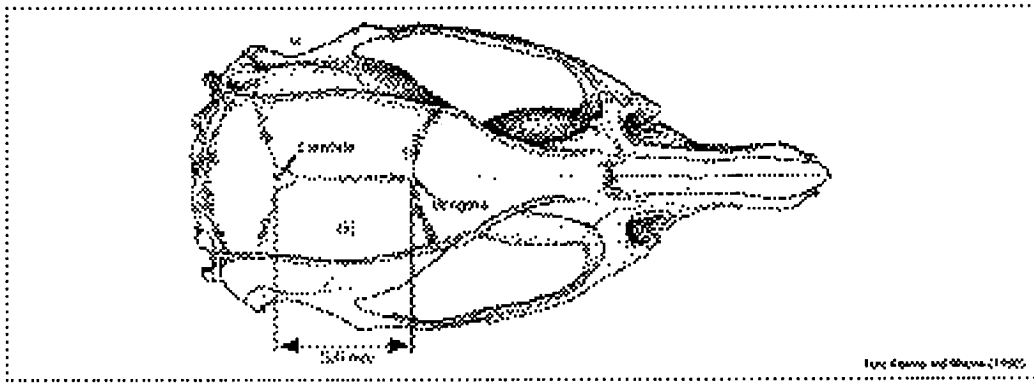


Fig.10: Skull of a mouse, with the borders of the different skull plates and the intersection pints of the sutures (bregma and lambda)

The tip of the cannula remained within the ventricle for additional 3-5 minutes to allow for diffusion of kainic acid. After the needle was removed some cerebrospinal fluid should appear, indicating that the ventricle was punctured. The wound was treated with antibiotic powder and sutured or closed by means of a Michel surgery clamp. During the following 30-90 minutes, epileptic seizures were observed as a sign of successful injection. Animals showing no exsudation of cerebrospinal fluid or not developing epileptic seizures were excluded from the following studies. At different time points post injection (6 hours to 6 months) mice were killed and the brains harvested for various experiments (see there) This procedure is shown in a series of pictures in plates I and II (p.91/92).

4.4.3. Co-administration of growth factors

If growth factors were co-administered, they were mixed in the desired dilution with kainic acid and were co-injected, whereby the maximum amount of liquid injected into the ventricle never exceeded 2 µl of total volume.

4.4.4. Extraction of hippocampi

The skull was opened as described above: two cuts were made along the brain base and one along the hemisphere midline. The bone of the skull was broken away and the brain was removed. Then the cerebellum was cut off with a spatula and the brain was divided in the two hemispheres. One of the spatula was placed between the brainstem and with a second one the cortex was "rolled out" towards the lateral side. Now the hippocampus was found on the outer edge and could be extracted from the tissue.

Hippocampi were frozen on a piece of aluminium foil on dry ice and stored at -80°C until further use. An illustrated description is given in plate III (p.93)

4.4.5. Minipumps

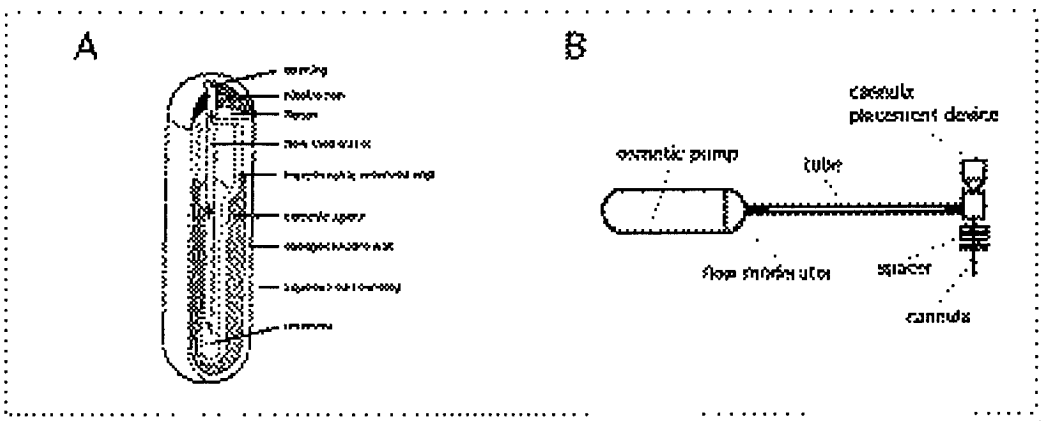


Fig. 11: **A)** schematic drawing illustrating the components of an Alzet minipump **B)** Assembly of minipump and catheter for chronic icv. infusion

a) Filling

The pumps were held in an upright position and filled with a 1 ml syringe connected to a blunt, 27 gauge, cannula. Growth factors were diluted in sterile PBS or sterile NaCl. The cannula was completely inserted into the pump corpus and retracted cautiously afterwards, with slight pressure applied to the plunger. To insert the catheter (about 2 cm), the plastic cap of the flow moderator was broken off, the unit of moderator and catheter was completely filled with the same solution as loaded into the pump. After checking for air bubbles in the unit, the flow moderator was placed into the pump body.

Pumps were "*in vitro* primed" in a 0,9% sterile NaCl solution for 6 hours at 37°C. During this time the distal end of the catheter was placed outside the solution to hinder the priming solution to enter the system.

b) Fixation of the pump

Mice were anesthetized with 7% chloralhydrate (0.01 ml/g body weight) and the skin over the skull and the neck was opened with a scalpel. Two holes were drilled for (i) chronic substance infusion into the left lateral ventricle by means of an osmotic pump and (ii) injection of kainic acid into the right lateral ventricle (see above). The coordinates from bregma for chronic infusion were as follows: -0.14 mm anterioposterior, +0.75 mm lateral and 2.5 mm dorsoventral. After the priming of the pump, the PBS-filled infusion cannula was connected to the catheter and the spacers were threaded around the infusion cannula so it could be placed 1 mm deep to the floor of the lateral ventricle. For this purpose the spacer was glued to the infusion cannula with a very small drop of cyanide super-glue. To fix the catheter to the skull a two-component polymer mixture, such as used for tooth prostheses, was placed around the cannula. After polymerisation the cannula was retracted, the skull was roughened with a diamond drill bit and the polymer-cannula composition was replaced on the skull with a drop of cyanide super-glue in between. After complete hardening of the glue a tunnel under the *panniculus carnosus* muscle of the dorsal skin was opened with a surgical probe and the pump body was placed between the scapulas.
A small (1 mm Ø) ring of sterling silver made from 0.8 mm wire was glued around the hole for later KA injection, the open wound was disinfected with antibiotic powder and closed with dentist`s polymer.

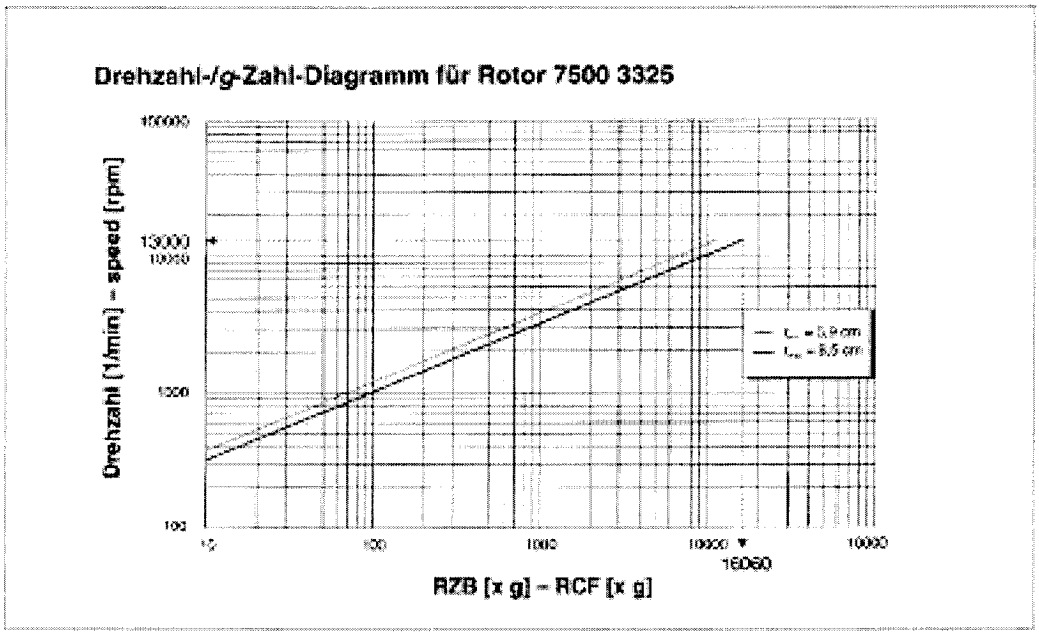
c) Lesion in minipump-implanted mice

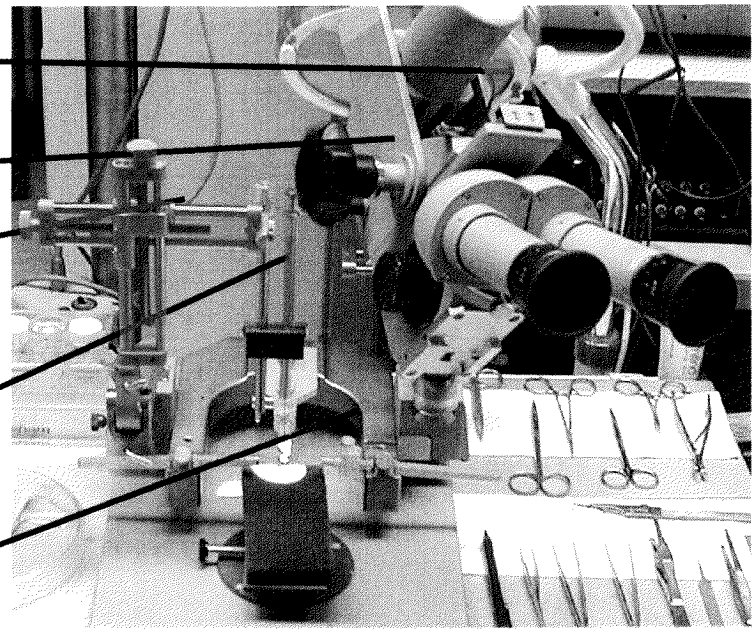
Two days after minipump implantation the mouse was placed again into the stereotaxic apparatus after appropriate anesthesia (see above), the silver ring was located through the skin and the skin was opened with a scalpel. The cannula was lowered through the hole until it reached the ventricle (3.2 mm) and KA was injected (0.3-0.5 µl). After retraction of the cannula the wound was

again treated with antibiotics and closed with dentist`s polymer. Continuous substance application through the minipump lasted for 5 days, then the mice were anesthetized, killed and the brains were removed as described above.

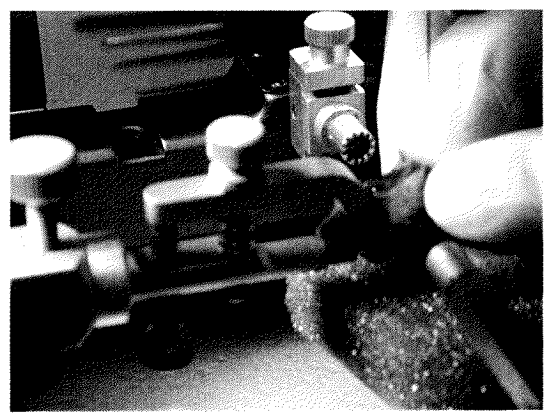
4.5. Appendix

In some of the protocols the centrifugation unit is rpm and not xg, since this unit is displayed on the centrifuges. In this case a Heraeus Biofuge with a Rotor 7500 3325 was used, and the number can be easily converted to xg by help of the following chart:



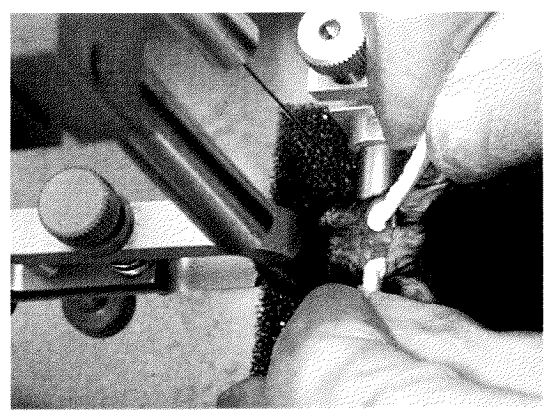


Mouse head was shaved from the ears to the eyes

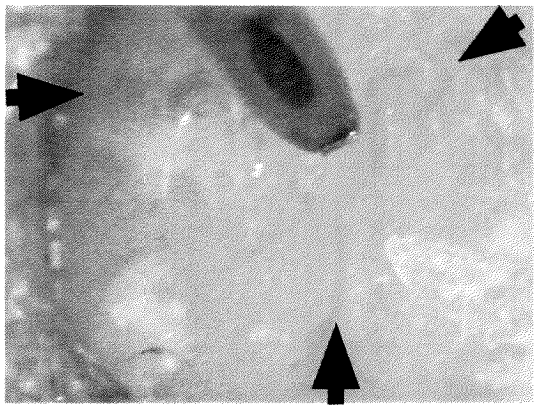


Head was fixed with a mouse adaptor using a palate fixation and ear bars.

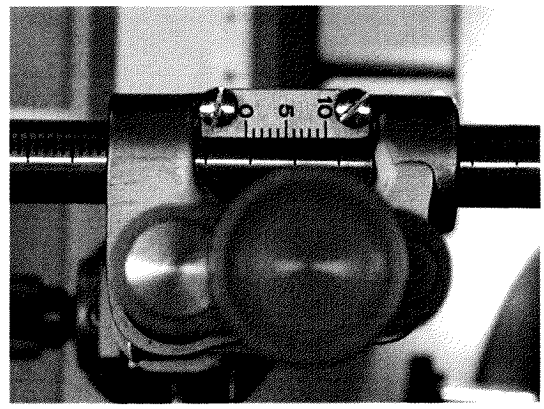
Scalp was opened 0.8 cm with a scalpel



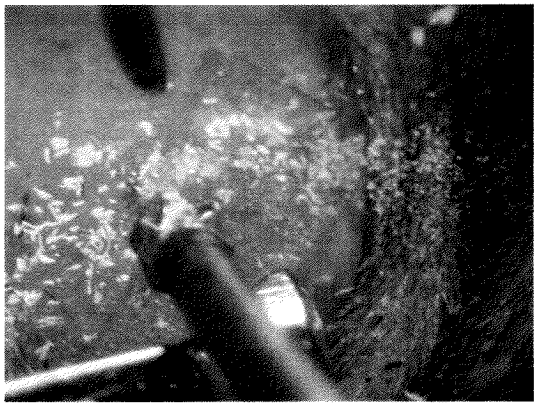
Periost was pushed away with two swobs



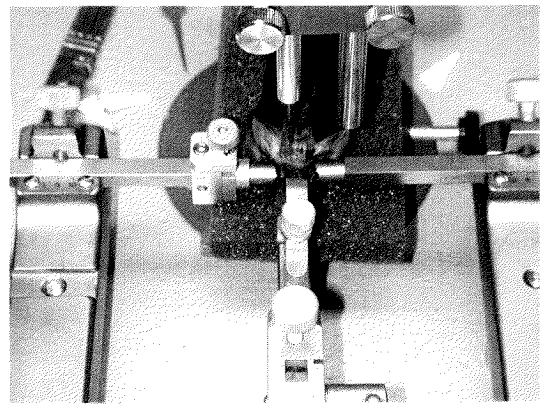
Bregma was taken as reference point (zero) to calculate coordinates



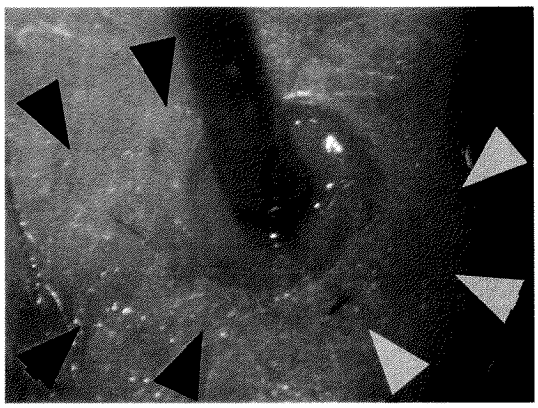
anterior/posterior -2.0 mm
lateral -2.9 mm



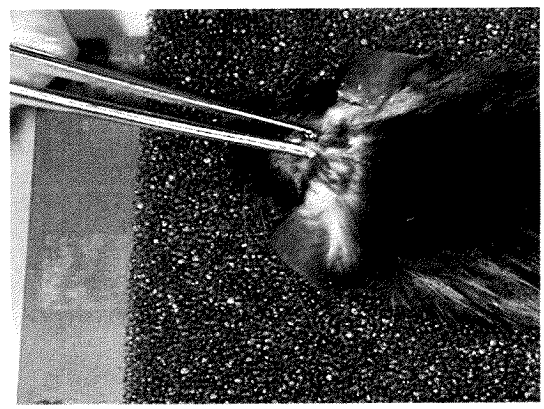
Point of the insertion was marked with a pencil and a hole was drilled in the skull



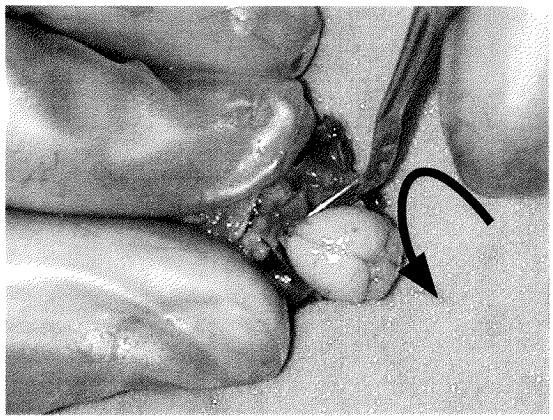
Cannula was inserted. dorsoventral -3.2 mm, counted from the dura, KA was injected over a minute. Cannula was retracted after 2 - 5 min



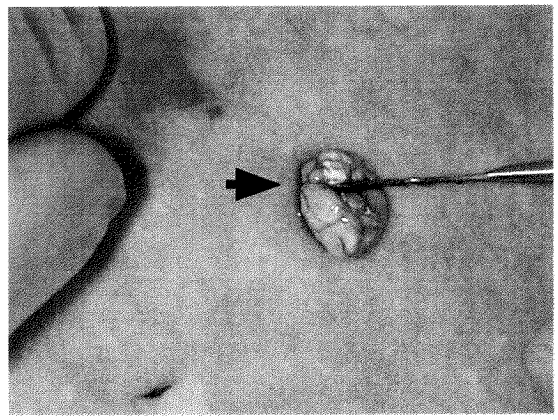
Appearance of cerebrospinal fluid during retraction of the cannula showed that the ventricle was punctured



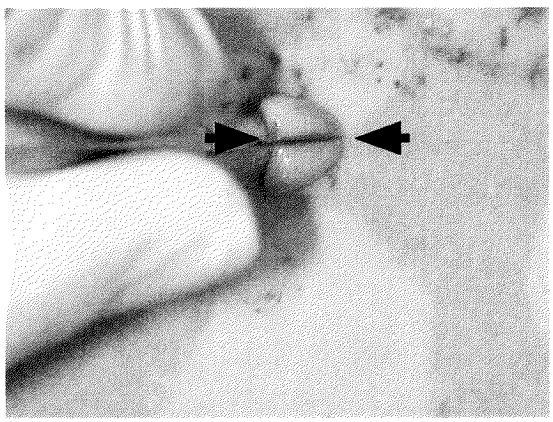
Wound was treated with antibiotic powder and sutured or closed with a Michel clamp.



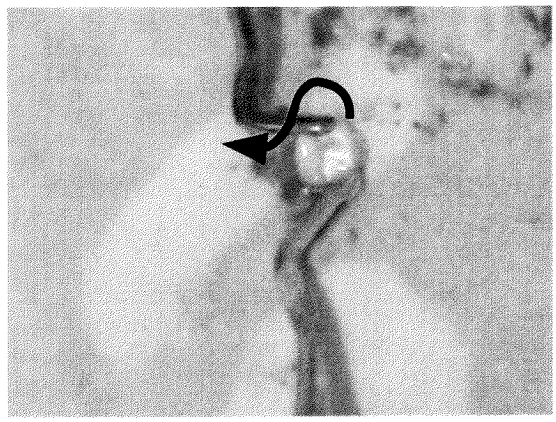
Brain was removed from the skull



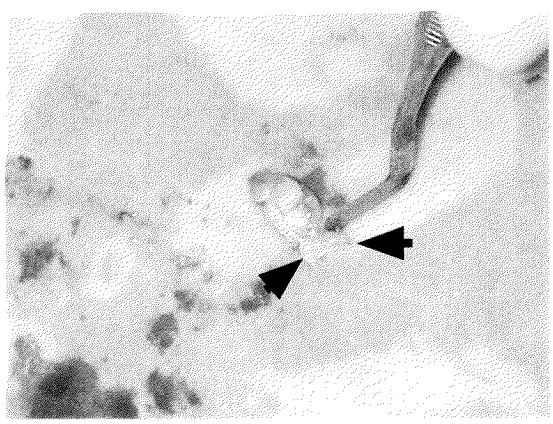
Cerebellum was cut off



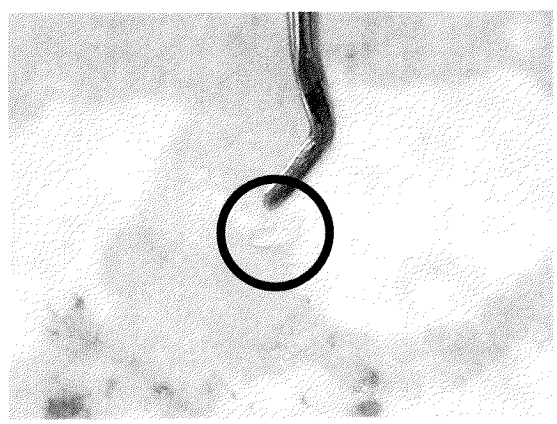
Hemispheres were divided



Hemispheres were rolled off from the brain stem to the outside



Hippocampus was cut off and cleaned from surrounding tissue



Isolated Hippocampi were frozen on a piece of aluminum folie and placed on dry ice

5. Publications

5.1 Nrf Publication

Submitted to the European Journal of Neuroscience

Upregulation and activation of the Nrf-1 transcription factor in the lesioned hippocampus: A novel neuroprotective mechanism against reactive oxygen species?

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Christian Alzheimer[2] and Sabine Werner[1]*

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a) Abstract

The Nrf-1 and Nrf-2 transcription factors play a pivotal role in the cellular defense against the toxic effects of reactive oxygen species (ROS). Although ROS are key effectors of neuronal death after ischemic and traumatic brain injury, it is not known whether Nrf-1 and Nrf-2 are involved in neuroprotective signaling. Here, we analyzed the temporal and spatial expression pattern of Nrf-1 and Nrf-2 after unilateral excitotoxic lesion of mouse hippocampus. In marked contrast to previous *in vitro* studies, where upregulation of these transcription factors on the mRNA level was never detected, we found a strong induction of Nrf-1 mRNA and protein expression in neurons of the lesioned hippocampus, accompanied by a weak elevation of Nrf-2 mRNA levels. The following observations underscore the functional significance of Nrf-1 in surviving neurons: Firstly, the newly formed Nrf-1 predominantly localized to the nucleus in the injured hippocampus. Secondly, expression of the cytoprotective enzyme, heme oxygenase-1, a major target of Nrf-1 and Nrf-2 action, was co-regulated with Nrf-1 in the same hippocampal neurons. Since Nrf-1 and Nrf-2 are potent inducers of various other cytoprotective proteins, our data implicate the induction of these factors as a critical step in neuronal survival after acute brain injury.

Introduction

Nrf-1 and Nrf-2 are important regulators of genes that are required for cytoprotection. They are members of the „cap`n collar" family of transcription factors which bind to the antioxidant response element in the promoters of several genes involved in the cellular stress response (Wasserman and Fahl, 1997). Binding of Nrf-1 or Nrf-2 to this element induces the expression of phase II detoxifying enzymes and oxidative stress-inducible proteins such as NAD(P)H:quinone oxidoreductase, glutathione S-transferase A2, UDP-glucuronosyl transferase, epoxide hydrolase, γ-glutamylcysteine synthetase (γ-GCS), heme oxygenase–1 (HO-1), A170 and MSP23 (Prestera *et al.*, 1995; Venugopal and Jaiswal, 1998; Ishii *et al.*, 2000). The functional importance of this induction was revealed by the analysis of knockout mice. Targeted disruption of Nrf-1 resulted in anemia and embryonic lethality (Chan *et al.*, 1998). Furthermore, and relevant to its potential protective effect, fibroblasts derived from these mice showed lower levels of glutathione and enhanced sensitivity to the toxic effects of oxidant compounds (Kwong *et al.*, 1999). In Nrf-2 knockout animals, induction of phase II detoxifying enzymes by a phenolic antioxidant was strongly reduced in the liver and intestine (Chan and Kan, 1999), and macrophages isolated from these mice had a profoundly impaired response to electrophilic and reactive oxgen species (ROS)-producing agents (Ishii *et al.*, 2000).

Given the strong cytoprotective effects of Nrf-1 and Nrf-2 in several organs, we wondered whether they play also a role in acute brain injury associated with increased formation of ROS. For this purpose, we employed a well-established model of local excitotoxic brain damage, where intracerebroventricular (icv.) injection of the rigid glutamate analogon, kainic acid (KA), results in a unilateral lesion of area CA3 of the hippocampus (Ben-Ari and Cossart, 2000). This model appears particularly suitable, since production of ROS emerged as a major step in the cascade of molecular events leading to neuronal death in glutamate excitotoxicity (Reynolds and Hastings, 1995; Gunasekar *et al.*, 1995; Boldyrev *et al.*, 2000; reviewed in Bast and Bär, 1997). Here we demonstrate a coordiated

induction of Nrf-1, Nrf-2 and HO-1 expression in the lesioned hippocampus, indicating that Nrf-1 and Nrf-2 promote protective pathways involved in detoxification of ROS.

Materials and methods

Hippocampal KA lesions. Male C57/Bl6 mice (22g) were housed and fed according to federal guidelines, and all surgical procedures were approved by the local government of Bavaria. KA lesions were produced as described (Tretter *et al.*, 1996, 2000). Briefly, animals were anesthetized by i.p. injection of 7% chloralhydrate (0.01 ml/g body weight). KA (0.25 µl, 1 µg/µl in phosphate buffered saline) was injected unilaterally into the right lateral ventricle using a stereotaxic apparatus (coordinates from bregma: -2.0 mm anteroposterior, -3.2 mm dorsoventral and -2.9 mm lateral).

Tissue preparation, RNA isolation and RNase protection assay. Mice were sacrificed at different time points after KA injection. Complete ipsilateral and contralateral hippocampi (4 mice per time point) were removed, pooled, and used for RNA isolation or for preparation of nuclear lysates. RNase protection assays were performed as described by Werner *et al.* (1992). Probe DNAs: HO-1 (Hanselmann *et al.* 2001), Nrf-1 (nt 988-1210 of the murine Nrf-1 cDNA; Accession No. U2053), and Nrf-2 (nt 1837-2123 of the murine Nrf-2 cDNA (Accession No. X78709). As a loading control, 1 µg of the RNA samples was loaded on a 1% agarose gel prior to hybridization and stained with ethidium bromide.

Immunofluorescence. 6 µm frozen sections from complete brains were incubated with polyclonal antibodies directed against Nrf-1 (Santa Cruz Biochemicals, Santa Cruz, CA; 1: 1000 diluted), HO-1 (Stressgen Biotechnologies Corp. Victoria, Canada; 1: 1000 diluted), glial fibrillary acidic protein (GFAP) (Progen Biotechnik, Heidelberg, Germany; 1: 50 diluted) or Map-2 (Leinco Technologies, St. Louis, MO; 1: 500 diluted). Antibody-binding cells were visualized using FITC- Cy™2- or Cy™3-coupled secondary antibodies (Jackson Immuno Research, West Grove, PA). Staining with Hoechst was performed to visualize nu-

clei. Slides were analyzed with a Zeiss Axioplan microscope or with a Leitz confocal microscope (TCSNT Laser).

Preparation of nuclear extracts and westernblot analysis. Nuclear extracts from non-injured and lesioned hippocampi were prepared as described by Andrews and Faller (1991). Alternatively, complete hippocampi were homogenized at 4°C with a motor-driven Teflon glass homogenizer, and nuclei were prepared according to Gorski *et al.* (1986). 10 µg aliquots of the lysates were analyzed by western blotting using the Nrf-1 antibody described above and an alkaline phosphatase detection system.

Results and Discussion

Stereotactic injection of the excitotoxin KA into the ventricle resulted in a severe lesion in the CA3 region of the ipsilateral but not of the contralateral hippocampus of mice (Tretter *et al.*, 1996; 2000; Fig. 2A). To determine a possible role of Nrf-1 and Nrf-2 in the injured brain, RNA from non-injured hippocampi and from ipsilateral and contralateral hippocampi was analyzed by RNase protection assay for the expression of these transcription factors. Nrf-1 mRNA was expressed at low levels in the non-injured hippocampus (Fig. 1A). Within 6 hours of KA-injection, a strong induction of Nrf-1 expression was seen in the injured ipsilateral hippocampus and also in the non-lesioned contralateral hippocampus. This peak of induction was followed by a decline within the next two days (Fig. 1A), which was more rapid in the contralateral hippocampus.

Expression of Nrf-2 mRNA was not detectable in the non-injured hippocampus. However, a slight but significant upregulation was observed after KA injection. The kinetics of this induction was identical to that of Nrf-1. This result was reproduced in three RNase protection assays using RNAs from independent injury experiments. The up-regulation of Nrf-1 and Nrf-2 expression is remarkable, since transcriptional regulation of these genes has not been demonstrated yet. By contrast, the activity is usually regulated by activation of preformed protein that translocates into the nucleus in response to ROS or electrophils (Itoh *et al.*, 1999).

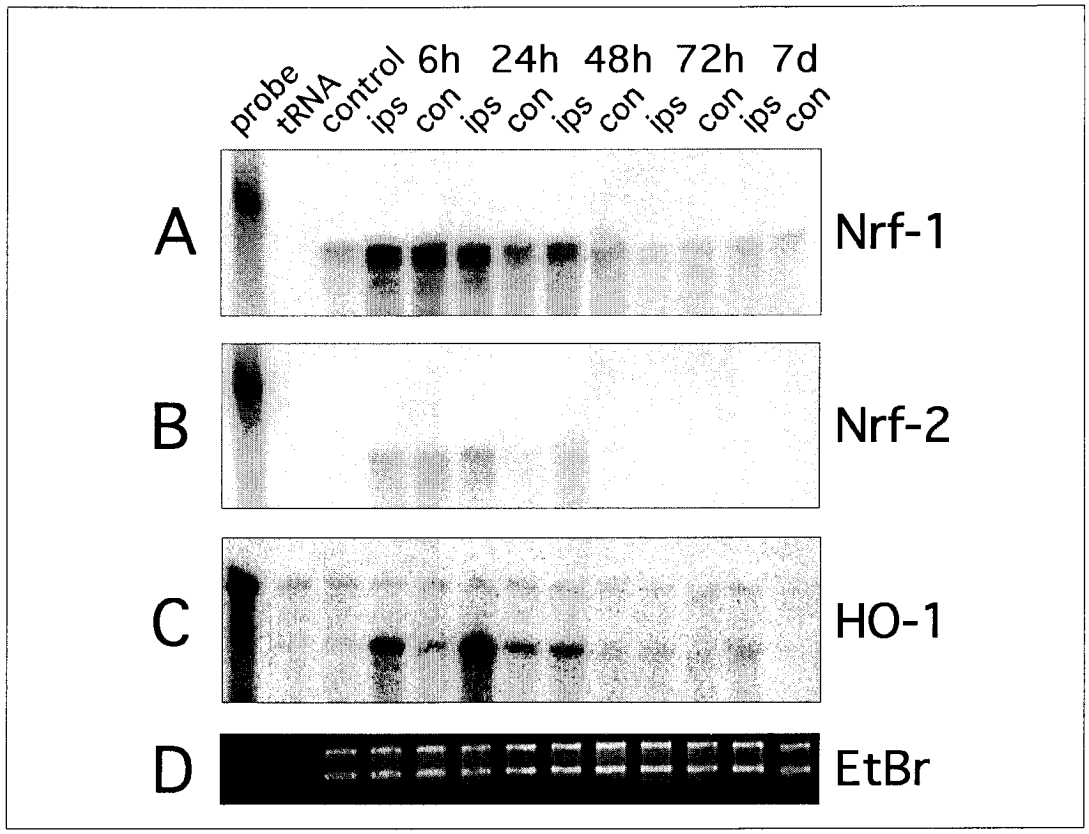

Fig. 1 Increased expression of Nrf-1, Nrf-2 and HO-1 mRNAs in the lesioned hippocampus after KA lesion. *10 μg total RNA from non-injured (control) and from the ipsilateral (ips) and contralateral (con) hippocampi of KA-injected mice were analyzed by RNase protection assay for the presence of **(A)** Nrf-1, **(B)** Nrf-2, and **(C)** HO-1 mRNAs. 1000 cpm of the hybridization probes were loaded in the lanes labeled „probe" and used as size markers. The same batch of RNAs was used for all RNase protection assays. 1 μg of the same RNAs was loaded on a 1% agarose gel prior to hybridization and stained with ethidium bromide **(D)**.*

It is worth noting that upregulation of Nrf-1 and Nrf-2 occurred not only on the damaged (ipsilateral) side, but also on the contralateral, non-injured side. Since the two hippocampi are interconnected by commissural fibers, epileptic discharges instigated by icv. KA in the ipsilateral hippocampus will spread to the other side. Hippocampal CA3 neurons are highly vulnerable to seizure activity *per se* (Ben-Ari and Cossart, 2000), so that neuronal loss does not necessarily require direct exposure to KA. The fact that we did not observe neuronal damage on the contralateral side despite transmitted epileptic activity (cf. Tretter *et al.*, 2000) might suggest that the induction of Nrf-1 and Nrf-2 in the contralateral hippocampus serves as a mechanism to protect the CA3 neurons of this side against the excessive glutamate release during seizure activity.

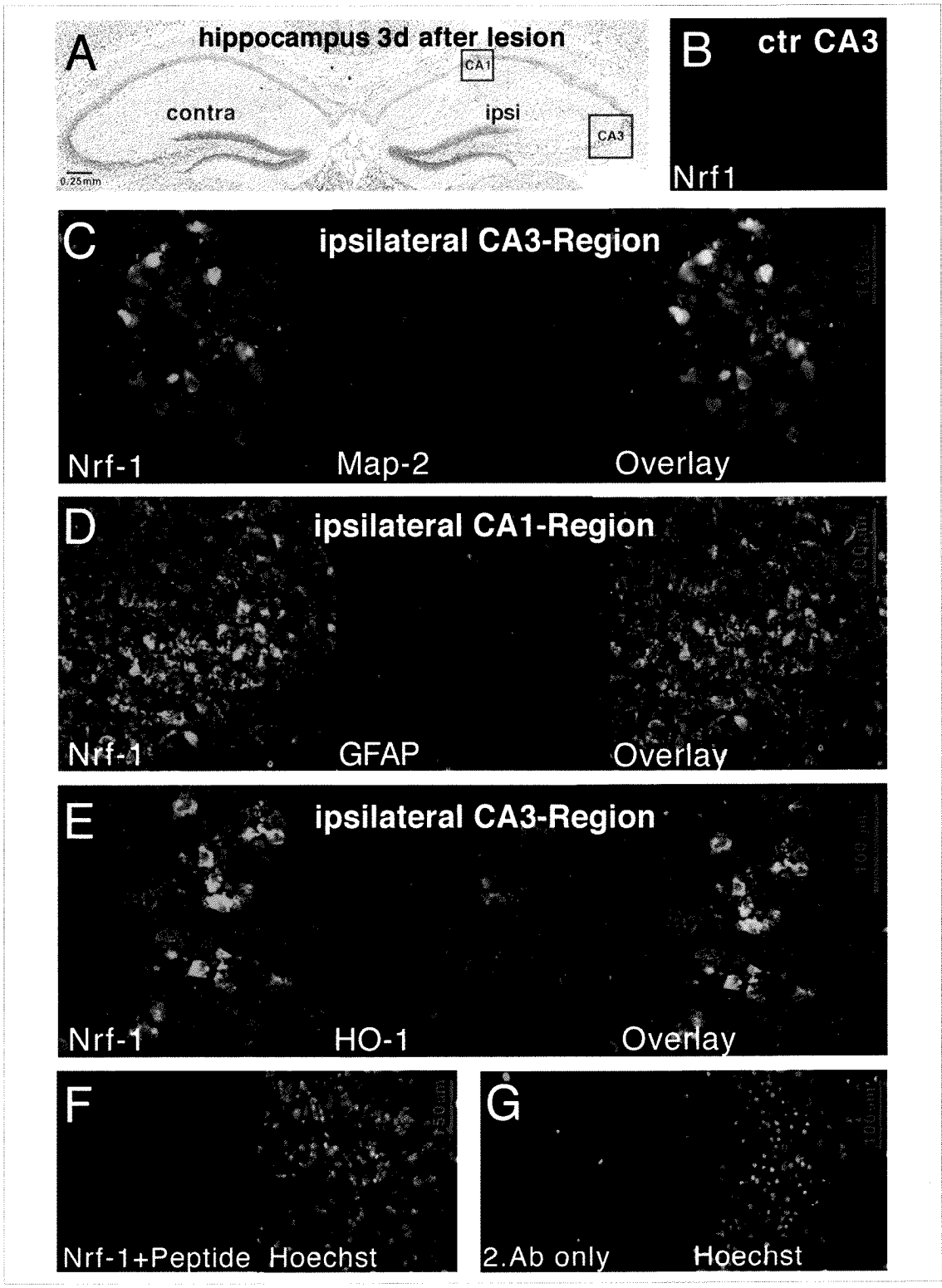

Fig.2 *Immunolocalization of Nrf-1 and HO-1 in the injured ipsilateral hippocampus.* ***Mice*** *were sacrificed before* ***(B)*** *or 3 days after KA injection* ***(A, C-G)****. The complete brains were isolated and embedded in tissue-freezing medium. 6 mm frozen sections were stained with cresyl violet* ***(A)*** *or incubated with antibodies directed against Nrf-1* ***(B-F)****, HO-1* ***(E)****, GFAP* ***(D)****, and Map-2* ***(C)*** *and visualized using FITC-, CyTM2-, or CyTM3-coupled secondary antibodies. Immunostaining without the first antibody is shown in* ***(G)****, immunostaining with the Nrf-1 antibody which had been pre-treated with the immunization peptide is shown in* ***(F)****.*

To further determine whether the upregulation of Nrf-1 and Nrf-2 expression after hippocampal lesion is of functional importance, we analyzed the expression of HO-1, a major target of Nrf-1 and Nrf-2 action. As shown in Fig.1C, the mRNA expression of this enzyme was strongly upregulated within 6-24 hours after KA injection, particularly at the ipsilateral side. Most importantly, this induction was slightly delayed compared to that of Nrf-1 and Nrf-2, suggesting that HO-1 is indeed a target of these transcription factors in the lesioned hippocampus.

To determine whether the increase in Nrf-1 mRNA expression seen after hippocampal injury correlates with elevated levels of the corresponding protein and to localize Nrf-1 within the wounded hippocampus, brain sections were stained with a polyclonal antiserum directed against Nrf-1. In addition, staining with antibodies against GFAP or MAP-2 was performed to identify whether Nrf-1 or Nrf-2 are expressed by astrocytes or neurons, respectively. Fig.2B shows the weak staining with the Nrf-1 antibody in the
hippocampus of animals that had not been injected with KA. However, strong signals were observed in the CA3 and CA1 regions of the lesioned (ipsilateral) hippocampus (Fig. 2C and D) and to a lesser extent on the non-lesioned (contralateral) side (not shown) of KA-injected animals. The specificity of these signals was confirmed by immunostaining without the primary antibody (Fig. 2G) or with the primary antibody which had been pre-treated with the peptide used for immunization (Fig. 2F). Co-staining with antibodies against the glial marker GFAP or the neuronal marker MAP-2 revealed that Nrf-1 is predominantly expressed by CA3 and CA1 neurons (co-staining shown for CA3 neurons) but not by glial cells (co-staining shown for CA1).

Since Nrf-1 and Nrf-2 are translocated from the cytosol to the nucleus upon activation (Itoh *et al.*, 1999), we determined the intracellular localization of Nrf-1. In a first step, nuclear lysates were prepared from normal and injured hippocampi and analyzed by western blotting with an Nrf-1 antibody. As shown in Fig. 3A, a protein of the expected size (81 kDa) was found in normal and injured hippocampal nuclei, with particularly high levels being present at the ipsilateral side.

The same result was obtained when nuclei were prepared by a different method (see Materials and Methods).

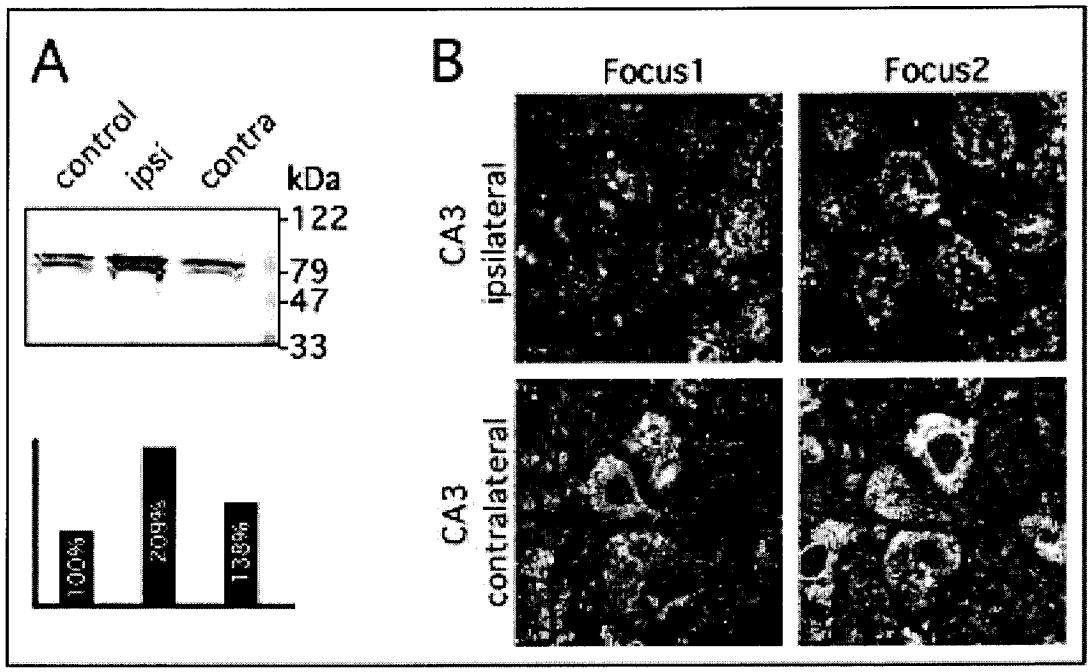

Fig. *3 Nrf-1 protein is present in the nucleus of neurons in the injured hippocampus.* ***(A)*** *Nuclear lysates (20 μg protein) from complete non-lesioned hippocampi and from the complete ipsilateral and contralateral hippocampi of KA-injected mice (48h after injection) were analyzed by western blotting for the presence of Nrf-1 protein. The signal intensities are shown schematically below. The intensity of the band in the control lane was arbitrarily set as 100%.* ***(B)****: Immunofluoresence staining of the ipsilateral (upper panel) andcontralateral (lower panel) hippocampus (CA3 region, 3d after KA injection) with an antibody against Nrf-1. Analysis was performed with a confocal microscope.*

The specificity of the 81 kDa band was verified by westernblotting with the Nrf-1 antibody that had been pre-treated with the immunization peptide. In this case the 81 kDa band disappeared (not shown). The nuclear localization was confirmed by confocal microscopy. Consistent with the western blot data, Nrf-1 was predominantly located in the nucleus at the ipsilateral side (Fig. 3B, upper panel), whereas a more cytoplasmic localization was seen at the contralateral side (Fig .3B, lower panel). The different staining is not a result of nuclear swelling as determined by propidium iodide staining of the nuclei (data not shown). These findings suggest that Nrf-1 is biologically active, particularly at the ipsilateral side.

To further determine whether the upregulation of Nrf-1 expression might be responsible for the increased levels of HO-1 in the injured hippocampus, we performed co-immunofluorescence with antibodies directed against Nrf-1 and HO-1. Interestingly, strong Nrf-1 and HO-1 signals were seen in the same neurons of the ipsilateral CA3 region (Fig. 2E), indicating that HO-1 is indeed a target of Nrf-1 in these cells. The upregulation of this enzyme after hippocampal lesion is consistent with previous findings from other groups who demonstrated increased HO-1 expression in glial cells and neurons of the rat hippocampus after KA treatment (Matsuoka *et al.*, 1998; Nakaso *et al.*, 1999a). However, we did not observe expression in glial cells, possibly reflecting the differences between the animal species used. In addition to HO-1, the oxidative stress-inducible proteins A170 and MSP23 that are targets of Nrf-2 action in macrophages (Ishii *et al.*, 2000) were shown to be upregulated by KA-mediated excitotoxicity in the rat brain (Nakaso *et al.*, 1999b; 2000), suggesting that the expression of these genes is also regulated by Nrf-1 and Nrf-2 in the lesioned hippocampus.

HO-1 is the rate-limiting enzyme in the degradation of heme, a process which is of particular importance in injured tissues where the disruption of blood vessels leads to release of hemoglobin and myoglobin from erythrocytes. The free heme can promote free radical formation and lipid peroxidation, resulting in severe tissue damage (reviewed by Dong, 2000). Furthermore, HO is important for the protection of cells from oxidative stress *in vitro* and *in vivo* (reviewed by Dong, 2000; Elbirt and Bonkovsky, 1999). One of the products of HO action, biliverdin, appears to be primarily responsible for this effect, since it is rapidly reduced to the potent anti-oxidant bilirubin. Taken together, our data suggest an important role of Nrf-1 and Nrf-2 in acute brain injury due to the induction of HO-1 and possibly other cytoprotective molecules that are important in the defense against ROS.

Acknowledgements. This work was supported by grants from the Swiss National Science Foundation (grant No. 61358.00 to S.W. and from the Bundesministerium für Bildung und Forschung (to C.A. and S.W.). C.A. is a Heisenberg fellow of the Deutsche Forschungsgemeinschaft

References

Andrews, N.C., & Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucl. Acids Res. **19**, 2499.

Bast, A. & Bär, P.R. (1997) The antioxidant/prooxidant balance in neurodegeneration and neuroprotection. In Bär, P.R. & Flint Beal, M. (eds.), *Neuroprotection in CNS diseases.* Marcell Dekker Inc., New York, pp.147-159.

Ben-Ari, Y. & Cossart, R. (2000) Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci.* **23**, 580-587.

Boldyrev, A., Song, R., Dyatlov, V. A., Lawrence, D. A. & Carpenter, D.O. (2000) Neuronal death and reactive oxygen species. *Cell. Mol. Neurobiol.* **20**, 433-450.

Chan, J.Y., Kwong, M., Lu, R., Chang, J., Wang, B., Yen, T.S. & Kan, Y.W. (1998). Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. *EMBO J.* **17**, 1779-1787.

Chan, K., & Kan, Y.W. (1999) Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc. Natl. Acad. Sci. USA* **96**, 12731-12736.

Dong, Z., Lavrovsky, Y., Venkatachalam, M.A., & Roy, A.K. (2000) Heme oxygenase-1 in tissue pathology. *Am. J. Pathol.* **156**, 1485-1488.

Elbirt, K.K., & Bonkovsky, H.L. (1999) Heme oxygenase: Recent advances in understanding its regulation and role. *Proc. Ass. Amer. Phys.* **111**, 438-447.

Gorski, K., Carneiro, M., and Schibler. (1986). Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* **47**, 767-776.

Gunasekar, P.G, Kanthasamy, A.G., Borowitz, J.L. & Isom, G.E. (1995) NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death. *J. Neurochem.* **65**, 2016-2021.

Hanselmann, C., Mauch, C., & Werner, S. (2001) A novel role of heme oxygenase in wound repair and psorisis? *Biochem. J.*, **353**, 459-466.

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, J.D., & 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.

Kwong, M., Kan, Y.W., & Chan, J.Y. (1999) The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress-inducing agents. *J. Biol. Chem.*, **274**, 37491-37498.

Matsuoka, Y., Kitamura, Y., Okazaki, M., Kakimura, J., Tooyama, I., Kimura H., &Taniguchi, T. (1998) Kainic acid induction of heme oxygenase in vivo and in vitro. *Neurosci.*, **85**, 1223-1233.

Nakaso, K., Kitayama, M., Kimura, K., Yanagawa, T., Ohama, E., Nakashima, K., Ishii, T., & Yamada, K. (1999a) Induction of heme oxygenase-1 in the rat brain by kainic acid-mediated excitotoxicity: the dissociation of mRNA and protein expression in hippocampus. *Biochem. Biophys. Res. Commun.*, **259**, 91-96.

Nakaso, K., Kitayama, M., Ishii, T., Bannai, S., Yanagawa, T., Kimura, K., Nakashima, K., Ohama, E., & Yamada, K. (1999b) Effects of kainate-mediated excitotoxicity on the expression of rat counterparts of A170 and MSP23 stress proteins in the brain. *Brain Res. Mol. Brain Res.*, **69**, 155-163.

Nakaso, K., Kitayama, M., Fukuda, H., Kimura, K., Yanagawa, T., Ishii, T., Nakashima, K., & Yamada, K. (2000) Oxidative stress-related proteins A170 and heme oxygenase-1 are differently induced in the rat cerebellum under kainate-mediated excitotoxicity. *Neurosci. Lett.*, **282**, 57-80.

Prestera, T., Talalay, P., Alam, J., Ahn, Y.I, Lee, P.J., & Choi, A.M. (1995). Parallel induction of heme oxygenase-1 and chemoprotective phase 2 enzymes by electrophiles and antioxidants: regulation by upstream antioxidant-resonse elements (ARE). *Mol. Med.*, **1**, 827-837.

Reynolds, I.J. & Hastings, T.G. (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J. Neurosci.,* **15**, 3318-3327.

Tretter, Y.P., Munz, B., Hübner, G., ten Bruggencate, G., Werner, S., & Alzheimer, C. (1996) Strong induction of activin expression after hippocampal lesion. *NeuroReport,* **7**, 1819-1823.

Tretter, Y.P., Hertel, M., Munz, B., ten Bruggencate, G., Werner, S., & Alzheimer, C. (2000) Induction of activin A is essential for the neuroprotective action of basic fibroblast growth factor in vivo. *Nat. Med.*, **6**, 812-815.

Venugopal, R., & Jaiswal, A.K.(1998) Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene,* **17**, 3145-3156.

Wasserman, W.W., & Fahl, W.E. (1997) Functional antioxidant responsive elements. *Proc. Natl. Acad. Sci. USA,* **94**, 5361-5366.

Werner, S., Peters, K.G., Longaker, M.T., Fuller-Pace, F., Banda, M., & Williams, L.T. (1992) Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc. Natl. Acad. Sci. USA*, **89**, 6896-6900.

List of abbreviations:

CNS: central nervous system

GFAP: glial fibrillary acidic protein

FCS: Fetal calf serum

HO-1: heme oxygenase 1

KA: kainic acid

Map-2: Microtubule associated protein 2

Nrf-1: NFE2-related factor 1

Nrf-2: NFE2-related factor 2

PBS: Phosphate-buffered saline

5.2 Activin Publication

The following experiments of the activin publication were performed by myself:

- All of the experiments in this paper were reproduced by myself.
- Some of the published figures show results of my work: Nissl stains (Fig. 3), RNase protection assay (Fig. 2e).
- Some of the mice used for the statistics (Fig 3e/j) were manipulated by myself.

Induction of activin A is essential for the neuroprotective action of basic fibroblast growth factor *in vivo*

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Exogenous application of neurotrophic growth factors has emerged as a new and particularly promising approach not only to promote functional recovery after acute brain injury but also to protect neurons against the immediate effect of the injury. Among the various growth factors and cytokines studied so far, the neuroprotective and neurotrophic profile of basic fibroblast growth factor (bFGF) is the best documented[1-5]. Using an animal model of acute excitotoxic brain injury[6], we report here that the neuroprotective action of bFGF, which is now being tested in stroke patients, depends on the induction of activin A, a member of the transforming growth factor-β superfamily. Our evidence for this previously unknown mechanism of action of bFGF is that bFGF strongly enhanced lesion-associated induction of activin A; in the presence of the activin-neutralizing protein follistatin, bFGF was no longer capable of rescuing neurons from excitotoxic death; and recombinant activin A exerted a neuroprotective effect by itself. Our data indicate that the development of substances influencing activin expression or receptor binding should offer new ways to fight neuronal loss in ischemic and traumatic brain injury.

Consistent with its neuroprotective action in other lesion models[1-5], basic fibroblast growth factor (bFGF) abolished the neuronal damage in the hippocampal CA3 region typically associated with intracerebroventricular application of kainic acid (KA). Histological staining of coronal hippocampus sections showed that the substantial loss of CA3 neurons in the ipsilateral hippocampus after injection of KA (Fig. 1*a*) was prevented when bFGF was co-injected with KA (Fig. 1*b*). Nerve cell counts in area CA3 of hippocampi from control mice ($n = 8$), KA-lesioned mice ($n = 20$) and mice co-injected with KA and bFGF ($n = 20$) indicated significant cell loss only in KA-treated mice ($P < 0.001$). Cell counts in the CA1 region did not demonstrate significant differences among the three groups of mice ($P > 0.05$). Activin β_A mRNA is strongly upregulated in this lesion model[7,8]. As none of the potential dimerization partners of the β_A chain were induced (the β_B or α chains), β_A mRNA most likely gives rise solely to homodimeric activin A ($\beta_A\beta_A$), but not to other members of the activin/inhibin family ($\beta_A\beta_B$: activin AB; $\beta_B\beta_B$: activin B; $\beta_A\alpha$: inhibin A). Autoradiograms showed that the β_A signal was much more prominent in the hippocampi of mice co-injected with bFGF than in the hippocampi of lesioned mice (Fig. 1*c* and *d*). The signal was particularly intense in the CA1 region, but *in situ* hybridization with a ^{35}S-labeled riboprobe demonstrated appreciable levels of β_A mRNA also in surviving CA3 neurons after longer exposure of the nuclear emulsion (data not shown). RNase protection assays from lesioned and co-injected mice further corroborated the considerable effect of bFGF on the intensity but not on the time course of β_A mRNA induction (Fig. 1*e* and *f*). Upregulation of activin β_A mRNA by bFGF only occurred after hippocampal lesion, whereas injection of the same batch of bFGF in control mice did not increase β_A mRNA levels (Fig. 1*f*), indicating that the increased expression of βA mRNA in response to bFGF depends on cellular events related to the injection of KA. Compared with KA-injected mice with hippocampal lesion, mice with bFGF-protected hippocampi had a higher density of activin-immunopositive neurons in the CA3 region (Fig. 2) as well as stronger activin signals in the CA1/CA2 region (data not shown). The increased expression of activin in the latter region should further increase the availability of activin in area CA3, given the high diffusibility of this molecule[9,10].

Given the considerable effect of bFGF on activin A expression in hippocampi exposed to KA, this cytokine might be involved in the neuroprotective effects ascribed to bFGF. In a first step, we examined whether exogenous recombinant activin A would prevent neuronal damage when co-injected with KA. Whereas a low concentration of activin A (0.5 μg/kg body weight) failed to abrogate neuronal damage in the CA3 region ($n = 3$, Fig. 3*e*), a higher concentration (2.5 μg/kg body weight) did afford neuroprotection ($n = 6$; Fig. 3*b* and *e*). In contrast, co-injection of KA with the same concentration of BSA did not prevent the lesion, demonstrating the specificity of the activin effect ($n = 3$; Fig. 3*a* and *e*). The therapeutic potential of exogenous activin A was confirmed in a second set of experiments in which the cytokine was continuously infused into the ventricle for 7 days by means of an osmotic minipump ($n = 7$; Fig. 3*d* and *e*). We obtained identical results in mice exposed to KA receiving continuous infusion of bFGF instead of activin A ($n = 4$; Fig. 3*e*). In contrast, minipump infusion of vehicle alone did not abrogate KA lesions ($n = 4$; Fig. 3*c* and *e*)

This finding demonstrated that exogenous activin A protects neurons against KA excitotoxicity *in vivo*, extending observations made in cell culture and other lesion models[11-13]. It also raised the question of whether upregulation of endogenous activin A is essential for the neuroprotective action of bFGF. To address this, we used the soluble activin-binding protein follistatin, which neutralizes activin *in vitro* and *in vivo*[14,15]. Injection of follistatin alone did not affect the density of CA3 neurons ($n = 3$;

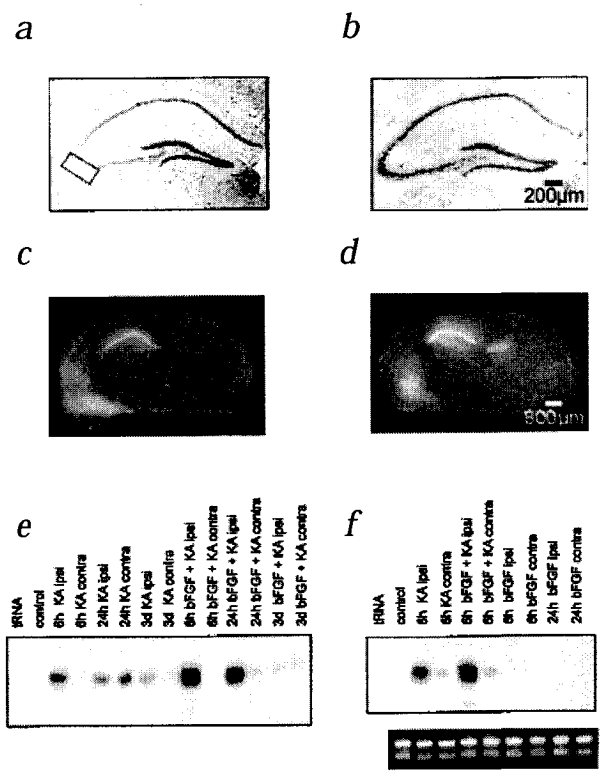

Fig. 1 Neuroprotection by bFGF is associated with increased expression of activin βA mRNA. Mice were injected with KA (***a*** and ***e***) or co-injected with KA and bFGF (***b*** and ***d***) and killed 24 h later. Sections from contralateral hippocampi did not show neuronal loss (data not shown). Lesion in the ipsilateral hippocampal CA3 region of mice injected with KA is prevented in mice co-injected with bFGF (box in *a*, area used for cell counts). *In situ* hybridization shows that the expression of activin βA mRNA in the ipsilateral hippocampus is stronger and more-extended in bFGF-protected mice (*d*) than in lesioned mice (*c*). ***e*** and ***f***, RNase protection assays using ipsilateral (ipsi) and contralateral (contra) hippocampi from mice injected with KA or bFGF, or co-injected with KA and bFGF and killed before (control) and at different times after injection (above gels). Bottom (*f*), RNA quality and loading control.

Fig. 3*j*). Both co-injection of follistatin with bFGF/KA ($n = 5$; Fig. 3*g* and *j*) and continuous infusion of follistatin for 7 days with bFGF/KA being administered 2 days after minipump implantation ($n = 4$; Fig. 3*i* and *j*) resulted in the consistent failure of bFGF to protect CA3 neurons against KA lesions. In contrast, co-injection ($n = 4$) or minipump infusion ($n = 7$) of vehicle containing BSA instead of follistatin did not affect the neuroprotective action of bFGF (Fig. 3*f*, *h* and *j*), demonstrating the specificity of the effect of follistatin. These results provide strong evidence for an essential function for endogenous activin A in the neuroprotective effect of exogenously administered bFGF.

Thus, within few hours of acute brain injury, expression of both bFGF and activin A is strongly upregulated[7,16,17]. As activin A induction is detected as early as 1 hour after injury[16], its upregulation is unlikely to depend solely on preceding bFGF expression. Instead, bFGF will further augment induction of activin A. Despite this presumed interaction, levels of endogenous activin A in area CA3 after lesion are apparently too low to protect endangered neurons against KA lesion. In contrast, the apparently higher activin level in the CA1 region (Fig. 1*c*) might explain at least in part why CA1 neurons are not vulnerable to the injection of KA. Only after administration of exogenous bFGF, endogenous activin A production in the CA3 region is boosted into a therapeutically relevant range. The injected follistatin neutralizes enough activin A in area CA3 to abrogate the neuroprotection produced by bFGF, but fails to functionally antagonize the much higher activin levels in area CA1.

In addition to binding to activin A, follistatin binds to bone morphogenetic proteins 2, 4 and 7 (ref. 18,19), which also belong to the transforming growth factor (TGF)-β superfamily. We therefore cannot entirely dismiss the possibility that these molecules contribute to the beneficial effects of bFGF, but we are not aware of any data indicating a bFGF-dependent or bFGF-independent induction of bone morphogenetic proteins after brain injury. Furthermore, the affinity of follistatin for bone morphogenetic proteins is substantially lower than its affinity for activin (dissociation constants, 23 nM and 0.5–0.7 nM, respectively)[19,20]. As follistatin does not bind TGF-β (ref. 14), this factor is apparently not involved in the neuroprotective effect of bFGF in our lesion model. Therefore, the effect of follistatin is most likely due to the inhibition of activin function.

Recently, a different type of interaction between bFGF and the TGF-β superfamily was reported from cultured midbrain dopaminergic neurons, in which the neurotrophic effect of bFGF was mediated by TGF-β1–3 (ref. 21). In contrast to activin A, which we have shown here to be of neuronal origin in lesioned hippocampus, the effect of the TGF-β molecules was mediated by co-cultured glial cells. Our study has demonstrated the clinical relevance of such an interaction between the FGF and TGF-β superfamilies *in vivo*. As it is becoming evident that activin A is essential for the neuroprotective effects of bFGF in acute brain injury, development of substances that influence activin expression or receptor binding should offer new therapeutic venues.

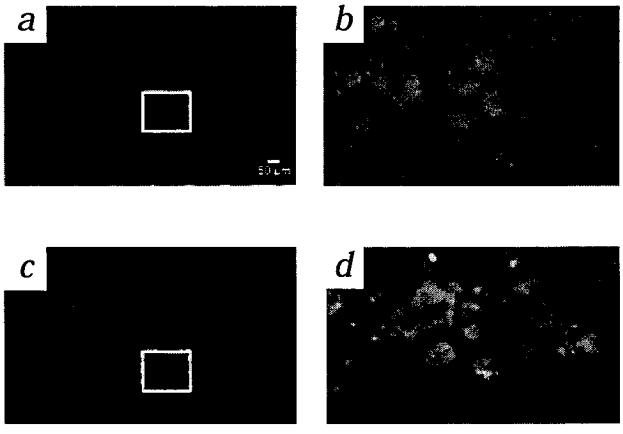

Fig. 2 Activin is of neuronal origin. CA3 regions of mice injected with KA (***a*** and ***b***) or co-injected with KA and bFGF (***e*** and ***d***) 24 h after lesion. *a* and *c*, Single staining with an activin-specific antiserum (red color) at lower magnification. *b* and *d*, Double immunofluorescence with antibodies against activin (red) and the neuronal marker protein MAP-2 (green) at higher magnification (whites squares (*a* and *c*), areas used for higher magnification). Activin-expressing neurons appear yellow/orange after superimposition. Activin-immunoreactive neurons in protected CA3 regions have higher density than those in lesioned CA3 regions.

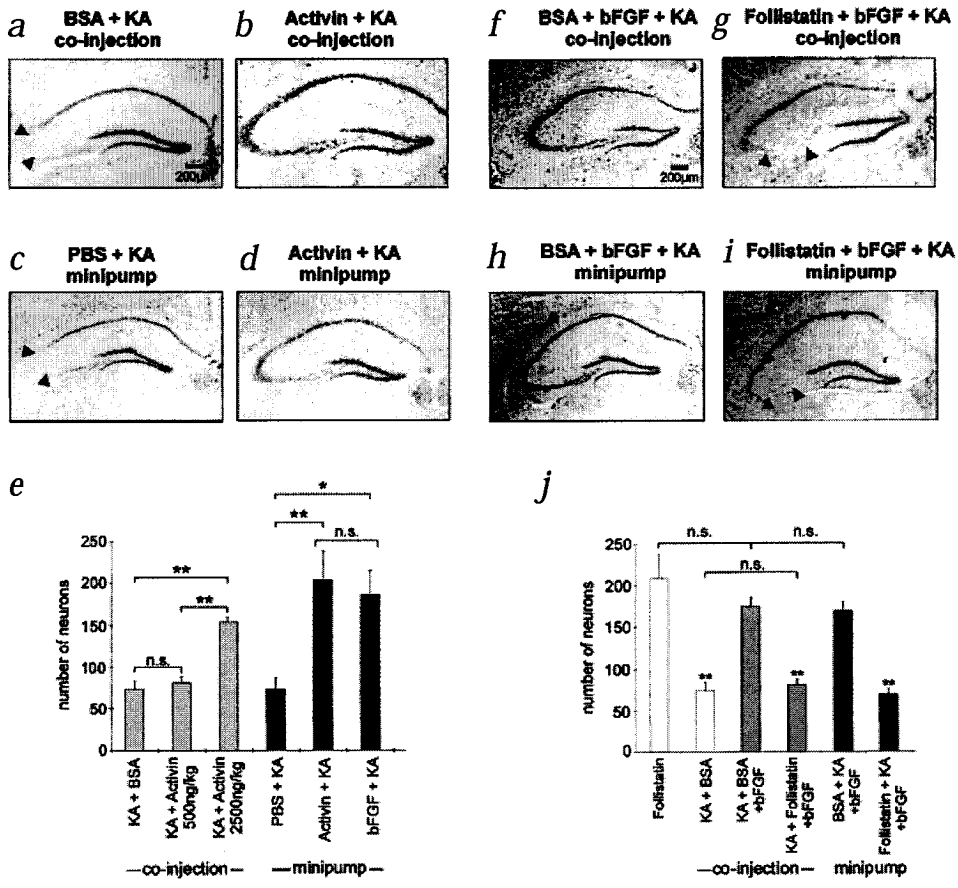

Fig. 3 Neuroprotective function of exogenous and endogenous activin. ***a*** and ***b***, Mice were injected with KA (with BSA dissolved in PBS added; *a*) or co-injected with KA and recombinant activin A (2.5 µg/kg body weight; *b*). Activin A protected against KA excitotoxicity (arrowheads (*a*), lesion). ***e*** and ***d***, Continuous intracerebroventricular infusion of activin A (*d*) rescues neurons from the excitotoxic cell death occurring under control conditions (arrowheads, *c*). ***e***, Effects of the different treatment protocols on neuronal survival in area CA3 of ipsilateral hippocampus. Grey bars, co-injection; filled bars, minipump. *, $P < 0.01$; **, $P < 0.001$; n.s., not significant. ***f*** and ***g***, bFGF and KA were co-injected together with BSA (dissolved in vehicle; *f*) or follistatin (*g*). ***h*** and ***i***, Alternatively, a combination of bFGF and KA was injected into the ventricle of mice receiving an intracerebroventricular infusion of BSA (dissolved in vehicle; *h*) or follistatin (*i*) through osmotic minipumps. Both methods of follistatin application abolish the neuroprotective effect of bFGF, as indicated by the appearance of typical CA3 lesions (arrowheads, *g* and *i*). ***j***, Quantification of neuronal survival in area CA3 of ipsilateral hippocampus. Grey bars summarize all co-injection experiments; filled bars summarize all minipump experiments; open bars, control experiments not shown as micrographs (follistatin alone, $n = 3$; co-injection of KA/BSA, $n = 3$). **, $P < 0.001$, compared with like-colored columns; n.s., not significant.

Also, unraveling the molecular targets of activin, few of which are known at present, should yield new insights into basic mechanisms of neuroprotection.

Methods

Hippocampal kainate lesions and intracerebroventricular substance application. Male C57Bl/6 mice (8–12 weeks old) were obtained from Charles River Laboratories (Sulzfeld, Germany). The mice were anesthetized by intraperitoneal injection of 7% chloral hydrate (0.01 ml/g body weight) and placed into a stereotaxic apparatus as described[7]. Activin A (0.5 or 2.5 µg/kg body weight, in PBS), bFGF (1 µg/kg body weight, in PBS), and follistatin (2.5 µg/kg, diluted in PBS from a stock solution in 1 N acetic acid) were co-injected in different combinations with 0.2 µl KA (1 µg/µl in PBS, pH 7.0) unilaterally into the right ventricle (coordinates from bregma: –2.0 mm antero–posterior, –3.8 mm dorso–ventral and –2.9 mm lateral). The substitution of active substances with BSA (2.5 µg/kg diluted in PBS or PBS and acetic acid) served to exclude unspecific effects of the injected volume and its protein content. Human bFGF was purchased from Roche Biochemicals (Mannheim, Germany). Recombinant human follistatin produced in CHO cells was provided by A.F. Parlow (National Hormone and Pituitary Program, Torrance, California). Recombinant human activin A was provided by D. Gospodarowicz (Chiron Corp., Emeryville, California). Human activin A is fully active on rodent cells[22]. Mice were anesthetized and killed at different times after the lesion (6 h, 24 h and 3 d). 'Sham-operated' (injection of solvents alone) and unoperated mice served as controls. Non-wounded and wounded ipsilateral and contralateral hippocampi were removed, immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA isolation. For *in situ* hybridizations, whole brains were removed, frozen on dry ice and stored at –80 °C.

Osmotic minipump implantation. Mice were anesthetized with 7% chloral hydrate. Osmotic minipumps (model 1007D; Alza, Palo Alto, California) filled with activin A (490 ng/kg per h in PBS), bFGF (230 ng/kg per h in PBS), follistatin (740 ng/kg per h in PBS and acetic acid) or PBS alone, were implanted subcutaneously into the back of the mouse, and a cannula connected through a catheter to the minipump was placed stereotactically in the left lateral ventricle (coordinates from bregma: –0.14 mm antero–posterior, –2.5 mm dorso–ventral and +0.76 mm lateral). Two days after implantation, KA alone or in combination with bFGF was injected into the right ventricle as described above. Continuous substance application through the minipump lasted for 5 more days, then the mice were anesthetized and killed. Brains were removed, frozen on dry ice and stored at –80 °C for further processing.

RNA isolation and RNase protection analysis. RNA isolation and RNase protection assays were done as described[23]. RNA was pooled from the hippocampi of two to four mice. As a loading control, 1 µg of the same set of RNA was separated by 1% agarose gel electrophoresis and stained with ethidium bromide. The RNase protection assay was done with three different sets of RNAs from independent injury experiments.

***In situ* hybridization.** *In situ* hybridization studies used a synthetic oligodeoxyribonucleotide probe essentially as described elsewhere[24]. Frozen coronal brain sections 10 μm in thickness were mounted on poly-L-lysine coated slides. Air-dried sections were post-fixed for 5 min in ice-cold 4% paraformaldehyde, washed in 1× PBS and dehydrated in 70% and 100% ethanol for 5 min. Sections were stored in a freezer at -80 °C. The following antisense oligonucleotide 48 nucleotides in length was used for activin β_A:5′-AATCCAGCAACTTGCCAACAGAAATC-CTCTCAGCCAAAGCAAGGGCAT-3′. The oligonucleotide was diluted to 0.3 pmol/μl and labeled by terminal desoxynucleotidyl transferase (Roche, Mannheim, Germany) with 10 pmol ^{32}P-dATP (110 TBq/mol (3,000 Ci/mmol); Hartmann Analytic, Braunschweig, Germany). Sections were hybridized overnight at 42 °C with 100 μl hybridization buffer (50% formamide, 4× SSC, 10% dextrane sulfate, 5× Denhardt's solution, 200 μg/ml sonicated salmon sperm DNA, 100 μg/ml long chain polyadenylic acid, 25 mM sodium phosphate, pH 7.0, and 1 mM sodium pyrophosphate) and 2× 10^5 counts per minute radiolabeled oligonucleotide per slide. After hybridization, sections were washed in 1× SSC at 55 °C for 30 min, then rinsed in 1× SSC, 0.1× SSC, 70% ethanol and 95% ethanol at room temperature. The air-dried sections were then exposed for 24 h at -80 °C to Hyperfilm™ βmax (Amersham, Braunschweig, Germany). After development, sections were counterstained with cresyl violet.

Quantification of neuronal survival. Brain sections (10 μm in thickness) containing the hippocampus and stained with cresyl violet were placed under an Olympus upright microscope. Using a computer-based image analysis program (Neurolucida; BioMetric Systems, Weiterstadt, Germany), a rectangular frame (200 × 400 μm) was superimposed onto the CA3 region of the hippocampi (Fig. 1*a*) or, in one series of nerve cell counts, onto the CA1 region, and two independent researchers unaware of the preceding *in vivo* treatment protocol counted the number of stained neurons within the frame. From each hippocampus, three sections were examined, taken at the following anteroposterior coordinates from bregma: -1.8 mm, -2.0 mm and -2.2 mm. Data are presented as histograms with error bars indicating s.e.m. Statistical analysis (one-way ANOVA with Bonferroni post-test comparison) was done with the use of Graphpad prism 2.0.

Immunofluorescence. Air-dried sections (prepared as described above) were fixed for 20 min in ice-cold acetone. Sections were incubated overnight at 4 °C using a 1:1,000 dilution of a polyclonal activin antiserum that does not cross-react with TGF-β (ref. 25) or with a fluorescein-isothiocyanate-coupled antibody directed against MAP-2 protein (1:500 dilution; Leinco Technologies, St. Louis, Missouri). Antibody-bound activin was visualized with antibody against rabbit IgG conjugated to Texas Red (1:50 dilution; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Sections treated without the primary antibodies were used as controls.

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1. Ay, H., Ay, I., Koroshetz, W.J. & Finklestein, S.P. Potential usefulness of basic fibroblast growth factor as a treatment for stroke. *Cerebrovasc. Dis.* **9**, 131–135 (1999).
2. Bethel, A., Kirsch, J.R., Koehler, R.C., Finklestein, S.P. & Traystman, R.J. Intravenous basic fibroblast growth factor decreases brain injury resulting from focal ischemia in cats. *Stroke* **28**, 609–615 (1997).
3. Kirschner, P.B. *et al.* Basic fibroblast growth factor protects against excitotoxicity and chemical hypoxia in both neonatal and adult rats. *J. Cereb. Blood Flow Metab.* **15**, 619–623 (1995).
4. Anderson, K.J., Dam, D. Lee, S. & Cotman, C.W. Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. *Nature* **332**, 360–361 (1988).
5. Peterson, D.A., Lucidi-Phillipi, C.A., Murphy, D.P. Ray, J. & Gage, F.H. Fibroblast growth factor-2 protects entorhinal layer II glutamatergic neurons from axotomy-induced death. *J. Neurosci.* **16**, 886–898 (1996).
6. Nadler, J.V., Perry, B.W., Gentry, C. & Cotman, C.W. Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. *J. Comp. Neurol.* **192**, 333–359 (1980).
7. Tretter, Y.P. *et al.* Strong induction of activin expression after hippocampal lesion. *Neuroreport* **7**, 1819–1823 (1996).
8. Munz, B., Hübner, G., Tretter, Y., Alzheimer, C. & Werner, S. A novel role of activin in inflammation and repair. *J. Endocrinol.* **161**, 187–193 (1999).
9. McDowell, N., Zorn, A.M., Crease, D.J. & Gurdon, J.B. Activin has direct long-range signaling activity and can form a concentration gradient by diffusion. *Curr. Biol.* **7**, 671–681 (1997).
10. Munz, B. *et al.* Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. *EMBO J.* **18**, 5205–5215 (1999).
11. Krieglstein, K., Suter-Crazzolara, C., Fischer, W.H. & Unsicker, K. TGF-β superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP$^+$ toxicity. *EMBO J.* **14**, 736–742 (1995).
12. Wu, D.D. *et al.* Expression of the activin axis and neuronal rescue effects of recombinant activin A following hypoxic-ischemic brain injury in the infant rat. *Brain Res.* **835**, 369–378 (1999).
13. Hughes, P.E., Alexi, T., Williams, C.E., Clark, R.G. & Gluckman, P.D. Administration of recombinant human activin-A has powerful neurotrophic effect on select striatal phenotypes in the quinolinic acid lesion model of Huntington's disease. *Neuroscience* **92**, 197–209 (1999).
14. Nakamura, T. *et al.* Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836–838 (1990).
15. de Winter, J.P. *et al.* Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. *Mol. Cell. Endocrinol.* **116**, 105–114 (1996).
16. Lai, M., Gluckman, P., Dragunow, M. & Hughes, P.E. Focal brain injury increases activin βA mRNA expression in hippocampal neurons. *Neuroreport* **8**, 2691–2694 (1997).
17. Riva, M.A., Donati, E., Tascedda, F., Zolli, M. & Racagni, G. Short- and long-term induction of basic fibroblast growth factor gene expression in rat central nervous system following kainate injection. *Neuroscience* **59**, 55–65 (1994).
18. Fainsod, A. *et al.* The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* **63**, 39–50 (1997).
19. Iemura, S.-I. *et al.* Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* 95, 9337–9342 (1998).
20. Sugino, H., Sugino, K., Hashimoto, O., Shoji, H. & Nakamura, T. Follistatin and its role as an activin-binding protein. *J. Med. Invest.* **44**, 1–14 (1997).
21. Krieglstein, K., Reuss, B., Maysinger, D. & Unsicker K. Transforming growth factor-β mediates the neurotrophic effect of fibroblast growth factor-2 on midbrain dopaminergic neurons. *Eur. J. Neurosci.* **10**, 2746–2750 (1998).
22. Yasuda, H. *et al.* Activin A: An autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *J. Clin. Invest.* **92**, 1491–1496 (1993).
23. Werner, S. *et al.* Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. *EMBO J.* **12**, 2635–2643 (1993).
24. Wisden, W., Morris, B.J. & Hunt, S.P. in *Molecular Neurobiology—A Practical Approach* (eds. Chad, J. & Wheal, H.) 205–225 (Oxford University Press, New York, 1991).
25. Hübner, G. & Werner, S. Serum growth factors and proinflammatory cytokines are potent inducers of activin expression in cultured keratinocytes. *Exp. Cell Res.* **228**, 106–113 (1996).

5.3 CTGF Publication

European Journal of Neuroscience, Vol. 12, pp. 376–380, 2000

SHORT COMMUNICATION
Connective tissue growth factor: a novel player in tissue reorganization after brain injury?

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Abstract

Recent studies have suggested a role of connective tissue growth factor (CTGF) in repair processes of the skin as well as in various types of fibrotic disease. However, a function of this molecule in central nervous system (CNS) repair has not been demonstrated yet. In this study we analysed the temporal and spatial expression pattern of CTGF after unilateral kainic acid lesions of the hippocampal CA3 region in mice. We found a strong induction of CTGF mRNA and protein expression in neurons and glial cells of the lesioned hippocampus. Interestingly, increased expression of this mitogen was accompanied by elevated levels of the extracellular matrix molecule fibronectin, which is a known target of CTGF action. Therefore, our data indicate a novel function of CTGF in postlesional restructuring of the hippocampus, where it possibly participates in glial scar formation.

Introduction

Connective tissue growth factor (CTGF) is a member of a rapidly growing protein family, now designated as the CCN (CTGF/cysteine-rich 61/nephroblastoma overexpressed) family (reviewed by Brigstock, 1999). CTGF is expressed in a wide variety of tissues and organs (Dammeier *et al.*, 1998a; Brigstock, 1999). It stimulates proliferation and chemotaxis of fibroblasts directly (Bradham *et al.*, 1991), and it enhances the mitogenic effect of other growth factors (Kireeva *et al.*, 1997). Furthermore, CTGF strongly stimulates expression of the extracellular matrix proteins collagen type I and fibronectin as well as of integrin α5 by fibroblasts (Frazier *et al.*, 1996). Due to these properties, CTGF plays an important role in connective tissue cell proliferation and extracellular matrix deposition. Interestingly, it seems to act as a mediator of transforming growth factor beta 1 (TGF-β1) in these processes (Kothapalli *et al.*, 1997, 1998). A series of studies have demonstrated a strong overexpression of CTGF in various types of fibrotic and inflammatory disease, including fibrotic skin disease (Igarashi *et al.*, 1995, 1996), lung and kidney fibrosis (Ito *et al.*, 1998; Lasky *et al.*, 1998), advanced arteriosclerotic lesions (Oemar *et al.*, 1997), and inflammatory bowel disease (Dammeier *et al.*, 1998b). In addition to these pathological situations, increased levels of CTGF mRNA and/or protein have been observed in healing skin wounds (Igarashi *et al.*, 1993; Dammeier *et al.*, 1998a). By contrast, a role of CTGF in repair processes of the central nervous system (CNS) has not been demonstrated yet. Because CTGF strongly promotes extracellular matrix gene expression, we speculated about a novel role of CTGF in glial scarring after injury to the CNS. To test this hypothesis we analysed the expression of CTGF and its target molecule fibronectin in a well-established model of local excitotoxic brain damage (Ben-Ari, 1985), where intracerebroventricular injection of the rigid glutamate analogon, kainic acid (KA), results in a unilateral lesion of area CA3 of the hippocampus. We report here that KA lesions induce CTGF mRNA and protein expression in the lesioned hippocampus, accompanied by an increased production of fibronectin. These data implicate CTGF in postlesional restructuring of CNS tissue, its predominant role being promotion of glial scar formation.

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Materials and methods

Hippocampal KA lesions

Male C57/Bl6 mice (22 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). They were housed and fed according to federal guidelines, and all surgical procedures were approved by the local government of Bavaria. KA lesions were produced as recently described (Tretter *et al.*, 1996). Briefly, animals were anaesthetized by i.p. injection of 7% chloralhydrate (0.1 mg/g body weight). KA (0.25 μL, 1 μg/μL in phosphate-buffered saline) was injected within 1 min unilaterally into the right lateral ventricle using a stereotaxic apparatus (coordinates – distances from bregma: –2.0 mm anteroposterior, –3.8 mm dorsoventral and –2.9 mm lateral) in conjunction with a Hamilton microlitre syringe.

Tissue preparation, RNA isolation and RNase protection assay

Mice were killed at different time points after KA injection. Complete ipsilateral and contralateral hippocampi were removed, immediately frozen in liquid nitrogen and stored at –70 °C until used for RNA isolation. Isolation of total RNA was performed as described by Chomczynski & Sacchi (1987). RNase protection assays were carried out according to Werner *et al.* (1992). Twenty-microgram RNA aliquots were hybridized with ^{32}P-labelled riboprobes complementary to murine CTGF (fisp12) and fibronectin. A 170-bp fragment of the murine CTGF cDNA (Dammeier *et al.*, 1998a) and a

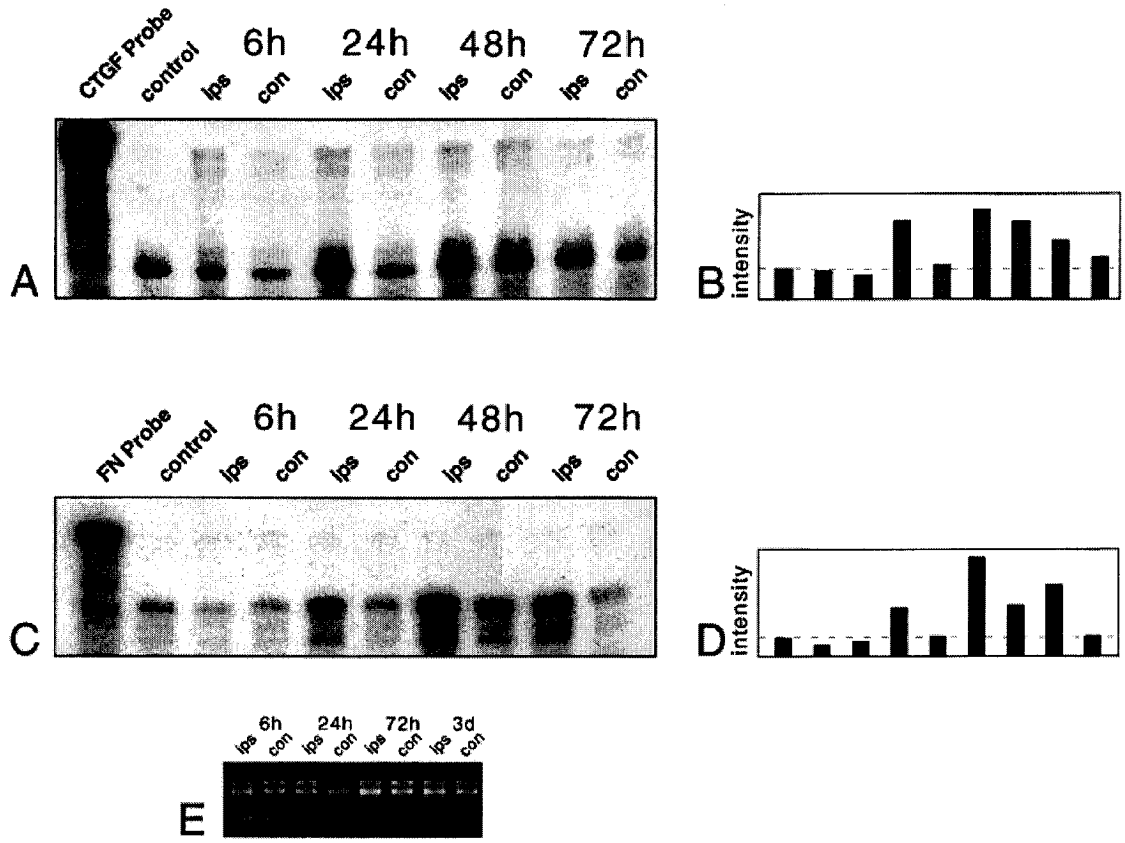

FIG. 1. RNase protection assay demonstrating increased levels of CTGF and fibronectin mRNAs in the lesioned hippocampus after KA lesion. RNA was isolated from non-injured hippocampi (control) and from the ipsilateral (ips) and contralateral (con) hippocampi at different time points after KA injection as indicated. Twenty micrograms of total RNA was analysed by RNase protection assay for the presence of (A) CTGF and (C) fibronectin mRNAs. Hybridization probes (1000 c.p.m.) were loaded in the lanes labelled 'probe' and used as size markers. The signal intensities (arbitrary units) as determined by phosphoimaging are shown in B and D. The same batch of RNAs was used for both RNase protection assays. One microgram of the same RNAs was loaded on a 1% agarose gel prior to hybridization and stained with ethidium bromide (E).

162-bp fragment from the 3′-end of the mouse fibronectin cDNA (kindly provided by Dr P. Ekblom) were used as templates. As a loading control, 1 μg of the RNA samples was loaded on a 1% agarose gel prior to hybridization and stained with ethidium bromide.

Immunohistochemistry and immunofluorescence

Complete brains were removed at different time points after KA injection and frozen in tissue-freezing medium (Jung, Nussloch, Germany). Six-micrometre frozen sections were fixed with cold acetone. For immunohistochemistry they were treated for 4 min at room temperature with 0.3% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase activity and subsequently incubated with a 1 : 300 dilution of a polyclonal fibronectin antibody (Dako, Hamburg, Germany). Antibody-binding cells were stained with the avidin–biotin–peroxidase complex system (Vector Laboratories, Burlingame, USA) using diaminobenzidine as a chromogenic substrate. After development, sections were stained with cresyl violet. For immunofluorescence, the sections were incubated with the fibronectin antibody (1 : 300 diluted), an affinity-purified rabbit polyclonal antiserum directed against a carboxyterminal peptide of CTGF (Dammeier *et al.*, 1998a) (1 : 300 diluted) or with an antibody directed against glial fibrillary acidic protein (GFAP, Progen Biotechnik, Heidelberg, Germany, 1 : 50 diluted). Antibody-binding cells were visualized with antirabbit-IgG-FITC (Roche Biochemicals, Mannheim, Germany) or antiguinea-pig-IgG-Texas Red (Jackson Immuno Research, West Grove, PA, USA, 1 : 50 diluted). Sections treated without the primary antibody or with the primary antibody which had been pretreated overnight at 4 °C with the peptide used for immunization were used as controls. Slides were analysed with a Zeiss Axioplan microscope or with a Leitz confocal microscope (TCSNT Laser).

Results and discussion

Stereotactic injection of the excitotoxin KA into the ventricle resulted in a severe lesion in the CA3 region of the ipsilateral but not contralateral hippocampus of mice (Tretter *et al.*, 1996). To determine the expression of CTGF in normal and lesioned hippocampus, mice were killed at different time points after KA injection. Ipsilateral and contralateral hippocampi were removed and used for RNA isolation. RNA from non-injured hippocampi was used as a control. As shown in Fig. 1A and B, a basal level of CTGF mRNA expression was observed in the non-injured hippocampus. Within 24–48 h of injection, a strong induction of CTGF expression was seen in the ipsilateral hippocampus, accompanied by less pronounced induction in the contralateral hippocampus after 48 h. This peak of induction was followed by a decline within the next 24 h (Fig. 1A and B). This

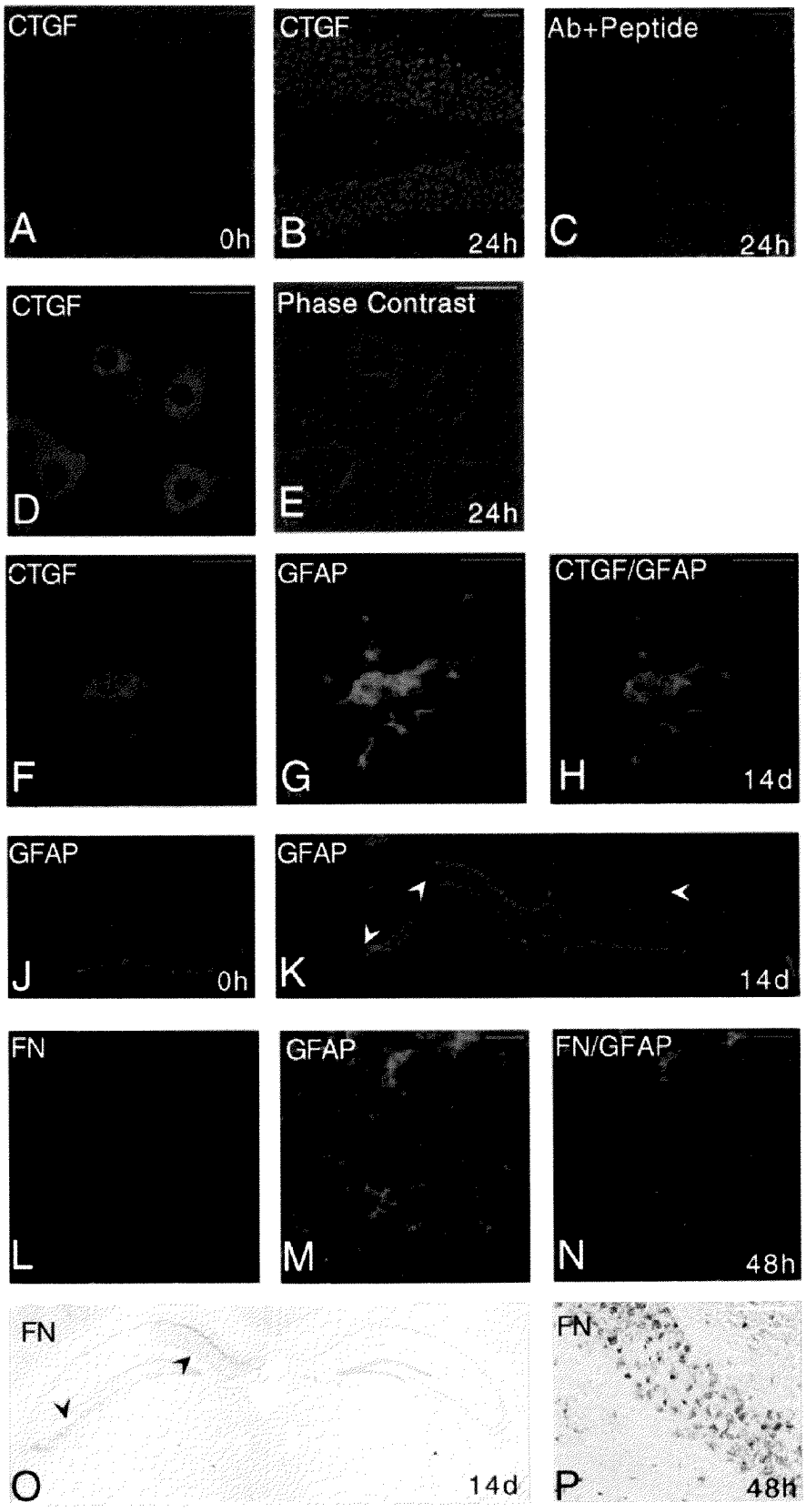

FIG. 2. Immunolocalization of CTGF and fibronectin in the injured ipsilateral hippocampus. Mice were killed before (0 h) or at different time points after KA injection as indicated. The complete brains were isolated and embedded in tissue-freezing medium. Six-micrometre frozen sections were incubated with antibodies against CTGF (A–F, H), GFAP (G, H, J, K, M and N) and fibronectin (L, N–P), and visualized using FITC- or Texas Red-coupled secondary antibodies (A–N) or a diaminobenzidine substrate kit (brown colour; O and P). Immunostaining with the CTGF antibody which had been pretreated with the immunization peptide is shown in C. A phase contrast picture is shown in E. The complete area around the hippocampus at day 14 after KA injection is shown in K and O. The left side of a non-injured hippocampus is shown in J. Areas of the ipsilateral dentate gyrus are shown in (A–H). An area above the dentate gyrus is shown in (L–N). An area of the CA3 region of the ipsilateral hippocampus is shown in P. The pictures shown in (D–H) were taken with a confocal microscope. Scale bars, 15 μm (D–H); 100 μm (A–C and P); 500 μm (J, K and O).

result was reproduced in three RNase protection assays using RNAs from independent injury experiments.

Because CTGF has been shown to stimulate the expression of extracellular matrix molecules in various cell types, we compared the expression of this growth factor with that of the extracellular matrix protein fibronectin. The latter has been identified as a target of CTGF action in fibroblasts (Frazier *et al.*, 1996). Furthermore, it is a major constituent of the glial scar which forms after lesion to the nervous system (for review see Stichel & Muller, 1998). As shown in Fig. 1C and D, the levels of fibronectin mRNA increased significantly within 24–48 h after injury, particularly in the ipsilateral hippocampus. Most importantly, high levels of fibronectin mRNA were still observed at day 3 after KA injection. This result was reproduced in three RNase protection assays using RNAs from independent injury experiments. In addition to fibronectin, collagen type Iα mRNA was also present in the normal and lesioned hippocampus, although at very low levels. However, a minor induction in the expression of this matrix molecule was also observed after hippocampal injury, whereby the kinetics of induction were similar to that seen for fibronectin (data not shown).

To determine whether the increase in CTGF and fibronectin mRNA expression seen after hippocampal injury correlates with elevated levels of the corresponding proteins and to localize CTGF within the wounded hippocampus, frozen sections from complete brains were stained with monospecific polyclonal antisera directed against CTGF (Dammeier *et al.*, 1998a) and fibronectin. In addition, staining with an antibody against GFAP, a marker for astrocytes, was performed to identify the glial scar and to determine whether CTGF is expressed by astrocytes. As shown in Fig. 2A, CTGF protein was not detectable in the non-lesioned hippocampus. However, strong signals were observed in the CA1 and CA3 region (not shown) as well as in the dentate gyrus (Fig. 2B) of the ipsilateral (lesioned) hippocampus as early as 24 h after KA injection. The specificity of these signals was confirmed by immunostaining without the primary antibody (data not shown) or with the primary antibody which had been pretreated with the peptide used for immunization (Fig. 2C). At higher magnification the majority of the CTGF-positive cells were identified as neurons (Fig. 2D and E). A confocal analysis revealed a strong cytoplasmic staining of these cells (Fig. 2D). Because a substantial amount of the CTGF-positive neurons in the pyramidal cell layer die within the first days after KA injection (Faherty *et al.*, 1999), the CTGF in the cytoplasm might be released and become available for other cells. At later stages of the repair process (day 14 after KA injection) CTGF was also found extracellularly as well as in cells with an astrocyte-like morphology (Fig. 2F). Indeed, double-staining with antibodies directed against CTGF (green) and GFAP (red) revealed the presence of CTGF protein in GFAP-positive astrocytes (Fig. 2F–H). These results demonstrate that the KA-induced lesion is accompanied by a strong induction of CTGF mRNA and protein expression in both neurons and astrocytes. The reason for the increased expression of CTGF after injury is presently unknown. The early increase in the mRNA levels of this growth factor after KA injection suggests a direct effect. Because expression of TGF-β1, the major CTGF-inducer, is also increased after KA lesion (Morgan *et al.*, 1993), it is well conceivable that the induction of TGF-β1 contributes to the elevated levels of CTGF at later stages of the repair process.

We subsequently localized fibronectin in the injured brain. As expected from the mRNA data, fibronectin was detected at particularly high levels in the CA3 region of the ipsilateral (lesioned) hippocampus at early stages (48 h) after KA injection (Fig. 2P). At later stages (14 days) a strong deposition of fibronectin had occurred, particularly within the CA1 and CA3 regions of the ipsilateral hippocampus (Fig. 2O). A high density of GFAP-positive astrocytes was also observed in these regions of the lesioned (Fig. 2K, left side; indicated by arrows) but not non-injured hippocampus (Fig. 2J), suggesting that the fibronectin is produced by these cells. Indeed, costaining with antibodies against fibronectin (green) and GFAP (red) revealed that fibronectin is associated with astrocytes (Fig. 2L–N). This finding is consistent with previous data showing astroglial fibronectin expression in the hippocampus of epileptic rats (Niquet *et al.*, 1994).

The induction of glial scar formation has been attributed to various growth factors, particularly to TGF-β1 (Logan *et al.*, 1994a,b). Because the effect of this growth factor on extracellular matrix production by fibroblasts has been shown to be mediated by CTGF (Kothapalli *et al.*, 1997, 1998), it seems possible that CTGF is also responsible for the effect of TGF-β1 on glial scar formation. This hypothesis is supported by the temporal and spatial coexpression of CTGF and fibronectin which indicates that the CTGF which is produced by neurons and astrocytes could stimulate fibronectin production by astrocytes in a paracrine and possibly also autocrine manner. These results shed new light on the orchestration of the scarring response, suggesting that CTGF might emerge as a major promoter of glial scar formation and organization. Future studies using CTGF antagonists, which have already been suggested to be effective in the treatment of other types of fibrotic processes (Grotendorst, 1997), should help to elucidate the role of CTGF in the scarring response of CNS tissue.

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Abbreviations

CNS, central nervous system; CTGF, connective tissue growth factor; GFAP, glial fibrillary acidic protein; KA, kainic acid; TGF-β, transforming growth factor β.

References

Ben-Ari, Y. (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience*, **14**, 375–403.

Bradham, D.M., Igarashi, A., Potter, R.L. & Grotendorst, G.R. (1991) Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J. Cell Biol.*, **114**, 1285–1294.

Brigstock, D.R. (1999) The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocrine Rev.*, **20**, 189–206.

Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **62**, 156–159.

Dammeier, J., Beer, H.-D., Brauchle, M. & Werner, S. (1998a) Dexamethasone is a novel potent inducer of connective tissue growth factor expression: implications for glucocorticoid therapy. *J. Biol. Chem.*, **273**, 18 185–18 190.

Dammeier, J., Brauchle, M., Falk, W., Grotendorst, G.R. & Werner, S. (1998b) Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *Int. J. Biochem. Cell. Biol.*, **30**, 909–922.

Faherty, C.J., Xanthoudakis, S. & Smeyne, R.J. (1999) Caspase-3-dependent neuronal death in the hippocampus following kainic acid treatment. *Brain Res. Mol. Brain Res.*, **70**, 159–163.

Frazier, K., Williams, S., Kothapalli, D., Klapper, H. & Grotendorst, G.R. (1996) Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J. Invest. Dermatol.*, **107**, 404–411.

Grotendorst, G.R. (1997) Identification and development of novel antifibrotic agents. *Exp. Opin. Invest. Drugs*, **6**, 777–781.

Igarashi, A., Okochi, H., Bradham, D.M. & Grotendorst, G.R. (1993) Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol. Cell. Biol.*, **4**, 637–645.

Igarashi, A., Nashiro, J., Kikuchi, K., Sato, S., Ihn, H., Grotendorst, G.R. & Takehara, K. (1995) Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J. Invest. Dermatol.*, **105**, 280–284.

Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Grotendorst, G.R. & Takehara, K. (1996) Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J. Invest. Dermatol.*, **106**, 729–733.

Ito, Y., Aten, J., Bende, R.J., Oemar, B.S., Rabelink, T.J., Weening, J.J. & Goldschmeding, R. (1998) Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int.*, **53**, 853–861.

Kireeva, M.L., Latinki, B.V., Kolesnikova, T.V., Chen, C.-C., Yang, G.P., Abler, A.S. & Lau, L.F. (1997) Cyr61 and Fisp12 are both ECM-associated signalling molecules: activities, metabolism, and localization during development. *Exp. Cell Res.*, **233**, 63–77.

Kothapalli, D., Frazier, K.S., Welply, A., Segarini, P.R. & Grotendorst, G.R. (1997) Transforming growth factor β induces anchorage-independent growth of NRK fibroblasts via a connective tissue growth factor-dependent pathway. *Cell Growth Differ.*, **8**, 61–68.

Kothapalli, D., Hayashi, N. & Grotendorst, G.R. (1998) Inhibition of TGF-β-stimulated CTGF gene expression and anchorage-independent growth by cAMP identifies a CTGF-dependent restriction point in the cell cycle. *FASEB J.*, **12**, 1151–1161.

Lasky, J.A., Ortiz, L.A., Tonthat, B., Hoyle, G.W., Corti, M., Athas, G., Lungarella, G., Brody, A. & Friedman, M. (1998) Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. *Am. J. Physiol.*, **275**, L365–L371.

Logan, A., Berry, M., Gonzalez, A.M., Frautschy, S.A., Sporn, M.B. & Baird, A. (1994a) Effects of transforming growth factor β1 on scar production in the injured central nervous system of the rat. *Eur. J. Neurosci.*, **6**, 355–363.

Logan, A., Oliver, J.J. & Berry, M. (1994b) Growth factors in CNS repair and regeneration. *Progr. Growth Factor Res.*, **5**, 379–405.

Morgan, T.E., Nichols, N.R., Pasinetti, G.M. & Finch, C.E. (1993) TGF-β1 mRNA increases in macrophage/microglial cells of the hippocampus in response to deafferentation and kainic acid-induced neurodegeneration. *Exp. Neurol.*, **120**, 291–301.

Niquet, J., Jorquera, I., Ben-Ari, Y. & Represa, A. (1994) Proliferative astrocytes may express fibronectin-like protein in the hippocampus of epileptic rats. *Neurosci. Lett.*, **180**, 13–16.

Oemar, B.S., Werner, A., Garnier, J.-M., Do, D.-D., Godoy, N., Nauck, M., März, W., Rupp, J., Pech, M. & Lüscher, T.F. (1997) Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. *Circulation*, **95**, 831–839.

Stichel, C.C. & Muller, H.W. (1998) The CNS lesion scar: new vistas on an old regeneration barrier. *Cell Tissue Res.*, **294**, 1–9.

Tretter, Y.P., Munz, B., Hübner, G., ten Bruggencate, G., Werner, S. & Alzheimer, C. (1996) Strong induction of activin expression after hippocampal lesion. *Neuroreport*, **7**, 1819–1823.

Werner, S., Peters, K.G., Longaker, M.T., Fuller-Pace, F., Banda, M. & Williams, L.T. (1992) Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc. Natl Acad. Sci. USA*, **89**, 6896–6900.

6. Conclusions and Outlook

One of the major goals of our laboratory is the identification and functional characterisation of novel players involved in tissue repair processes. During the past few years, a series of genes have been identified that are regulated by skin injury and that play crucial roles in the regulation of cutaneous wound repair. Interestingly, many of these genes were also shown to be involved in the repair of the injured gut and liver, suggesting that different organs use similar mechanisms to heal their wounds. Thus it was particularly interesting to determine, whether the same genes are also involved in a very different type of tissue repair process, namely that of the injured CNS. The results presented in this thesis demonstrate that all three genes that have been analysed previously in the skin (Nrf-2, activin and CTGF genes) are also regulated by injury to the CNS. This finding demonstrates that the chosen strategy can indeed lead to the identification of new players in neuroprotection or tissue reorganisation after hippocampal lesion.

Nrf-1 and Nrf-2

Recent results obtained in the skin wound healing model revealed a strong increase in the expression of Nrf-2 after injury. By contrast, Nrf-1, although highly expressed in normal and wounded skin, was not regulated by wounding (S.Braun, unpublished data). Interestingly, hippocampal injury was also accompanied by up-regulation of Nrf-2 expression. In contrast to the skin, however, we also found increased levels of Nrf-1 in the injured hippocampus, and the basal and injury-regulated expression of the latter was much higher compared to Nrf-2. Thus, members of the same family are obviously involved in the repair/reorganization of both organs, although the individual family members appear to exert their function in a tissue-specific member.

Both Nrf-1 and Nrf-2 play important roles in the detoxification of ROS. This process is important in the injured skin where high levels of these aggressive molecules are produced early after injury by leukocytes in the wound tissue (Clark, 1996). In the brain, ROS are key effectors of neuronal cell death (Sée *et*

al., 2001), indicating that the up-regulation of Nrf-1 and Nrf-2 expression is an essential neuroprotective mechanism. This hypothesis is supported by my preliminary findings that demonstrated increased expression of both transcription factors after co-injection of KA and the neuroprotective growth factor bFGF. Thus it seems possible that the up-regulation of Nrf-1 and Nrf-2 is part of the neuroprotective effect of bFGF.

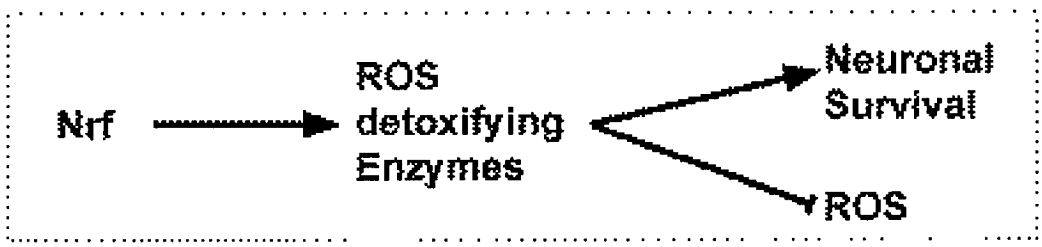

***Fig. 12**: Possible roles of Nrf-1 and Nrf-2 in the regulation of ROS-detoxifying enzymes and in neuronal survival*

To determine whether Nrf-1 and Nrf-2 are indeed neuroprotective, it would be interesting to lesion the hippocampus of mice with reduced or increased Nrf activities in the brain. Unfortunately, Nrf-1 knockout mice are not viable (Chan *et al.*, 1998) and can, therefore, not be used for hippocampal injury studies. Thus, it would be essential to generate a tissue-specific Nrf-1 knockout mouse, in which the Nrf-1 gene is only deleted in hippocampal neurons. In contrast to the Nrf-1 knockout mouse, Nrf-2 null mice are viable and could be used for hippocampal injury experiments (Chan *et al.*, 1996), although compensation exerted by the highly expressed Nrf-1 appears likely.

To inhibit both Nrf-1 and Nrf-2, a dominant-negative mutant of either transcription factor should be expressed specifically in hippocampal neurons of transgenic mice using the CAM kinase II promoter. Such a mutant lacks the transactivation domains and should, therefore, compete with wild-type Nrf-1 and Nrf-2 for binding to the ARE sequence. A cDNA encoding such a mutant has been generated in our laboratory, and this approach is currently being employed to analyse Nrf-2 function in the skin. In addition, increased Nrf-1 or Nrf-2 activity in the hippocampus could be achieved by overexpressing a

constitutively active mutant of either transcription factor in the hippocampus, and these mutants are also available in the laboratory. The use of such animals would allow us to determine whether the levels of active Nrf-1 and Nrf-2 affect neuronal death after hippocampal lesion.

Since the generation of transgenic mice is very time-consuming, it might be worthwhile to try a transient expression of these Nrf mutants in the hippocampus. In this case, eukaryotic expression vectors containing the mutant cDNAs could be directly injected into the ventricle. In preliminary experiments with a vector including the gene for green fluorescent protein, I could show that it is generally possible to express a protein in this way in the CA3 region of the hippocampus.

Recently, Kobayashi *et al.* (1999) described a third member of the Nrf family, Nrf-3, which also binds to AREs. The biological function of this protein has as yet not been characterised. Thus it would be interesting to investigate if this member of the Nrf family is also regulated after the hippocampal lesion and if it plays a role in neuroprotection.

Activin

In the second part of my thesis, activin A was identified as a novel neuroprotective growth factor, which mediates the neuroprotective effect of bFGF. However, it is as yet unclear, whether activin acts directly on neurons, or or whether the observed neuroprotective effect is indirect and involves other factors produced by glial cells. To address this question, transgenic mice are currently being generated which express either a dominant-negative or a constitutively active activin receptor in hippocampal neurons. The analysis of these animals should reveal whether the activation or inhibition of neuronal activin receptors affects the extent of the KA lesion as well as the neuroprotective effect of bFGF.

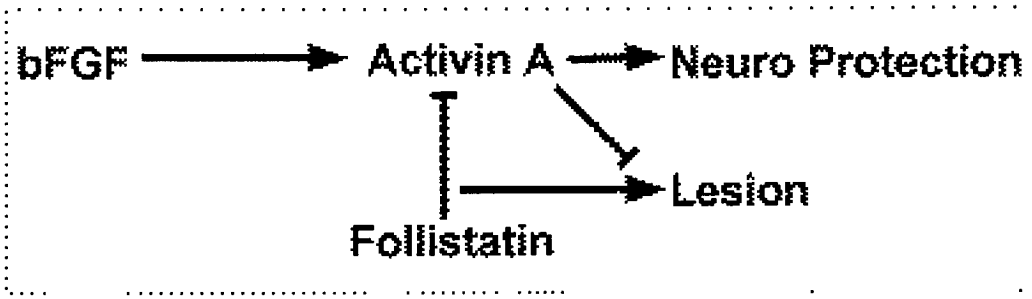

Fig.13: *Activin A mediates the neuroprotective effect of bFGF. The soluble activin binding-protein follistatin prevents the neuroprotective effects of both activin and bFGF.*

CTGF

A major problem in CNS repair is the rapid formation of a glial scar after lesion, which prevents neuronal regeneration. In the third part of my thesis I identified a putative novel player in glial scar fromation, CTGF. The latter has been shown to induce expression of extracellular matrix proteins in fibroblasts, and overexpression of CTGF is a characteristic feature of fibrotic process in various organs (reviewed by Brigstock, 1999). The strong up-regulation of CTGF only 6 h after hippocampal lesion together with the subsequent expression of a major extracellular matrix protein, fibronectin, strongly suggests a role of CTGF in glial scar formation.

The reason for the observed up-regulation of CTGF in the brain is as yet unknown. However, a recent publication reported on a role of ROS in the induction of CTGF expression in a lens epithelial cell line (Park *et al.*, 2001). Due to the abundance of ROS in the lesioned brain, a role of ROS in CTGF induction in the hippocampus appears possible. Thus it would be interesting to determine, whether modulation of Nrf-1 or –2 activity (see above) influences the expression of CTGF.

To determine the role of CTGF in glial scarring, it would be interesting to co-inject KA together with CTGF or with neutralising antibodies to this growth factor. Unfortunately, these experiments are not yet possible, since purified CTGF as well as neutralising antibodies are not yet available. Alternatively, I tried to inject antisense oligonucleotides either with or without liposomes.

However, this was as yet not successful due to the limited diffusion and/or uptake of the oligonucleotides as demonstrated by use of BrdU-labelled oligonucleotides.

Finally, the generation of CTGF knockout mice or transgenic mice that overexpress CTGF in the hippocampus should help to clarify the role of this growth factor in hippocampal repair and/or glial scarring.

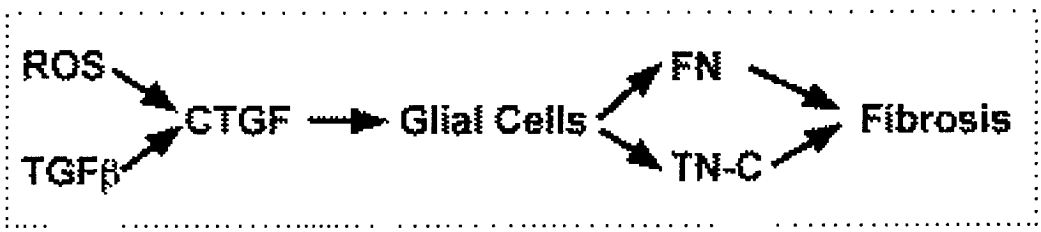

Fig.14: *CTGF induced by TGF β or ROS is inducing fibrosis in the brain and many other organs (FN=Fibronectin , TN-C=Tenascin-C)*

7. Bibliography

7.1. Journal articles

Abe, K., Iri, Y., Takayanagi, M. and Saito, H. (1991) Involvement of protein kinase activation in neurotrophic effects of basic fibroblast growth factor in cultured brain neurons. *Jpn J Pharmacol*, **56**, 563-566.

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

Amaral, D.G. and Witter, M.P. (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience*, **31**, 571-591.

Anderson, K.J., Dam, D., Lee, S. and Cotman, C.W. (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. *Nature*, **332**, 360-361.

Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res*, **19**, 2499.

Asashima, M., Kinoshita, K., Ariizumi, T. and Malacinski, G.M. (1999) Role of activin and other peptide growth factors in body patterning in the early amphibian embryo. *Int Rev Cytol*, **191**, 1-52.

Ay, H., Ay, I., Koroshetz, W.J. and Finklestein, S.P. (1999) Potential usefulness of basic fibroblast growth factor as a treatment for stroke. *Cerebrovasc Dis*, **9**, 131-135.

Baird, A. (1994) Potential mechanisms regulating the extracellular activities of basic fibroblast growth factor (FGF-2). *Mol Reprod Dev*, **39**, 43-48.

Bansal, R. and Pfeiffer, S.E. (1997) FGF-2 converts mature oligodendrocytes to a novel phenotype. *J Neurosci Res*, **50**, 215-228.

Ben-Ari, Y. (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neurosc ence*, **14**, 375-403.

Ben-Ari, Y. and Cossart, R. (2000) Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci*, **23**, 580-587.

Bethel, A., Kirsch, J.R., Koehler, R.C., Finklestein, S.P. and Traystman, R.J. (1997) Intravenous basic fibroblast growth factor decreases brain injury resulting from focal ischemia in cats. *Stroke*, **28**, 609-615; discussion 615-606.

Bettler, B. and Mulle, C. (1995) Review: neurotransmitter receptors. II. AMPA and kainate receptors. *Neuropharmacology*, **34**, 123-139.

Boldyrev, A., Song, R., Dyatlov, V.A., Lawrence, D.A. and Carpenter, D.O. (2000) Neuronal cell death and reactive oxygen species. *Cell Mol Neurobiol*, **20**, 433-450.

Bondy, S.C. and Lee, D.K. (1993) Oxidative stress induced by glutamate receptor agonists. *Brain Res*, **610**, 229-233.

Bradham, D.M., Igarashi, A., Potter, R.L. and Grotendorst, G.R. (1991) Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol*, **114**, 1285-1294.

Brigstock, D.R. (1999) The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev*, **20**, 189 206.

Brigstock, D.R., Steffen, C.L., Kim, G.Y., Vegunta, R.K., Diehl, J.R. and Haring, P.A. (1997) Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin regulated Mr 10,000 forms of connective tissue growth factor. *J Biol Chem*, **272**, 20275-20282.

Carcamo, J., Weis, F.M., Ventura, F., Wieser, R., Wrana, J.L., Attisano, L. and Massague, J. (1994) Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol Cell Biol*, **14**, 3810-3821.

Chan, J.Y., Kwong, M., Lu, R., Chang, J., Wang, B., Yen, T.S. and Kan, Y.W. (1998) Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. *Embo J*, **17**, 1779-1787.

Chan, K. and Kan, Y.W. (1999) Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci U S A*, **96**, 12731-12736.

Chan, K., Lu, R., Chang, J.C. and Kan, Y.W. (1996) NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc Natl Acad Sci U S A*, **93**, 13943-13948.

Chen, Y., Bhushan, A. and Vale, W. (1997) Smad8 mediates the signaling of the ALK-2 [corrected] receptor serine kinase. *Proc Natl Acad Sci U S A*, **94**, 12938-12943.

Choi, D.W. (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci*, **11**, 465 469.

Choi, D.W. (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci*, **18**, 58-60.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, **162**, 156-159.

Dammeier, J., Beer, H.D., Brauchle, M. and Werner, S. (1998a) Dexameth sone is a novel potent inducer of connective tissue growth factor expression. Implications for glucocorticoid therapy. *J Biol Chem*, **273**, 18185-18190.

Dammeier, J., Brauchle, M., Falk, W., Grotendorst, G.R. and Werner, S. (1998b) Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *Int J Biochem Cell Biol*, **30**, 909-922.

de Winter, J.P., ten Dijke, P., de Vries, C.J., van Achterberg, T.A., Sugino, H., de Waele, P., Huylebroeck, D., Verschueren, K. and van den Eijnden-van Raaij, A.J. (1996) Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. *Mol Cell Endocrinol*, **116**, 105-114.

Dietrich, W.D., Alonso, O., Busto, R. and Finklestein, S.P. (1996) Posttreatment with intravenous basic fibroblast growth factor reduces histopathological damage following fluid-percussion brain injury in rats. *J Neurotrauma*, **13**, 309-316.

Dong, Z., Lavrovsky, Y., Venkatachalam, M.A. and Roy, A.K. (2000) Heme ox genase-1 in tissue pathology: the Yin and Yang. *Am J Pathol*, **156**, 1485 1488.

Ebendal, T., Bengtsson, H. and Soderstrom, S. (1998) Bone morphogenetic proteins and their receptors: potential functions in the brain. *J Neurosci Res*, **51**, 139-146.

Elbirt, K.K. and Bonkovsky, H.L. (1999) Heme oxygenase: recent advances in understanding its regulation and role. *Proc Assoc Am Physicians*, **111**, 438-447.

Faherty, C.J., Xanthoudakis, S. and Smeyne, R.J. (1999) Caspase-3-dependent neuronal death in the hippocampus following kainic acid treatment. *Brain Res Mol Brain Res*, **70**, 159-163.

Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H. and Blum, M. (1997) The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech Dev*, **63**, 39-50.

Fang, J., Yin, W., Smiley, E., Wang, S.Q. and Bonadio, J. (1996) Molecular cloning of the mouse activin beta E subunit gene. *Biochem Biophys Res Commun*, **228**, 669-674.

Fann, M.J. and Patterson, P.H. (1994) Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. *Proc Natl Acad Sci U S A*, **91**, 43-47.

Finklestein, S.P., Apostolides, P.J., Caday, C.G., Prosser, J., Philips, M.F. and Klagsbrun, M. (1988) Increased basic fibroblast growth factor (bFGF) i munoreactivity at the site of focal brain wounds. *Brain Res*, **460**, 253-259.

Frazier, K., Williams, S., Kothapalli, D., Klapper, H. and Grotendorst, G.R. (1996) Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol*, **107**, 404-411.

Fridovich, I. (1974) Superoxide dismutases. *Adv Enzymol Relat Areas Mol Biol*, **41**, 35-97.

Fridovich, I. (1978) The biology of oxygen radicals. *Science*, **201**, 875-880.

Fridovich, I. (1999) Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann N Y Acad Sci*, **893**, 13-18.

Fujikawa, D.G., Shinmei, S.S. and Cai, B. (2000) Seizure-induced neuronal necrosis: implications for programmed cell death mechanisms. *Epilepsia*, **41**, S9-13.

Gamer, L.W., Wolfman, N.M., Celeste, A.J., Hattersley, G., Hewick, R. and Rosen, V. (1999) A novel BMP expressed in developing mouse limb, spinal cord, and tail bud is a potent mesoderm inducer in Xenopus embryos. *Dev Biol*, **208**, 222-232.

Goldschmeding, R., Aten, J., Ito, Y., Blom, I., Rabelink, T. and Weening, J.J. (2000) Connective tissue growth factor: just another factor in renal fibrosis? *Nephrol Dial Transplant*, **15**, 296-299.

Gorski, K., Carneiro, M. and Schibler, U. (1986) Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell*, **47**, 767-776.

Gremo, F. and Presta, M. (2000) Role of fibroblast growth factor-2 in human brain: a focus on development. *Int J Dev Neurosci*, **18**, 271-279.

Grotendorst, G.R. (1997) Connective tissue growth factor: a mediator of TGF beta action on fibroblasts. *Cytokine Growth Factor Rev*, **8**, 171-179.

Gunasekar, P.G., Kanthasamy, A.G., Borowitz, J.L. and Isom, G.E. (1995) NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death. *J Neurochem*, **65**, 2016-2021.

Hanselmann, C., Mauch, C. and Werner, S. (2001) Haem oxygenase-1: a novel player in cutaneous wound repair and psoriasis? *Biochem J*, **353**, 459 466.

Heldin, C.H., Miyazono, K. and ten Dijke, P. (1997) TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature*, **390**, 465-471.

Hertel, M., Tretter, Y., Alzheimer, C. and Werner, S. (2000) Connective tissue growth factor: a novel player in tissue reorganization after brain injury? *Eur J Neurosci*, **12**, 376-380.

Hollmann, M. and Heinemann, S. (1994) Cloned glutamate receptors. *Annu Rev Neurosci*, **17**, 31-108.

Hoetter, G.,Neidhardt, H., Schneider, C. and Pohl, J. (1995) Cloning of a new member of the TGF-beta family: a putative new activin beta C chain. *Biochem Biophys Res Commun*, **206**, 608-613.

Hubner, G., Alzheimer, C. and Werner, S. (1999) Activin: a novel player in tissue repair processes. *Histol Histopathol*, **14**, 295-304.

Hubner, G. and Werner, S. (1996) Serum growth factors and proinflammatory cytokines are potent inducers of activin expression in cultured fibroblasts and keratinocytes. *Exp Cell Res*, **228**, 106-113.

Hughes, P.E., Alexi, T., Williams, C.E., Clark, R.G. and Gluckman, P.D. (1999) Administration of recombinant human Activin-A has powerful neurotrophic effects on select striatal phenotypes in the quinolinic acid lesion model of Huntington's disease. *Neuroscience*, **92**, 197-209.

Iemura, S., Yamamoto, T.S., Takagi, C., Uchiyama, H., Natsume, T., Shim saki, S., Sugino, H. and Ueno, N. (1998) Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc Natl Acad Sci U S A*, **95**, 9337-9342.

Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Gr tendorst, G.R. and Takehara, K. (1996) Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J Invest Dermatol*, **106**, 729-733.

Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Grotendorst, G.R. and Takehara, K. (1995) Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol*, **105**, 280-284.

Igarashi, A., Okochi, H., Bradham, D.M. and Grotendorst, G.R. (1993) Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell*, **4**, 637-645.

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

Ito, Y., Aten, J., Bende, R.J., Oemar, B.S., Rabelink, T.J., Weening, J.J. and Goldschmeding, R. (1998) Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int*, **53**, 853-861.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M. and 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.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D. and Y mamoto, 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.

Jaiswal, A.K. (1994) Human NAD(P)H:quinone oxidoreductase2. Gene structure, activity, and tissue-specific expression. *J Biol Chem*, **269**, 14502-14508.

Johnsen, O., Murphy, P., Prydz, H. and Kolsto, A.B. (1998) Interaction of the CNC-bZIP factor TCF11/LCR-F1/Nrf1 with MafG: binding-site selection and regulation of transcription. *Nucleic Acids Res*, **26**, 512-520.

Katayama, T., Shiota, K. and Takahashi, M. (1990) Activin A increases the number of follicle-stimulating hormone cells in anterior pituitary cultures. *Mol Cell Endocrinol*, **69**, 179-185.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**, 239-257.

Kim, H., Seo, J.Y. and Kim, K.H. (2000) NF-kappaB and cytokines in pancreatic acinar cells. *J Korean Med Sci*, **15 Suppl**, S53-54.

Kireeva, M.L., Latinkic, B.V., Kolesnikova, T.V., Chen, C.C., Yang, G.P., Abler, A.S. and Lau, L.F. (1997) Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. *Exp Cell Res*, **233**, 63-77.

Kirschner, P.B., Henshaw, R., Weise, J., Trubetskoy, V., Finklestein, S., Schulz, J.B. and Beal, M.F. (1995) Basic fibroblast growth factor protects against excitotoxicity and chemical hypoxia in both neonatal and adult rats. *J Cereb Blood Flow Metab*, **15**, 619-623.

Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Haya hi, N. and Yamamoto, M. (1999) Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. *J Biol Chem*, **274**, 6443-6452.

Koketsu, N., Berlove, D.J., Moskowitz, M.A., Kowall, N.W., Caday, C.G. and Finklestein, S.P. (1994) Pretreatment with intraventricular basic fibroblast growth factor decreases infarct size following focal cerebral ischemia in rats. *Ann Neurol*, **35**, 451-457.

Kothapalli, D., Frazier, K.S., Welply, A., Segarini, P.R. and Grotendorst, G.R. (1997) Transforming growth factor beta induces anchorage-independent growth of NRK fibroblasts via a connective tissue growth factor dependent signaling pathway. *Cell Growth Differ*, **8**, 61-68.

Kothapalli, D., Hayashi, N. and Grotendorst, G.R. (1998) Inhibition of TGF-beta stimulated CTGF gene expression and anchorage-independent growth by cAMP identifies a CTGF-dependent restriction point in the cell cycle. *Faseb J*, **12**, 1151-1161.

Krieglstein, K., Reuss, B., Maysinger, D. and Unsicker, K. (1998) Short communication: transforming growth factor-beta mediates the neurotrophic effect of fibroblast growth factor-2 on midbrain dopaminergic neurons. *Eur J Neurosci*, **10**, 2746-2750.

Krieglstein, K., Suter-Crazzolara, C., Fischer, W.H. and Unsicker, K. (1995) TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. *Embo J*, **14**, 736-742.

Kwong, M., Kan, Y.W. and Chan, J.Y. (1999) The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress inducing agents. Role for Nrf1 in gamma-gcs(l) and gss expression in mouse fibroblasts. *J Biol Chem*, **274**, 37491-37498.

Lai, M., Gluckman, P., Dragunow, M. and Hughes, P.E. (1997) Focal brain injury increases activin betaA mRNA expression in hippocampal neurons. *Neuroreport*, **8**, 2691-2694.

Lasky, J.A., Ortiz, L.A., Tonthat, B., Hoyle, G.W., Corti, M., Athas, G., Lugarella, G., Brody, A. and Friedman, M. (1998) Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. *Am J Physiol*, **275**, L365-371.

Lippoldt, A., Andbjer, B., Rosen, L., Richter, E., Ganten, D., Cao, Y., Petterson, R.F. and Fuxe, K. (1993) Photochemically induced focal cerebral ischemia in rat: time dependent and global increase in expression of basic fibroblast growth factor mRNA. *Brain Res*, **625**, 45-56.

Liu, Z. and Holmes, G.L. (1997) Basic fibroblast growth factor-induced seizures in rats. *Neurosci Lett*, **233**, 85-88.

Logan, A., Berry, M., Gonzalez, A.M., Frautschy, S.A., Sporn, M.B. and Baird, A. (1994a) Effects of transforming growth factor beta 1 on scar production in the injured central nervous system of the rat. *Eur J Neurosci*, **6**, 355-363.

Logan, A., Frautschy, S.A. and Baird, A. (1991) Basic fibroblast growth factor and central nervous system injury. *Ann N Y Acad Sci*, **638**, 474-476.

Logan, A., Oliver, J.J. and Berry, M. (1994b) Growth factors in CNS repair and regeneration. *Prog Growth Factor Res*, **5**, 379-405.

Marini, M.G., Chan, K., Casula, L., Kan, Y.W., Cao, A. and Moi, P. (1997) hMAF, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J Biol Chem*, **272**, 16490-16497.

Mark, R.J., Keller, J.N., Kruman, I. and Mattson, M.P. (1997) Basic FGF attenuates amyloid beta-peptide-induced oxidative stress, mitochondrial dy unction, and impairment of Na+/K+-ATPase activity in hippocampal neurons. *Brain Res*, **756**, 205-214.

Mathews, L.S. (1994) Activin receptors and cellular signaling by the receptor serine kinase family. *Endocr Rev*, **15**, 310-325.

Mathews, L.S. and Vale, W.W. (1991) Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell*, **65**, 973-982.

Matsuoka, Y., Kitamura, Y., Okazaki, M., Kakimura, J., Tooyama, I., Kimura, H. and Taniguchi, T. (1998) Kainic acid induction of heme oxygenase in vivo and in vitro. *Neuroscience*, **85**, 1223-1233.

Mattson, M.P. and Goodman, Y. (1995) Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. *Brain Res*, **676**, 219-224.

McCarthy, S.A. and Bicknell, R. (1993) Inhibition of vascular endothelial cell growth by activin-A. *J Biol Chem*, **268**, 23066-23071.

McIntosh, L.J., Cortopassi, K.M. and Sapolsky, R.M. (1998) Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. *Brain Res*, **791**, 215-222.

Meier, C.L. and Dudek, F.E. (1996) Spontaneous and stimulation-induced synchronized burst afterdischarges in the isolated CA1 of kainate-treated rats. *J Neurophysiol*, **76**, 2231-2239.

Moi, P., Chan, K., Asunis, I., Cao, A. and Kan, Y.W. (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.

Monaghan, D.T., Holets, V.R., Toy, D.W. and Cotman, C.W. (1983) Anatomical distributions of four pharmacologically distinct 3H-L-glutamate binding sites. *Nature*, **306**, 176-179.

Morgan, T.E., Nichols, N.R., Pasinetti, G.M. and Finch, C.E. (1993) TGF-beta 1 mRNA increases in macrophage/microglial cells of the hippocampus in r sponse to deafferentation and kainic acid-induced neurodegeneration. *Exp Neurol*, **120**, 291-301.

Morrison, R.S., Sharma, A., de Vellis, J. and Bradshaw, R.A. (1986) Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. *Proc Natl Acad Sci U S A*, **83**, 7537-7541.

Mulcahy, R.T., Wartman, M.A., Bailey, H.H. and Gipp, J.J. (1997) Constitutive and beta-naphthoflavone-induced expression of the human gamma glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem*, **272**, 7445 7454.

Munz, B., Hubner, G., Tretter, Y., Alzheimer, C. and Werner, S. (1999a) A novel role of activin in inflammation and repair. *J Endocrinol*, **161**, 187-193.

Munz, B., Smola, H., Engelhardt, F., Bleuel, K., Brauchle, M., Lein, I., Evans, L.W., Huylebroeck, D., Balling, R. and Werner, S. (1999b) Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. *Embo J*, **18**, 5205-5215.

Nadler, J.V., Perry, B.W. and Cotman, C.W. (1978) Intraventricular kainic acid preferentially destroys hippocampal pyramidal cells. *Nature*, **271**, 676 677.

Nadler, J.V., Perry, B.W., Gentry, C. and Cotman, C.W. (1980) Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. *J Comp Neurol*, **192**, 333-359.

Nakagami, Y., Saito, H. and Matsuki, N. (1997) Basic fibroblast growth factor and brain-derived neurotrophic factor promote survival and neuronal circuit formation in organotypic hippocampal culture. *Jpn J Pharmacol*, **75**, 319-326.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990) Activin-binding protein from rat ovary is follistatin. *Science*, **247**, 836-838.

Nakaso, K., Kitayama, M., Fukuda, H., Kimura, K., Yanagawa, T., Ishii, T., N kashima, K. and Yamada, K. (2000) Oxidative stress-related proteins A170 and heme oxygenase-1 are differently induced in the rat cerebellum under kainate-mediated excitotoxicity. *Neurosci Lett*, **282**, 57 60.

Nakaso, K., Kitayama, M., Kimura, K., Yanagawa, T., Ohama, E., Nakashima, K., Ishii, T. and Yamada, K. (1999) Induction of heme oxygenase-1 in the rat brain by kainic acid-mediated excitotoxicity: the dissociation of mRNA and protein expression in hippocampus. *Biochem Biophys Res Commun*, **259**, 91-96.

Niquet, J., Jorquera, I., Ben-Ari, Y. and Represa, A. (1994) Proliferative astr cytes may express fibronectin-like protein in the hippocampus of epileptic rats. *Neurosci Lett*, **180**, 13-16.

Oda, S., Nishimatsu, S., Murakami, K. and Ueno, N. (1995) Molecular cloning and functional analysis of a new activin beta subunit: a dorsal mesoderm inducing activity in Xenopus. *Biochem Biophys Res Commun*, **210**, 581 588.

Oemar, B.S., Werner, A., Garnier, J.M., Do, D.D., Godoy, N., Nauck, M., Marz, W., Rupp, J., Pech, M. and Luscher, T.F. (1997) Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. *Circulation*, **95**, 831-839.

Ogawa, Y., Schmidt, D.K., Nathan, R.M., Armstrong, R.M., Miller, K.L., Sawa mura, S.J., Ziman, J.M., Erickson, K.L., de Leon, E.R., Rosen, D.M. and et al. (1992) Bovine bone activin enhances bone morphogenetic protein induced ectopic bone formation. *J Biol Chem*, **267**, 14233-14237.

Okazaki, M.M. and Nadler, J.V. (1988) Protective effects of mossy fiber lesions against kainic acid-induced seizures and neuronal degeneration. *Neuroscience*, **26**, 763-781.

Olney, J.W., Adamo, N.J. and Ratner, A. (1971) Monosodium glutamate effects. *Science*, **172**, 294.

Olney, J.W. and Sharpe, L.G. (1969) Brain lesions in an infant rhesus monkey treated with monsodium glutamate. *Science*, **166**, 386-388.

Ornitz, D.M. and Itoh, N. (2001) Fibroblast growth factors. *Genome Biol*, **2**.

Otto, D., Frotscher, M. and Unsicker, K. (1989) Basic fibroblast growth factor and nerve growth factor administered in gel foam rescue medial septal neurons after fimbria fornix transection. *J Neurosci Res*, **22**, 83-91.

Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F. and Gage, F.H. (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci*, **19**, 8487 8497.

Palmer, T.D., Ray, J. and Gage, F.H. (1995) FGF-2-responsive neuronal pr genitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci*, **6**, 474-486.

Paradis, V., Dargere, D., Vidaud, M., De Gouville, A.C., Huet, S., Martinez, V., Gauthier, J.M., Ba, N., Sobesky, R., Ratziu, V. and Bedossa, P. (1999) Expression of connective tissue growth factor in experimental rat and human liver fibrosis. *Hepatology*, **30**, 968-976.

Park, S.K., Kim, J., Seomun, Y., Choi, J., Kim, D.H., Han, I.O., Lee, E.H., Chung, S.K. and Joo, C.K. (2001) Hydrogen peroxide is a novel inducer of connective tissue growth factor. *Biochem Biophys Res Commun*, **284**, 966-971.

Perez, Y., Morin, F., Beaulieu, C. and Lacaille, J.C. (1996) Axonal sprouting of CA1 pyramidal cells in hyperexcitable hippocampal slices of kainate treated rats. *Eur J Neurosci*, **8**, 736-748.

Peterson, D.A., Lucidi-Phillipi, C.A., Murphy, D.P., Ray, J. and Gage, F.H. (1996) Fibroblast growth factor-2 protects entorhinal layer II glutamatergic neurons from axotomy-induced death. *J Neurosci*, **16**, 886 898.

Phillips, D.J. (2000) Regulation of activin's access to the cell: why is mother nature such a control freak? *Bioessays*, **22**, 689-696.

Piek, E., Heldin, C.H. and Ten Dijke, P. (1999) Specificity, diversity, and regul ation in TGF-beta superfamily signaling. *Faseb J*, **13**, 2105-2124.

Poole, T.J., Finkelstein, E.B. and Cox, C.M. (2001) The role of FGF and VEGF in angioblast induction and migration during vascular development. *Dev Dyn*, **220**, 1-17.

Prestera, T., Talalay, P., Alam, J., Ahn, Y.I., Lee, P.J. and Choi, A.M. (1995) Parallel induction of heme oxygenase-1 and chemoprotective phase 2 enzymes by electrophiles and antioxidants: regulation by upstream antioxidant-responsive elements (ARE). *Mol Med*, **1**, 827-837.

Reuss, B., Hertel, M., Werner, S. and Unsicker, K. (2000) Fibroblast growth factors-5 and -9 distinctly regulate expression and function of the gap junction protein connexin43 in cultured astroglial cells from different brain regions. *Glia*, **30**, 231-241.

Reynolds, I.J. and Hastings, T.G. (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J Neurosci*, **15**, 3318-3327.

Riser, B.L., Denichilo, M., Cortes, P., Baker, C., Grondin, J.M., Yee, J. and Narins, R.G. (2000) Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc Nephrol*, **11**, 25-38.

Riva, M.A., Donati, E., Tascedda, F., Zolli, M. and Racagni, G. (1994) Short- and long-term induction of basic fibroblast growth factor gene expression in rat central nervous system following kainate injection. *Neuroscience*, **59**, 55-65.

Riva, M.A., Gale, K. and Mocchetti, I. (1992) Basic fibroblast growth factor mRNA increases in specific brain regions following convulsive seizures. *Brain Res Mol Brain Res*, **15**, 311-318.

Rowntree, S. and Kolb, B. (1997) Blockade of basic fibroblast growth factor retards recovery from motor cortex injury in rats. *Eur J Neurosci*, **9**, 2432 2441.

Rushmore, T.H. and Pickett, C.B. (1993) Glutathione S-transferases, structure, regulation, and therapeutic implications. *J Biol Chem*, **268**, 11475-11478.

Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D. and Fischer, W.H. (1990) Activin is a nerve cell survival molecule. *Nature*, **344**, 868 870.

Schwob, J.E., Fuller, T., Price, J.L. and Olney, J.W. (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. *Neuroscience*, **5**, 991-1014.

See, V. and Loeffler, J.P. (2001) Oxidative stress induces neuronal death by recruiting a protease and phosphatase-gated mechanism. *J Biol Chem*, **6**, 6.

Shimasaki, S., Koga, M., Esch, F., Mercado, M., Cooksey, K., Koba, A. and Ling, N. (1988) Porcine follistatin gene structure supports two forms of mature follistatin produced by alternative splicing. *Biochem Biophys Res Commun*, **152**, 717-723.

Sladeczek, F., Pin, J.P., Recasens, M., Bockaert, J. and Weiss, S. (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature*, **317**, 717-719.

Stichel, C.C. and Muller, H.W. (1998) The CNS lesion scar: new vistas on an old regeneration barrier. *Cell Tissue Res*, **294**, 1-9.

Sugino, H., Sugino, K., Hashimoto, O., Shoji, H. and Nakamura, T. (1997) Follistatin and its role as an activin-binding protein. *J Med Invest*, **44**, 1-14.

Surveyor, G.A., Wilson, A.K. and Brigstock, D.R. (1998) Localization of connective tissue growth factor during the period of embryo implantation in the mouse. *Biol Reprod*, **59**, 1207-1213.

Takami, K., Iwane, M., Kiyota, Y., Miyamoto, M., Tsukuda, R. and Shiosaka, S. (1992) Increase of basic fibroblast growth factor immunoreactivity and its mRNA level in rat brain following transient forebrain ischemia. *Exp Brain Res*, **90**, 1-10.

Thannickal, V.J. and Fanburg, B.L. (1995) Activation of an H2O2-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. *J Biol Chem*, **270**, 30334-30338.

Thannickal, V.J., Hassoun, P.M., White, A.C. and Fanburg, B.L. (1993) E hanced rate of H2O2 release from bovine pulmonary artery endothelial cells induced by TGF-beta 1. *Am J Physiol*, **265**, L622-626.

Tretter, Y.P., Hertel, M., Munz, B., ten Bruggencate, G., Werner, S. and Alzheimer, C. (2000) Induction of activin A is essential for the neuroprotective action of basic fibroblast growth factor in vivo. *Nat Med*, **6**, 812-815.

Tretter, Y.P., Munz, B., Hubner, G., ten Bruggencate, G., Werner, S. and Al heimer, C. (1996) Strong induction of activin expression after hippocampal lesion. *Neuroreport*, **7**, 1819-1823.

Venugopal, R. and Jaiswal, A.K. (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.

Venugopal, R. and Jaiswal, A.K. (1998) Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene*, **17**, 3145-3156.

Walicke, P., Cowan, W.M., Ueno, N., Baird, A. and Guillemin, R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc Natl Acad Sci U S A*, **83**, 3012-3016.

Walicke, P.A. and Baird, A. (1988) Neurotrophic effects of basic and acidic fibroblast growth factors are not mediated through glial cells. *Brain Res*, **468**, 71-79.

Wang, Q., Keutmann, H.T., Schneyer, A.L. and Sluss, P.M. (2000) Analysis of human follistatin structure: identification of two discontinuous N-terminal sequences coding for activin A binding and structural consequences of activin binding to native proteins. *Endocrinology*, **141**, 3183-3193.

Wasserman, W.W. and Fahl, W.E. (1997) Functional antioxidant responsive elements. *Proc Natl Acad Sci U S A*, **94**, 5361-5366.

Werner, S., Peters, K.G., Longaker, M.T., Fuller-Pace, F., Banda, M.J. and Williams, L.T. (1992) Large induction of keratinocyte growth factor expre sion in the dermis during wound healing. *Proc Natl Acad Sci U S A*, **89**, 6896-6900.

Werner, S., Weinberg, W., Liao, X., Peters, K.G., Blessing, M., Yuspa, S.H., Weiner, R.L. and Williams, L.T. (1993) Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. *Embo J*, **12**, 2635-2643.

Wild, A.C., Moinova, H.R. and Mulcahy, R.T. (1999) Regulation of gamma glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J Biol Chem*, **274**, 33627-33636.

Wu, D.D., Lai, M., Hughes, P.E., Sirimanne, E., Gluckman, P.D. and Williams, C.E. (1999) Expression of the activin axis and neuronal rescue effects of recombinant activin A following hypoxic-ischemic brain injury in the infant rat. *Brain Res*, **835**, 369-378.

Yasuda, H., Mine, T., Shibata, H., Eto, Y., Hasegawa, Y., Takeuchi, T., Asano, S. and Kojima, I. (1993) Activin A: an autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *J Clin Invest*, **92**, 1491-1496.

7.2. Books

Franklin, KBJ., Paxinos G.
The Mouse Brain in Stereotaxic Coordinates
Academic Press 1997

7.3. Internet sites

Online Mouse Brain Atlas:
Rosen GD, Williams AG, Capra JA, Connolly MT, Cruz B, Lu L, Airey DC, Kulkarni K, Williams RW (2000) The Mouse Brain Library @ www.mbl.org. Int Mouse Genome Conference 14: 166.

GenBank Database:
http://www.ncbi.nlm.nih.gov/

Nature medicine online:
http://medicine.nature.com/

European Journal of Neuroscience:
http://www.blackwell-science.com/~cgilib/jnlpage.bin?Journal=EJN&File=EJN&Page=aims

8. Curriculum Vitae

Name: **Hertel**
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Born: 25.04.1970 in Stuttgart/Germany

Family status: unmarried/no children

Pre-University education:

1977-1981 elementary schools in Stuttgart and near Munich
1981-1983 grammar school "Obermenziger Gymnasium"
1983-1988 middle school "Carl-Spitzweg-Realschule" finished with the "Mittlere Reife"
1988-1992 grammer school "Rupprecht Gymnasium", finished with the "**Abitur**"
1993 "Ergänzungsprüfung Mathematik" Kantonale Maturitätskommision Basel-Stadt

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1992-1994 **University of Basel** /Switzerland, Biologie II at the "Biozentrum Basel"

1994-1997 **University of Würzburg**/Germany, biology with main focus on genetics, cell- and developmental biology and neurobiology finished with the **diploma .**

practical training:

Department of Genetics (Würzburg), head Professor Martin Heisenberg, in the laboratory of Professor Erich Buchner:
"Restriction mapping of *Drosophila* Synapsin".

Max-Planck-Institute of Biochemistry, Department of Molecular Biology, director Professor Axel Ullrich
"Some aspects of cloning and transfection of Heregulin 3"

Department of botany I (Würzburg), Professor Hartmut Gimmler,
"Characterisation of a new yeast-like organism by its capability to ferment different sugars"

Diploma thesis: Max-Planck-Institute for Neurobiology, department of Neuro chemistry, director Professor Yves-Alain Barde,
"Purification and Biological Characterisation of the BDNF Precursor Protein" (about 12 month)

Ph.D. thesis: ETH-Zurich, Institute for Cell Biology, Professor Sabine Werner and University Munich, Institute of Physiology, P.D. Christian Alzheimer: "Novel players in neuroprotection and tissue reorganisation after hippocampal lesion" (since October 1997)

Publications:

Moritz Hertel, Yvonne Tretter, Christian Alzheimer, and Sabine Werner (2000) Connective tissue growth factor: A novel player in tissue reorganization after brain injury.
Eur. J. Neurosci, 12, 376-380.

Yvonne Tretter, **Moritz Hertel**, Barbara Munz, Gerrit ten Bruggencate, Sabine Werner and Christian Alzheimer. (2000) Induction of activin A is essential for the neuroprotective action of bFGF in vivo.
Nat. Med. 6, 812-815.

Bernhard Reuss, **Moritz Hertel**, Sabine Werner and Klaus Unsicker (2000). Fibroblast growth factors -5 and -9 distinctly regulat expression and function of the gap function protein connexin43 in cultured astroglial cells from different brain regions.
Glia, 30, 231-241.

Barbara Munz, Yvonne P. Tretter, **Moritz Hertel**, Felix Engelhardt, Christian Alzheimer, and Sabine Werner.(2001) The roles of activin in repair processes of the skin and the brain. Mol. Cell. Endocrinol. 180, 169-177

submitted: **Moritz Hertel**, Susanne Braun, Silke Durka, Christian Alzheimer and Sabine Werner
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