

# **Neurofilament proteins in the postnatal rat hippocampus. Developmental expression and changes in experimental epilepsy.**

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**TURKU 2008**

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ISBN 978-951-29-3586-4 (PRINT)

ISBN 978-951-29-3587-1 (PDF)

ISSN 0355-9483

Painosalama Oy – Turku, Finland 2008

*To my family*



# ABSTRACT

Francisco R. López Picón

## Neurofilament proteins in the postnatal rat hippocampus. Developmental expression and changes in experimental epilepsy.

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Neurofilament proteins (NFs) are the major components of the intermediate filaments of the neuronal cytoskeleton. The three different NF proteins; the low (NF-L), medium (NF-M), and high (NF-H) molecular weight proteins are distributed throughout the axon, perikaryon, and dendrites. NF proteins play an important role in neuronal development, and plasticity, and seem to contribute to the pathophysiology of several diseases. However, the detailed expression patterns of NF proteins in the course of postnatal maturation, and in response to seizures in the rat have remained unknown. In this work, I have studied the developmental expression and cellular distribution of the three NF proteins in the rat hippocampus during the postnatal development. The reactivity of NF proteins in response to kainic acid (KA)-induced status epilepticus (SE) was studied in the hippocampus of 9-day-old rats, and using *in vitro* organotypic hippocampal slices cultures prepared from P6-7 rats.

The results showed that NF-L and NF-M proteins are expressed already at the postnatal day 1, while the expression of NF-H mainly occurred during the second postnatal week. The immunoreactivity of NF proteins varied depending on the cell type and sub-cellular location in the hippocampus. In adult rats, KA-induced SE typically results in severe and permanent NF degradation. However, in our P9 rats KA-induced SE resulted in a transient increase in the expression of NF proteins during the first few hours but not degradation. No neuronal death or mossy fiber sprouting was observed at any time after SE. The *in vitro* studies with OHCs, which mimic the *in vivo* developing models where a local injection of KA is applied (e.g. intrahippocampal), indicated that NF proteins were rapidly degraded in response to KA treatment, this effect being effectively inhibited by the treatment with the AMPA receptor antagonist CNQX, and calpain inhibitor MDL-28170. These compounds also significantly ameliorated the KA-induced region-specific neuronal damage. The NMDA receptor antagonist and the L-type Ca<sup>2+</sup> channel blocker did not have any significant effect.

In conclusion, the results indicate that the developmental expression of NF in the rat hippocampus is differentially regulated and targeted in the different hippocampal cell types during the postnatal development. Furthermore, despite SE, the mechanisms leading to NF degradation and neuronal death are not activated in P9 rats unlike in adults. The reason for this remains unknown. The results in organotypic hippocampal cultures confirm the validity of this *in vitro* model to study development processes, and to perform pharmacological studies. The results also suggest that calpain proteases as interesting pharmacological targets to reduce neuronal damage after acute excitotoxic insults.

**Keywords:** neurofilament; epilepsy; hippocampus; development; neuronal damage; calpain.

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## ABBREVIATIONS

|        |   |
|--------|---|
| AD     | Alzheimer's disease   |
| AIF    | apoptosis inducing factor                                     |
| ALS    | amyotrophic lateral sclerosis                                 |
| AMPA   | $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |
| APAF-1 | apoptotic protease-activating factor 1                        |
| CAD    | caspase-activated DNase                                       |
| CaMKII | calmodulin-dependent protein kinase II                        |
| CDK-5  | cyclin-dependent kinase 5                                     |
| CMT    | Charcot-Marie-Tooth disease                                   |
| CNQX   | 6-cyano-7-nitroquinoxaline-2,3-dione                          |
| CNS    | central nervous system  |
| CR     | Cajal-Retzius cells   |
| DAB    | 3,3'-diaminobenzidine   |
| DG     | dentate gyrus   |
| DIV    | days <i>in vitro</i>  |
| DTT    | dithiothreitol  |
| EC     | entorhinal cortex   |
| EDTA   | ethylenediaminetetracetic acid                                |
| EPSC   | excitatory postsynaptic currents                              |
| ER     | endoplasmic reticulum   |
| ERK    | extracellular signal-regulated kinase                         |
| FJB    | Fluoro-Jade B   |
| GAD    | glutamate decarboxylase                                       |
| GFAP   | glial fibrillary acid protein                                 |
| GluR   | glutamate receptor  |
| GSK 3  | glycogen synthetic kinase 3                                   |
| ICAD   | inhibitor of caspase-activated DNase                          |
| i.c.v. | intra cerebroventricular                                      |
| IEG    | immediate early genes   |
| IF     | intermediate filament   |
| i.h.   | intra hippocampal   |
| ILAE   | international league against epilepsy                         |
| IP     | intraperitoneal   |
| KA     | kainic acid   |
| KSP    | Lysine-Serine-Proline   |
| LTP    | long term potentiation  |

|         |                                   |
|---------|-----------------------------------|
| MAP     | microtubule associated protein    |
| MF      | mossy fiber                       |
| MK-801  | dizocilpine                       |
| MRI     | magnetic resonance imaging        |
| NF      | neurofilament                     |
| NFP     | phosphorylated neurofilament      |
| NIF     | nifedipine                        |
| NMDA    | N-methyl-D-aspartate              |
| NUDEL   | Nuclear distribution gene E-like  |
| OHC     | organotypic hippocampal cultures  |
| P       | postnatal                         |
| PARP    | poly (ADP-ribose) polymerase      |
| PBS     | phosphate-buffered saline         |
| PFA     | paraformaldehyde                  |
| PKA     | protein kinase A                  |
| PKC     | protein kinase C                  |
| PMSF    | phenylmethylsulphonyl fluoride    |
| pp      | perforant pathway                 |
| PP2A    | protein phosphatase 2A            |
| PTX     | picrotoxin                        |
| PTZ     | pentylenetetrazol                 |
| PVDF    | polyvinylidene difluoride         |
| SC      | Schaffer collaterals              |
| SDS     | sodium dodecyl sulfate            |
| SE      | status epilepticus                |
| Sg      | stratum granulare                 |
| Sm      | stratum moleculare                |
| SNAP-25 | synaptosome associated protein 25 |
| So      | stratum oriens                    |
| Sp      | stratum pyramidale                |
| Sr      | stratum radiatum                  |
| SSSE    | self-sustained status epilepticus |
| SOD 1   | superoxide dismutase 1            |
| TLE     | temporal lobe epilepsy            |
| TM      | transmembrane domain              |

## LIST OF ORIGINAL COMMUNICATIONS

- I** Holopainen IE, Lauren HB, Romppanen A, Lopez-Picon FR. (2001). *Changes in neurofilament protein-immunoreactivity following kainic-acid treatment of organotypic hippocampal slice cultures*. J Neurosci Res., 66: 620-629.
- II** Lopez-Picon FR, Uusi-Oukari M, Holopainen IE. (2003). *Differential expression and localization of the phosphorylated and nonphosphorylated neurofilaments during the early postnatal development of the rat hippocampus*. Hippocampus, 13: 767-779.
- III** Lopez-Picon FR, Puustinen N, Kukko-Lukjanov TK, Holopainen IE. (2004). *Resistance of neurofilaments to degradation, and lack of neuronal death and mossy fiber sprouting after kainic acid-induced status epilepticus in the developing rat hippocampus*. Neurobiol Dis., 17: 415-426.
- IV** Lopez-Picon FR, Kukko-Lukjanov TK, Holopainen IE. (2006). *Calpain inhibitor MDL-28170 and AMPA/KA receptor antagonist CNQX inhibit neurofilament degradation and enhance neuronal survival in kainic acid-treated hippocampal slice cultures*. Eur J Neurosci., 23: 2686-2694.

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# 1. INTRODUCTION

Neurofilament (NF) proteins, the major components of the neuronal intermediate filaments, are expressed in the majority of mature neurons in the central nervous system (CNS). The three different proteins; low [NF-L (68 kDa)], medium [NF-M (160 kDa)] and high [NF-H (200 kDa)] molecular weight, are separate gene products, and are distributed through the axon, perikaryon and dendrites (Czosnek et al., 1980; Czosnek et al., 1980; Van der Zee et al., 1997). NF proteins play a crucial role in the neuronal growth, organization and plasticity (Nixon, 1992; Julien and Mushynski, 1998; Sanchez et al. 2000), and are known to be important pathological hallmarks in different neurological diseases (Gong et al., 2000; Menzies et al., 2002; Jordanova et al., 2003; Trimmer et al., 2004). In spite of the importance of the NF proteins, the studies of their role in development, neuronal plasticity and in several neuronal pathologies are scarce. Furthermore, the detailed expression, distribution and their function in response to seizures are unknown in the developing hippocampus.

Epilepsy is a disease that affects about 0.8% of the population having the highest incidence during the early childhood and in the elderly (Pitkänen and Sutula, 2002). This disorder manifests by recurrent unprovoked seizures that result from an electrical imbalance in the brain (Fisher et al., 2005). Several factors have been described as triggers for some of the epilepsies, for example structural abnormality in the brain, brain injury, infection, and genetic mutations. However, most of the epilepsy cases remain of unknown etiology.

To investigate different pathologies, and in special neurological diseases, animal models have been extremely useful. Advantages of experimental animal models of epilepsy and especially of status epilepticus (SE) include a robust development of hippocampal sclerosis and granule cell synaptic reorganization during the latent period, and chronic epilepsy once it is established. The latent period is important because it provides a window of opportunity to test antiepileptogenic treatments. Most importantly, SE models have increased our understanding of human temporal lobe epilepsy (TLE). Particularly, intriguing and still mostly unknown, are the aetiology and outcome of epilepsy in the developing brain. The most important questions about the exact mechanisms of the neuronal damage remain unsolved, thus successful neuroprotectant drugs have not been so far discovered.

The main objectives of this thesis were to study the normal developmental expression of the NF proteins in the early postnatal rat hippocampus. Furthermore to elucidate the seizure-induced changes in levels and cellular localization of NF proteins using both *in vivo* and *in vitro* models of epilepsy in the developing rat hippocampus. Moreover, we aimed to unfold the importance of NF proteins and calpain proteases in KA-induced neuronal death using organotypic hippocampal cultures as *in vitro* models of experimental epilepsy.

## 2. REVIEW OF THE LITERATURE

### 2.1 Neurofilaments

Neurofilaments (NFs) are a class of intermediate filaments (IF) (10 nm) specific for the neuronal cytoskeleton being abundantly distributed throughout the axon, perikaryon, and dendrites (Dahl, 1983; Trojanowski et al., 1986; Van der Zee et al., 1997). The NFs in vertebrates are composed of three subunit proteins with low 68 kDa (NF-L), medium 160 kDa (NF-M) and high 200 kDa (NF-H) molecular weights (Kaufmann et al., 1984), which are separate gene products and immunocytochemically distinct (Czosnek et al., 1980).

#### 2.1.1 Molecular biology of neurofilaments

Based on the sequence homology and exon-intron structure of the genes encoding each subunit, NFs,  $\alpha$  internexin and nestin comprise the type IV IF family. Other IF families include types I and II (keratins), type III (desmin, glial fibrillary acidic protein [GFAP], vimentin) and type V (nuclear lamins). The NF subunit proteins retain the principal structural feature shared by most IFs, a central 40 kDa core domain (Lewis and Cowan, 1985; Geisler et al., 1989).

| Species                     | NF-L           |            | NF-M           |            | NF-H           |            |
|-----------------------------|----------------|------------|----------------|------------|----------------|------------|
|                             | <u>Protein</u> | <u>DNA</u> | <u>Protein</u> | <u>DNA</u> | <u>Protein</u> | <u>DNA</u> |
| vs. <i>Homo sapiens</i>     | 95.9           | 88.3       | 91.7           | 88.0       | 79.2           | 78.5       |
| vs. <i>Mus musculus</i>     | 98.9           | 96.4       | 97.2           | 95.2       | 90.3           | 91.7       |
| vs. <i>Pan troglodytes</i>  | 96.3           | 88.7       | 90.3           | 86.6       | 78.7           | 78.4       |
| vs. <i>Canis familiaris</i> | 96.9           | 89.5       | 92.5           | 88.6       | 83.1           | 81.2       |
| vs. <i>Gallus gallus</i>    | 81.3           | 78.8       | 81.3           | 80.0       | 55.2           | 61.0       |

**Table 1.** NF protein and DNA homology (%) between rat and the NFs of other mammals and non mammal species. Data gathered using the protein and DNA sequence database in <http://www.ncbi.nlm.nih.gov>.

Core domains contain a distinctive primary sequence which forms coiled-coil structures with core domains of the other subunits and account for the self-assembly properties of the IFs including NF (Gardner et al., 1984). Core domains of NF proteins are flanked on

one side by a small (10 kDa) NH<sub>2</sub>-terminal domain, and on the other side by a COOH tail domain.

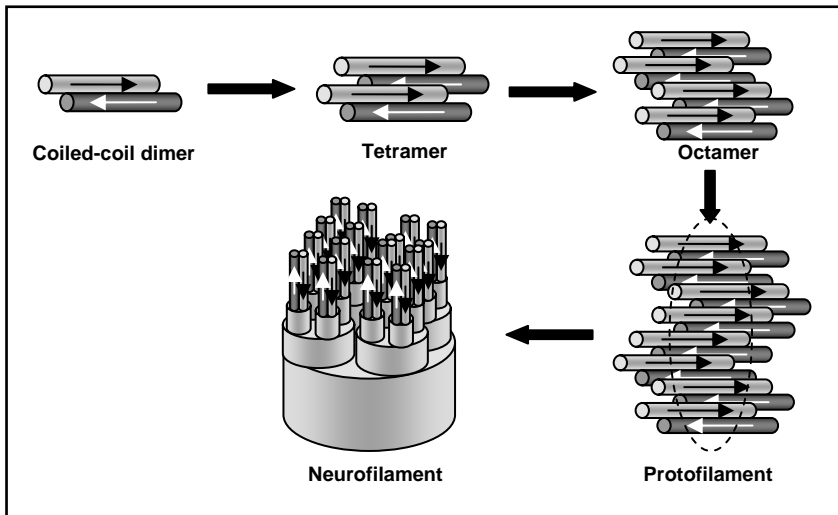
NF genes and protein in different mammalian species have high levels of homology. Table 1 shows the protein and DNA identity comparing rat (*Rattus norvegicus*), with human (*Homo sapiens*), mouse (*Mus musculus*), chimpanzee (*Pan troglodytes*), dog (*Canis familiaris*), and chicken (*Gallus gallus*).

The genomic context of the NF protein genes follows a similar pattern between the rat and human, although located in different chromosomes. NF-L and NF-M genes are in the same chromosomal location, 15p12 in rat, and 8p21 in human. NF-H genes are localized in loci 14q21 and 22q12.2 in the rat and human, respectively. (Gene locations obtained from the Homologene database in <http://www.ncbi.nlm.nih.gov>).

### **2.1.2 Structure and assembly of neurofilaments**

NFs are obligate heteropolymers *in vivo*, requiring the assembly of the key subunit NF-L together with either NF-M or NF-H in the correct stoichiometry (Ching and Liem, 1993; Lee et al., 1993). In the absence of NF-L, no filaments are formed. The central rod domain of the NF proteins, which is approximately 310 amino acids in length and contains two coiled-coil sub-domains, is responsible for the heterodimerization and the formation of filaments. The dimerization is formed in an antiparallel fashion, and it is thought to occur in an incremental way until the formation of the 10 nm filament as depicted in Fig. 1.

Very little is known about the actual molecular mechanism and control of the formation of the NF filaments. Nguyen et al. (2004) provided the first evidence that NUDEL protein associates with soluble pools of the NF-L and NF-H subunits, as well as with the polymerized NF structures in the adult CNS thus facilitating the polymerization of NFs. The C-terminal half of NUDEL binds directly to the rod domain of the NF-L subunit, whereas the association of NUDEL and NF-H is indirect. Importantly, NUDEL does not assemble with the final NF structures (Nguyen et al., 2004). Through these associations, NUDEL impacts on the assembly and homeostasis of the neurofilamentous network. These functions have direct repercussions for the architecture and integrity of neurons *in vivo*, and may be involved in neurodegeneration featuring NF defects.



**Figure 1.** Schematic picture of the NF assembly in antiparallel fashion forming coiled-coil dimers up to 10 nm filaments. Based on the hypothesized formation of filaments as discussed in Herrmann and Aebi, (1998) and Herrmann and Foisner, (2003).

The studies at the amino acid level have shown that there exists a remarkable spatial separation of the NF-H, NF-M and NF-L tails in the NF structure. In very recent studies by Zhulina et al. (2007) it was shown that the electrostatic interactions are a dominant force that controls the NF brush-like structure. In dephosphorylated NFs, confined (NF-H) and flower-like (NF-M) conformations for the projection domains have been described. Furthermore, it was shown that the ionization of the Lys-Ser-Pro (KSP) repeats triggers a conformational transition in the NF-H tail. This transition leads to the expulsion of its terminal domain to the periphery of the NF brush. They proposed that the phosphorylation of the NF proteins in axons can both increase the interfilament distance, and stabilize the cross-bridges between NFs (Zhulina and Leermakers, 2007a, b).

### 2.1.3 Transport of neurofilaments

During development and structural maintenance of neurons in maturity, neurons must transport component to and from the processes, axons and dendrites, usually from the location of synthesis in the perikarya. The axonal transport, the most widely studied, is usually divided in the “slow” and “fast” axonal transport (Nixon, 1998; Brady, 2000; Gallant, 2000). These transport rates occur through motor protein complexes that

translocates at different speeds [for review, see (Hirokawa and Takeda, 1998; Brady, 2000)].

The known motor proteins that mediate the fast axonal transport “running” along the microtubules are the kinesins and dyneins. The anterograde axonal transport of NFs is partially regulated by the microtubule associated motor kinesin (Yabe et al., 1999; Prahlad et al., 2000; Xia et al., 2003). Kinesin translocates along the microtubules in the direction of the axonal growth or “plus” end participating in the anterograde transport (Hirokawa and Takeda, 1998; Susalka and Pfister, 2000). The retrograde transport of NF occurs by interaction with the microtubule dynein-like motors, which run toward the “minus” end (Brady, 2000; Shea and Flanagan, 2001).

The transport of NFs have been observed not to occur in a continuous mode, but in short bursts of rapid transport that are interrupted by prolonged pauses (Roy et al., 2000; Wang et al., 2000; Ackerley et al., 2003). It has recently been proposed that dynein mediates retrograde NF transport within axons, and also the delivery of NFs from perikarya into axons. This is regulated by multiple NF phosphorylation events that modulate the NF interaction with dynein (Motil et al., 2006).

It has been shown that NFs binds to myosin (Rao et al., 2002) which also participates in the axonal transport (Morris and Hollenbeck, 1995; Bridgman, 2004; Brown and Bridgman, 2004). Furthermore, some NF subunits may undergo axonal transport via myosin-mediated interactions with the actin cortex (Jung et al., 2004).

## **2.1.4 Phosphorylation of neurofilaments**

### **2.1.4.1 Mechanisms of phosphorylation**

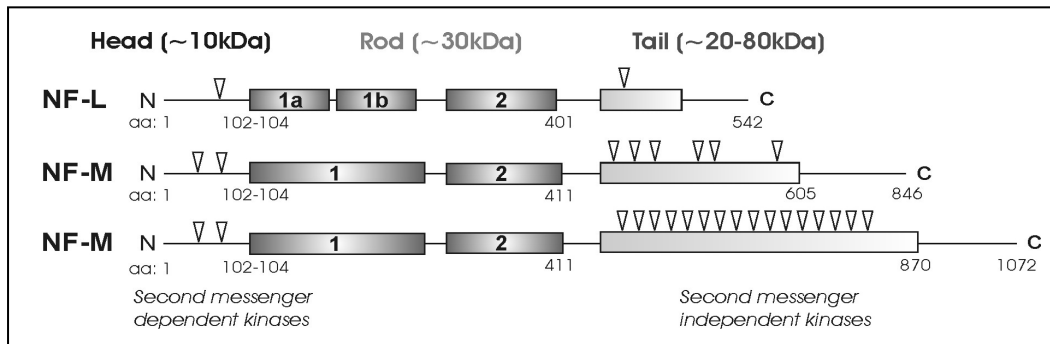
Phosphorylation is the major post-transcriptional modification in the NF proteins. Almost all (>99%) of the assembled NFs in myelinated internodal regions are known to be stoichiometrically phosphorylated in the KSP repeat domains (Hsieh et al., 1994). In contrast, the KSP repeats of NFs in cell bodies, dendrites and nodes of Ranvier are less phosphorylated (de Waegh et al., 1992; Cole et al., 1994; Hsieh et al., 1994).

The NF proteins are extensively phosphorylated in their COOH-terminal regions (see Fig. 2); the phosphate is present mainly as phosphoserine except for small amounts of phosphothreonine. The phosphorylation sites, which are KSP amino acid repeats, are highly conserved in the different species (Schlaepfer, 1987). In the case of NF-H, more



than 40 KSP existing repeats in the COOH-terminal domain can be separated in two categories, KSPXX and KSPXK (X in any amino acid except Lys), which are believed to be regulated by different protein kinases (Elhanany et al., 1994).

Several studies suggest that among numerous candidate NF protein kinases [glycogen synthetic kinase (GSK 3), extracellular signal-regulated kinase (ERK), stress-activated protein kinases, protein kinase A (PKA), protein kinase C (PKC)], and cyclin-dependent kinase-5 (cdk5) preferentially phosphorylate the KSPXK repeats (Shetty et al., 1993; Hisanaga et al., 1994; Sun et al., 1996). In contrast, ERKs and GSK appear to regulate KSPXX repeats (Pant and Veeranna, 1995). The extensive phosphorylation of these COOH-terminal domains, which project outward from the filament core, is considered to play an important role in the NF cross-linking, thus stabilizing the neuronal cytoskeleton.



**Figure 2.** Head, rod and tail domains of NF-L, NF-M and NF-H with indication of the location specific phosphorylation by different groups of kinases. The inverted triangles show the phosphorylation sites. Modified from Sihag et al., (2007).

Particular interest has been increasingly paid to the NH<sub>2</sub>-terminal head domain (Fig.2) of the NF proteins (Sihag and Nixon, 1990). Currently, at least 13 phosphorylation sites have been found in the NF-L head domain by *in vitro* studies. PKA, PKC, Rho-kinase, and calmodulin-dependent protein kinase II (CaMKII) have been described as the kinases recognizing those sites (Sihag and Nixon, 1991; Cleverley et al., 1998; Sihag et al., 1999; Hashimoto et al., 2000; Nakamura et al., 2000). Usually NF-M and NF-H undergo posttranslational hyperphosphorylation upon entry into the axons. NF-M phosphorylation shows the polarized character in the growing axons; a proximal-distal gradient with most intense phosphorylation in the axons of peripheral nerves, and little or no phosphorylation in the perikarya and dendrites (Pant and Veeranna, 1995).

NF phosphorylation plays multiple roles; its major function is believed to be the regulation of axonal radial growth. Extensive phosphorylation of KSP repeats in the tail domain of NF-M and NF-H occurs primarily in axons (Lee et al., 1988; Nixon, 1992). This results in side-arm formation, increased inter NF spacing, radial growth of axons, and increased conduction velocity (de Waegh et al., 1992; Nakagawa et al., 1995; Yin et al., 1998). Phosphorylation of KSP motifs triggered by a Schwann cell signal has been proposed as a key determinant, locally controlling NF accumulation, interfilament spacing, and radial growth of myelinated axons (de Waegh et al., 1992; Sanchez et al., 1996; Yin et al., 1998; Sanchez et al., 2000). Recent studies using NF-H null mice or mice transfected with a NF-H tailless mutant, suggest that neither NF-H nor its phosphorylated tail is essential for determining neuronal calibre (Rao et al., 1998; Rao et al., 2003). These studies, together with those using NF-M knockout mice (Elder et al., 1999a; Kriz et al., 2000), indicate that NF-M plays an important and profound role in regulating the axonal diameter.

Phosphorylation also plays a critical role in inhibiting NF assembly in the perikaryon. N-terminal head domain phosphorylation by protein kinase A (PKA) at Ser55 in NF-L or Ser46 in NF-M inhibits assembly of NF subunits into a heteropolymer *in vitro* and *in vivo* (Sihag and Nixon, 1991; Hisanaga et al., 1994; Nakamura et al., 2000). Consistent with these studies, phosphorylation/dephosphorylation of Ser55 and Ser57 in NF-L regulates NF assembly and disassembly (Gibb et al., 1996; Gibb et al., 1998). Prevention of NF-L Ser55 dephosphorylation through a Ser-to-Asp mutation, mimicking permanent phosphorylation, resulted in pathological accumulation of NF aggregates in neuronal cell bodies (Gibb et al., 1998). These studies suggested that the transient head domain phosphorylation in cell bodies regulates NFP polymerization and filament formation (Ching and Liem, 1999). Moreover, dephosphorylation of the head domains is prerequisite for NF assembly in axons.

So transient phosphorylation of head domain motifs of NF-L and NF-M by PKA and PKC in cell bodies can prevent NF assembly and C-terminal phosphorylation in perikarya (Lee et al., 1993; Hisanaga et al., 1994; Ching and Liem, 1999), protecting neurons from abnormal accumulation of phosphorylated NF aggregates in cell bodies.

#### **2.1.4.2 Protective effects of phosphorylation of neurofilaments**

Recent studies suggest that NFPs have a protective effect for neuronal survival. Couillard-Després et al. (1998) showed that when crossing transgenic mice

overexpressing human NF-H with mice expressing a mutant superoxide dismutase (SOD1G37R), the SOD1 mutant progeny lifespan increased by up to 65%.

The hypothesis of Nguyen et al. (2001, 2003) suggests that perikaryal NFs can alleviate the toxicity of mutant SOD1 by sequestering the p25/Cdk5 complex and by acting as a phosphorylation sink for deregulated kinase activity thereby reducing the potential toxicity of hyperphosphorylation of tau and of other cdk substrates, such as retinoblastoma (Nguyen et al., 2001; Nguyen et al., 2003). Many questions still remain, such as how to reconcile apparently conflicting functions of NFs.

### **2.1.5 Degradation of neurofilaments**

Many neurons extend their axons over great distances, up to 1 m in humans, to form synapses with appropriate receptor cells. To maintain the physiological functions of the nerves, certain proteins need to have long lifetimes to span the axon. NFs are among them. In the transport process, NF degradation is not detected. Different experiments indicate that the cytoskeletal proteins are metabolically stable during their passage through the axon, and essentially all of these proteins complete their trip to the axon terminus. So it is believed that NF degradation may only occur at synapses, where dephosphorylation of NFs by the protein phosphatase 2A (PP2A) precedes NF degradation. This suggests that phosphorylation has a protective function in protecting the NFs from degradation (Gong et al., 2003). The normal levels of the NFs must also be balanced with mechanisms of turnover and degradation. It has been shown that at the axonal terminus, the half residence time of NF is around 2 days (Paggi and Lasek, 1987).

The disintegration of NFs during pathological conditions is accounted for by the presence of Ca<sup>2+</sup>-activated protease (calpains) in the axoplasm. Calpains can degrade many different proteins, including important axonal cytoskeletal proteins such as spectrin, microtubule-associated protein (MAP)-1, tau, tubulin and NFs, as well as several protein constituents of myelin (e.g. myelin basic protein, myelin-associated glycoprotein and proteolipid protein) (Kampfl et al., 1997).

The breakdown and turnover of NF could be accounted for by the activation of calpain protease in nerve terminals. It is possible that the recycling of NF degradation products represents an important feed-back mechanism regulating NF production. The success or failure of axonal regeneration may be determined by intrinsic neuronal factors that control the production of the axonal cytoskeleton. NFs synthesis has also been detected

in axons, but accounts for less than 1% of the total axonal NFs (Grant and Pant, 2000; Sotelo-Silveira et al., 2000), and the role of this small amount of newly synthesized NFs is unknown.

### **2.1.6 Neurofilaments in development**

Although most populations of mature neurons express NF proteins, their amount and spatial distribution may vary in neuronal cell types and regions of the CNS (Trojanowski et al., 1986). During the development, the expression of NF proteins is suggested to be unique for each NF protein. In the embryonic rat brain, NF-L and NF-M proteins are co-expressed, while the appearance of NF-H is delayed to a very late embryogenetic or postnatal period (Schlaepfer, 1987). In humans, however, the three NF proteins are expressed already at the ninth gestational week (Arnold and Trojanowski, 1996). Furthermore, the phosphorylation of NFs, in particular that of NF-H, occurs late in ontogenesis and is perhaps related to the axonal maturation (Fischer and Shea, 1991).

Several studies of NF mRNA expression in different species have been published. In the mouse hippocampus, in addition to the prenatal expression of the NF-L mRNA, its fast increased during the postnatal (P) period was shown (Kure and Brown, 1995). In contrast, NF-H mRNA was not detected at embryonic stages, and it was expressed at low levels prior to P9, then increasing 2.5-fold between P9 and P11. Thereafter the levels further increased up to P28 (Kost et al., 1992). In a recent mRNA study in the hamster forebrain (Kost et al., 1992), NF-L and NF-M mRNAs were expressed at very low concentrations in the embryonic brain whereas their expression dramatically increased postnatally up to P28, and then declined.

### **2.1.7 Consequences of neurofilament knockout and overexpression**

The targeted disruption of NF genes in mice has shown the importance of NF protein subunits in the CNS. The physiological consequences of the gene inactivation have mainly been studied in motorneurons and other neurons with large axons. In the **NF-L** *-/-* mice, NF-M and NF-H decreased to ~5% of the normal levels, and the rats were unable to form filaments without NF-L. As a result, the mice show severe axonal hypotrophy and a lack of large myelinated axons (Zhu et al., 1997). The **NF-H** *-/-* mice had minor effects in the NF number and in the calibre of motor axons, although increase in NF-M and microtubules were observed, indicating a possible compensatory effect (Rao et al., 1998; Zhu et al., 1998; Kriz et al., 2000). Despite not affecting the axonal calibre, a

decrease in the speed of conduction of action potential, and reduction of myelin thickness have been detected in the **NF-H** *-/-* mice (Kriz et al., 2000; Perrot et al., 2007). The **NF-M** *-/-* mice showed that NF-M is more important than NF-H for the axonal radial growth. The NF-M knockout resulted in increase in NF-H, and decrease in NF-L and total NFs in the axons (Elder et al., 1999a; Elder et al., 1999b; Jacomy et al., 1999). In the **double knockout mice NF-M** *-/-*, **NF-H** *-/-* accumulation of unassembled NF-L in the neuronal perikarya was detected, ~25% loss of the ventral root axons, no NF formation in axons, and two-fold increase in microtubules (Elder et al., 1999b; Jacomy et al., 1999).

Given the importance of the stoichiometry of the NF proteins, also overexpressing mice have been studied. Fourfold **overexpression of NF-L** lead to amyotrophic lateral sclerosis (ALS)-like pathology, and most mice died within the first 3 postnatal weeks (Xu et al., 1993). **NF-M and NF-H overexpression** do not induce such important consequences as the NF-L overexpression, although changes in the axonal calibre and accumulation of NF in the perikarya have been detected (Julien et al., 1995; Wong et al., 1995; Marszalek et al., 1996).

### 2.1.8 Neurofilaments and diseases

Many alterations can potentially lead to accumulation of NFs, including deregulation of NF synthesis, defective axonal transport, abnormal phosphorylation and proteolysis. Abnormal accumulation of NFs is detected in many human neurodegenerative disorders, including among others amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), dementia with Lewy bodies, Parkinson's disease (PD) and diabetes. Studies in transgenic mice also showed that NFs affect the dynamics and function of other cytoskeletal elements, such as microtubules and actin filaments (Zhu et al., 1998; Ahlijanian et al., 2000). It is widely believed that NF abnormalities in neurodegenerative disorders are the hallmark of neuronal dysfunction.

**Amyotrophic lateral sclerosis (ALS)** is a progressive neurodegenerative disease that affects mainly motor neurons eventually leading to neuron death and loss of related muscle movements. A key neuropathological hallmark of ALS is the intraneuronal aggregates of NFs in degenerating motor neurons (Bruijn et al., 2004). Although the reason for the NF aggregates is unclear, transgenic mouse models which overexpress any of the NF subunits (see the previous section) will provoke motor neuropathy characterized by the presence of abnormal NF accumulations resembling those found in ALS (Julien et al., 1998).

It has been proposed that such alterations in NF homeostasis are directly relevant to the pathogenesis of ALS (Williamson et al., 1998). Interestingly, abnormal NF inclusions are often associated with decreases in levels of NF-L mRNA. NF-L mRNA is selectively reduced by up to 70% in degenerating neurons of ALS and AD patients (Wong et al., 2000; Menzies et al., 2002). Also codon deletions and insertions in the KSP regions of NF-H including a large deletion of five KSP repeats, have been detected in sporadic cases of ALS (Tomkins et al., 1998; Al-Chalabi et al., 1999).

**Alzheimer's disease (AD)** is a neurodegenerative disease established as the leading cause of dementia. Cytoskeletal disruption is a prominent feature in the pathogenesis of AD (Ahlijanian et al., 2000; Nunomura et al., 2001). Neurofibrillary tangles are the hallmark of AD and they are composed of abnormally modified tau, NFs and other cytoskeletal proteins. However, the biochemical processes of the formation of the tangles are not known. In AD, inappropriate hyperphosphorylation of proteins such as tau and NF is prominent, which is likely due to perturbation in the balance between kinases and phosphatases (Gong et al., 2000). This abnormal hyperphosphorylation may cause protein aggregation in NFs in AD neurons.

**Parkinson's disease (PD)** is a progressive disorder caused by degeneration of the dopaminergic neurons in the substantia nigra of the brain resulting in decreased dopamine availability. Clinically, PD is characterized by severe motor symptoms, including uncontrolled tremor, postural imbalance, slowness of movement, and rigidity (Lotharius and Brundin, 2002).

One of the major pathological hallmarks of PD is the accumulation of protein inclusions, known as Lewy bodies, in the substantia nigra. Lewy bodies are composed of numerous proteins, including the essential constituent of  $\alpha$ -synuclein protein, the three NFs, ubiquitin and proteasome subunits (Galloway et al., 1992; Trimmer et al., 2004). Electron microscopy and biochemical evidence indicate that the abnormally phosphorylated NFs form a non-membrane-bounded compacted precipitate in the neuronal soma in the affected neurons. The precipitation of the NF proteins in some cases is caused by mutation in the NF genes. Mutations of NF-L in the rod domain 2 might cause a peripheral motor nerve axonal loss, while mutations in the rod domain 2 of NF-M cause central dopaminergic loss (Hill et al., 1993).

**Charcot-Marie-Tooth disease (CMT)** is an inherited neurological disorder that affects both motor and sensory neurons. Patients slowly lose normal use of their extremities and also suffer from sensory loss. There are several types of CMT, but the simplest classification is into types 1, 2 and 3. Types 1 and 3 are due to demyelination, and type 2 is an axonal disease. Several families have been identified in which heterozygosity for mutations of the NF-L gene on chromosome 8 are associated with CMT2 (Mersiyanova et al., 2000; De Jonghe et al., 2001; Jordanova et al., 2003). The first of these changes is a Pro8Glu mutation, and the second change is a Leu333Pro mutation in the highly conserved rod domain 2B.

The NF-L mutations in CMT2 disrupt the NF assembly and the axonal transport (Fabrizi et al., 2007), and this probably underlies the disease mechanism. Some types of CMT are caused by defects in proteins expressed in the Schwann cell, including the connexin 32 genes (Braathen et al., 2007). Such mutations result in aberrant myelination and altered NF phosphorylation. In such cases, therefore, abnormalities of NF phosphorylation occur downstream of the primary pathological process but are still upstream of the end point.

## 2.2 The hippocampus

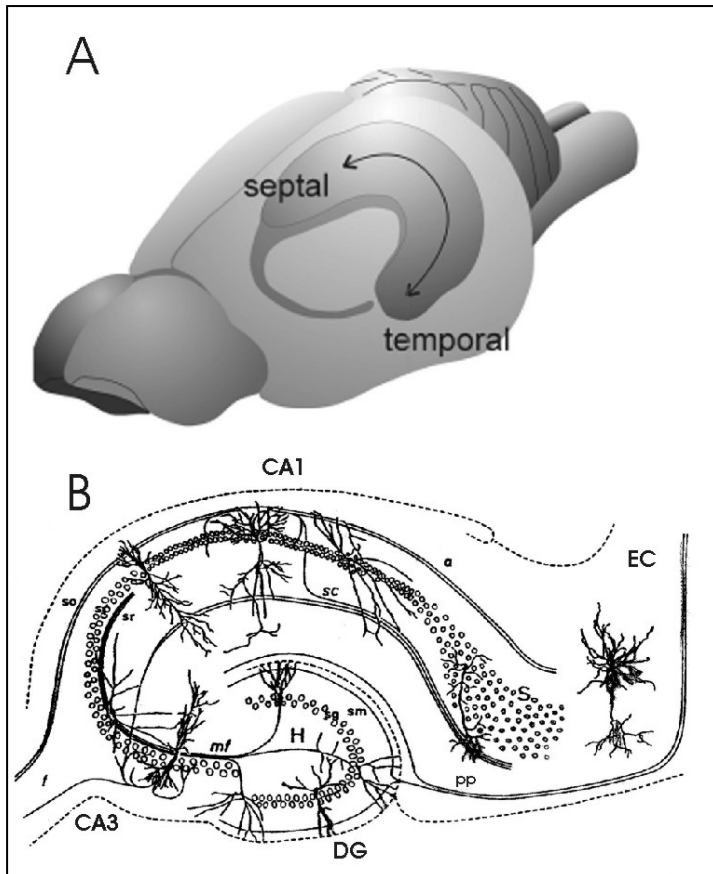
The hippocampus is a brain structure situated in the inner temporal lobe of the brain and it is involved in many important brain functions such as learning and memory. There are also various clinical conditions that result in morphological alterations in the hippocampus. Most of the factors that cause these diseases are not known, but it is clear that various parts of the hippocampus are differently vulnerable to damage. In TLE and ischemia in humans, for example, remarkable cell loss has been found in the CA1 area of the hippocampus (Cendes et al., 1995; Bernasconi et al., 2003).

The apparent simplicity of the neuronal circuitry of the hippocampus has attracted the research from different disciplines of neurobiology, including anatomy, physiology, pharmacology, and molecular biology. Because of its relative simple and regular structure, the hippocampus has been employed frequently as a model system for studies of developmental questions in neurobiology. Fig. 3 shows a schematic representation of the hippocampal structure.

## 2.2.1 Development and cytoarchitecture of the hippocampus

### 2.2.1.1 Dentate gyrus

The dentate gyrus (DG) comprises three regions or areas. Closest to the hippocampal fissure is a relatively cell free region called the molecular layers which contains the dendrites of the granule cells together with the inputs from the entorhinal cortex and commissural fibers. The principal region of the DG is the granule cell layer, which lies deep to the molecular layer and is made up primarily of densely packed granule cells. The granule cell layer and molecular layers form a “V” or “U” shaped structure that encloses the hilar region (also called polymorphic layer), which constitutes the third layer of the DG (Bayer, 1980a).



**Figure 3.** (A) The C-shaped hippocampus is shown in a transparent shell of the rat brain indicating the septotemporal or longitudinal axis. Adapted from Witter and Amaral (2004).

(B) Schematic representation of the hippocampal structure from Caesar and Aertsen (1991). Abbreviations: DG: dentate gyrus; EC: entorhinal cortex; f: fimbria; H: hilus; mf: mossy fiber; pp: perforant pathway; S: subiculum; sc: Schaffer collaterals; sg: stratum granulare; sm: stratum moleculare; so: stratum oriens; sp: stratum pyramidale; sr: stratum radiatum.

The neuronal proliferation has been intensively studied in the rat which has a gestational period of 21 days. The main granular cell proliferation starts in the late embryonic period



(E16) and is extended up to the postnatal days 21-25 (Bayer, 1980b, a). The proliferation of the granule cells also continues at a slow rate well into adult life (Bayer et al., 1982; Ehninger and Kempermann, 2008). Interestingly, such newly born cells appear to become functionally integrated in the hippocampal network (Hastings and Gould, 1999; Markakis and Gage, 1999; van Praag et al., 2002). The proliferation of the granular cells follows a gradient in which new cells lay in a deeper position. This gradient is in contrast to the most usual inside-out pattern observed in the cortical regions.

### **2.2.1.2 Pyramidal layers of the hippocampus**

The organization of the layers is similar for the CA1-CA3 regions. The main cellular layer is called the pyramidal cell layer. The layer located in the outside edge of the structure is called stratum oriens. In the CA3 field, but not in CA2 or CA1, a narrow acellular zone located just above the pyramidal cell layer is occupied by the mossy fiber axons originating from the DG granule cells. This narrow layer is called stratum lucidum.

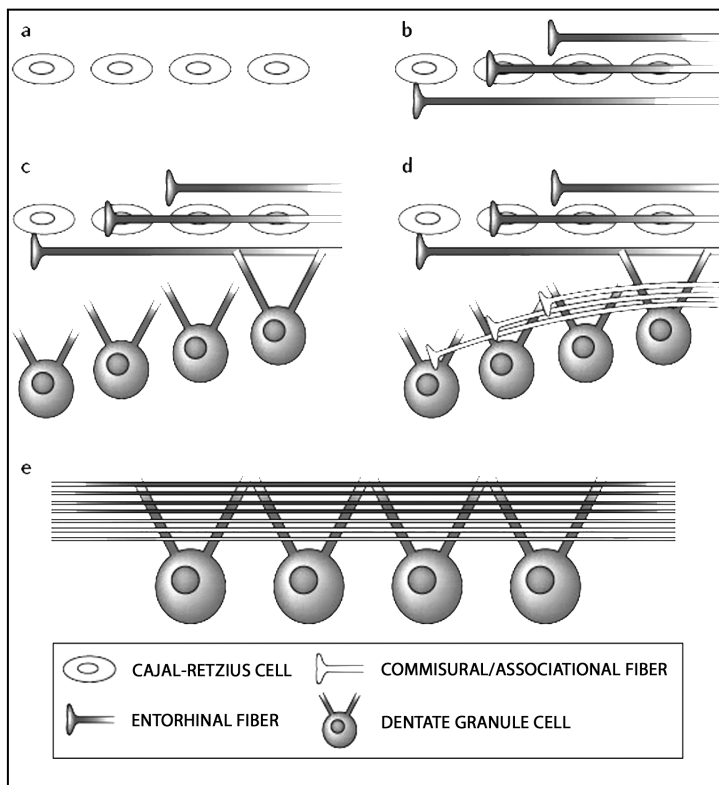
Above the stratum lucidum in CA3, and immediately beneath the pyramidal cell layer in CA2 and CA1, is the stratum radiatum. The following layer of the hippocampus is called the stratum lacunosum moleculare. The pyramidal cell proliferation of CA1-CA3 regions occurs entirely during the embryonic period E14-19 (Bayer, 1980,a, b), and their maturation extends postnatally.

### **2.2.1.3 Formation and layering of the hippocampus**

The precise mechanisms guiding the location and layering of the different cell types in the hippocampus have started recently to be unfolded. One of the most important and known molecules controlling the layering is reelin. Reelin is a large extracellular matrix protein that is secreted by Cajal-Retzius cells located in the marginal zone, and it serves as a molecular guiding cue for the subventricular migrating neurons during embryonic development in the cortex and hippocampus (Fig. 4) (D'Arcangelo et al., 1995; Del Rio et al., 1997; Frotscher et al., 2003). Reelin binds to a series of receptors and activates a defined signaling pathway thoroughly reviewed by Tissir and Goffinet (2003). In humans, deficiency in reelin is the cause of the Norman–Roberts-type lissencephaly (Hong et al., 2000). Reelin-deficient (*reeler*) mice have architectonic anomalies in most areas of the CNS, especially in the cortex and hippocampal formation (Yip et al., 2000; Rice and Curran, 2001; Yip et al., 2004). Cortical neurons in mutant mice are generated

in normal numbers and at the normal time although young neurons are unable to migrate past their predecessors, and maturation of the cortex proceeds from outside to inside.

As mention above, neurogenesis in the DG persists into postnatal life, which implies that late-generated granule cells have to migrate through and integrate into a fully differentiated dentate neuronal network. Accordingly, in contrast to most other brain regions, there is a persisting radial glial scaffold in the postnatal DG which also forms a template for migrating granule cells (Forster et al., 2002; Frotscher et al., 2003; Weiss et al., 2003; Forster et al., 2006a).



**Figure 4.** (a) Tangentially migrating Cajal-Retzius (CR) cells populate the marginal zone of the DG early in development. (b) CR cells serve as early targets of entorhinal fibers in the outer molecular layers. (c) DG granule cells are born in the hilar region of the DG and migrate to the marginal zone a process controlled by reelin-secreting CR cells. (d) Commisural associational fibers arrive to the molecular layers after the entorhinal fibers, and grow to their definitive targets, the granule cells. (e) The characteristic laminated structure of the DG formed. The granule cell layers, the commissural / associational fibers in the inner molecular layer, and the entorhinal

fibers in the outer molecular layers. Drawing obtained from Forster et al., (2006b).

With still many unknown factors, the studies of the DG suggest that reelin acts as a positional cue and exerts its effects on both radial glial cells and neurons. Fig. 4 shows a schematic sequence of events in the layering of the DG during development as suggested by Forster et al., (2006).

## 2.2.2 Hippocampal connectivity

### 2.2.2.1 Intrinsic connectivity of the hippocampus

The connectivity of the hippocampus can be divided in intrinsic and extrinsic connections. **In the intrinsic connections** the DG can be considered as the first step. The cells of the DG do not project outside of the hippocampal formation. Rather, they project via their axons, called mossy fibers, to cells in its own region (Claiborne et al., 1986), but mainly to the proximal dendrites of the pyramidal cells of the CA3 region of the hippocampus (Gaarskjaer, 1981). Collaterals of the CA3 pyramidal cells project to other levels of CA3 to CA1 and to subcortical regions. The CA3 pyramidal cell axons (Schaffer collaterals) constitute the major input system to the CA1 which terminate mainly in the stratum radiatum (Ishizuka et al., 1990). The projections of the pyramidal cells of the CA1 region project predominantly to the subiculum (Finch et al., 1983), and the subiculum, in turn, projects to the entorhinal cortex (EC) (Beckstead, 1978, Amaral et al., 1984).

**The associational and commissural connections of DG, CA3 and CA1** follow clear and distinct patterns. The inner third of the molecular layer of the **DG** receives a projection exclusively from cells in the polymorphic layer originated both on the ipsilateral and contralateral sides of the hippocampus (Deller et al., 1995; Deller et al., 1996).

In the **CA3 region**, cells located proximally communicate only with other cells in the proximal portion of CA3. **Associational projections** arising from mid and distal portions of CA3, project extensively along the septotemporal axis (Ishizuka et al., 1990). Furthermore in the rat, but not in the monkey (Amaral et al., 1984; Demeter et al., 1985), the CA3 pyramidal cells give rise to **commissural projections** to the CA3, CA2, and CA1 regions of the contralateral hippocampal formation. The same CA3 cells give rise to both ipsilateral and commissural projections (Swanson et al., 1980).

Unlike the CA3 field, **pyramidal cells in CA1** do not appear to give rise to collaterals that distribute within the CA1 area and there is only a **weak associational connection** (Tamamaki et al., 1987; Alkon et al., 1991). Furthermore, only a weak **commissural projection** to the contralateral CA1 appears to be present (van Groen and Wyss, 1990).

This difference in intrinsic organization between CA3 and CA1 has been taken to indicate that they may serve different functional processes within the hippocampal formation related to learning and memory (Treves and Rolls, 1992).

### **2.2.2.2 Extrinsic connectivity of the hippocampus**

#### *a) Extrinsic inputs to the hippocampus*

**The major input to the DG** and in turn to the hippocampus arises from the entorhinal cortex (EC) through the so-called perforant pathway. The perforant pathway originates in the neurons located in the layers II and III of the EC (Witter et al., 1988; Witter et al., 1989) although a smaller component of the projection originates in the deeper layers. These fibers travel from the EC through the subiculum and mainly terminate in the outer two-thirds of the molecular layer of the DG (Van Hoesen and Pandya, 1975). Despite the DG being the “traditional” target of the entorhinal–hippocampal fibers, there is evidence that the EC also projects to the other areas of the hippocampus and the subiculum as well (Witter et al., 1989; Naber et al., 2001).

The other inputs to the DG are subcortical originating mainly from the supramammillary region of the posterior hypothalamus, from the septal nuclei, and from various monoaminergic nuclei in the brain stem, especially the locus coeruleus and the raphe nuclei (Haring and Davis, 1983; Gaykema et al., 1990; Vertes, 1992; Yoshida and Oka, 1995; Lubke et al., 1997).

**The extrahippocampal inputs to the CA3 region** arise from different portions of the subcortical regions. Projections from the septal nucleus (Yoshida and Oka, 1995) terminate most heavily in the stratum oriens and to a lesser extent in the stratum radiatum (Nyakas et al., 1987; Gaykema et al., 1990). Also projections from the basal nucleus of the amygdala terminate abundantly in the stratum oriens and radiatum (Pikkarainen et al., 1999). The CA3 field also receives inputs from the noradrenergic nucleus locus coeruleus and nucleus subcoeruleus (Datta et al., 1998). Serotonergic fibers also reach the CA3 region and appear to terminate preferentially on interneurons (Freund et al., 1990; Vertes et al., 1999).

The **CA1 region receives extra hippocampal inputs** from a variety of cortical and subcortical structures. One of the main inputs arises from the EC through the perforant pathway as discussed above for the DG. Also the basal nucleus in the amygdaloid area projects to the stratum oriens, radiatum and lacunosum of CA1 (Pikkarainen et al., 1999;

Pitkänen et al., 2000; Kemppainen et al., 2002). For a complete review of connections between amygdala and hippocampus see Pitkänen *et al.* (2000).

### b) Extrinsic outputs of the hippocampus

The **DG** gives rise to no extrinsic projections other than the mossy fiber projection to the CA3 field of the hippocampus.

The most important **projection from the CA3 outside the hippocampus** is to the lateral septal nucleus (Swanson and Cowan, 1977). The CA3 projection is bilateral and topographically organized. Septal portions of CA3 project dorsally to the lateral septal nucleus and progressively more temporal portions of CA3 project more ventrally (Gaykema et al., 1991; Risold and Swanson, 1997).

**The CA1 field** has more extrinsic projections than the CA3. The CA1 field also projects to the lateral septal area and to a number of other subcortical and cortical areas. In that sense CA1 is similar to the subiculum, with which it shares most of its projections (van Groen and Wyss, 1990; Jay and Witter, 1991; Naber and Witter, 1998). The organization of projections to the lateral septal area is similar to the projections originating in CA3 but terminating at levels more rostral than those from CA3 (Risold and Swanson, 1997; Naber and Witter, 1998).

## **2.2.3 Organotypic hippocampal slice cultures**

Organotypic hippocampal cultures have been introduced as an interesting and successful system to study *in vitro* a fully functional hippocampus in contrast to cell cultures. In the *in vivo* hippocampus, the excitation enters the DG granule cell dendrites via the perforant pathway, runs along the granule cell axons, the mossy fibers, exciting CA3 pyramidal cell dendrites, which convey the information through the Schaffer collaterals to CA1 neurons, and the excitation exits the hippocampus through the alveus and the fimbria. This information-processing organization is in principle preserved in cultured hippocampal slices with exception of the inputs from the EC and the outputs from the hippocampus.

Hippocampal slice cultures are in general prepared from 6–7-day-old (P 6–7) rats (Stoppini et al., 1991) although both younger and older rats (from P4 up to P30) have been used. When the slices are prepared from rats of P6–P7, the hippocampus is still in an immature state and the DG granule cells are still proliferating. The other main cell

types in the hippocampus, the CA1-CA3 pyramidal cells, are in a more mature state since they proliferate prenatally (Schlessinger et al., 1975; Bayer, 1985; Bayer and Altman, 1987).

It has been shown in several studies that the hippocampal structure and the morphology of the hippocampus are well preserved in OHCs. Furthermore, the neuronal maturation, receptor expression, dendritic and axonal formation together with receptor expression, resembles that seen *in vivo* (Dailey et al., 1994; Frotscher and Heimrich, 1995; Gahwiler et al., 1997; Holopainen and Lauren, 2003). All those morphological features were assessed during the first 2-3 weeks after the preparation of the OHC. The cytoskeletal maturation has also been shown to be similar between the *in vitro* OHC and their *in vivo* counterparts (Hartel and Matus, 1997).

After 3 weeks *in vitro*, several different features develop in the slices when comparing to the *in vivo* counterpart. A lower number of dendritic spines and pronounced axonal sprouting in DG granular cells have been described in OHCs than *in vivo*. The sprouted axons form recurrent synapses with the dendrites of the granular cells (Coltman et al., 1995; Frotscher et al., 1995). This mossy fiber sprouting in conjunction with the preservation of the main excitatory loop in the hippocampus contributes to the progressively increased excitatory activity of granule cells during the culture time (Gutierrez and Heinemann, 1999; Bausch and McNamara, 2000; Lindroos et al., 2005).

## **2.3 Neuronal kainate receptors**

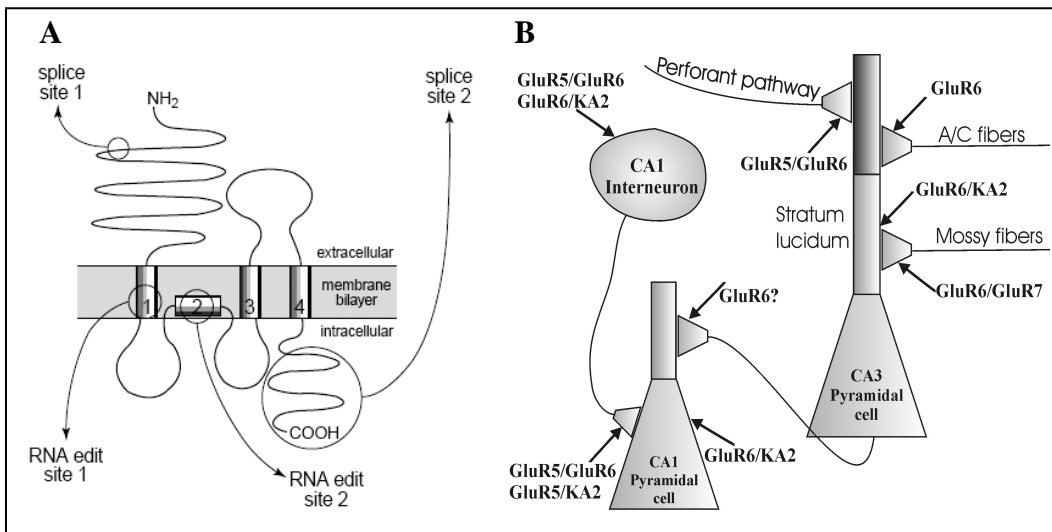
Fast excitatory neurotransmission in the mammalian nervous system is mainly mediated by glutamate. Glutamate has also a crucial role during the ontogeny of the nervous system, participating in the outgrowth of processes, formation and elimination of synapses, and in the activity-dependent fine tuning of connectivity in several brain areas. Alterations of glutamatergic neurotransmission have been related to the neuronal damage observed after episodes like ischemia, as well as to the etiology of a series of neurological conditions including epilepsy, and ALS (Lerma, 2003).

### **2.3.1 KA receptor subunits and composition**

Glutamate receptors (GluR) are integral membrane proteins which are responsible for mediating information transfer at most excitatory synapses in the brain. Ionotropic glutamate receptors belong to three receptor families, named after the agonists that activate them: N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole

propionic acid (AMPA) and kainic acid (KA) receptors. These receptors are among the most studied molecules in the CNS. Similarly to the AMPA and NMDA receptors, KA receptors are tetrameric combinations of five subunits: GluR5, GluR6, GluR7, KA1 and KA2. Of these, GluR5–7 are all known to form homomeric or heteromeric functional channels (Egebjerg et al., 1991; Schiffer et al., 1997), while KA1 and KA2 only form functional channels by coexpression with other subunits (Werner et al., 1991; Herb et al., 1992).

GluR5 was the first mammalian KA receptor subunit to be cloned (Bettler et al., 1990), and another four KA receptor subunits (GluR6, GluR7, KA1 and KA2) were soon identified after that (Egebjerg et al., 1991; Werner et al., 1991; Herb et al., 1992; Sakimura et al., 1992). These subunits can be divided into two groups on the basis of



**Figure 5.** **A)** Schematic representation of the KA receptor subunits indicating the trans-membrane domains intra- and extracellular domains, and the splicing and editing sites. Obtained from Chittajallu et al. (1999). **B)** A schematic representation of the expression and subcellular localization of KA receptors in the mossy fibers, CA3, C1, and interneurons of the hippocampal network based on (Pinheiro and Mulle, 2006). Abbreviation: A/C fibers, associational-commisural fibers.

their structural homology and affinity for KA. The low-affinity subunits, GluR5–GluR7, display 75% homology while the high-affinity subunits, KA1 and KA2, are 68% homologous. The homology between GluR5–GluR7 and KA1/KA2 is much lower

(~45%). The KA receptor subunits show ~40% sequence homology to the AMPA receptor subunits GluR1–GluR4.

All KA receptor subunits share a common topology and possess an extracellular N-terminus, three transmembrane domains (TM1, TM3 and TM4), with one region which forms a loop within the membrane (TM2) which leads to an intracellular loop (Wo and Oswald, 1995). The extracellular N-terminus, together with the extracellular loop between TM3 and TM4, forms the ligand binding domain, and the re-entrant loop of TM2 forms the lining of the pore region of the ion channel (Fig. 5A).

KA receptors also undergo both splice variation and RNA editing giving rise to a large number of possible receptors with differing pharmacological and functional properties. Fig. 5A shows the positions in the receptor subunits where splice variations and RNA editing have been described. The **RNA editing site 1** resulting in amino acid substitution Ile536Val, occurs in the TM1 domain and it has been identified in GluR6. Further editing in this region leads to a Tyr540Cys substitution (Kohler et al., 1993). The **RNA editing in site 2** for GluR5 (residue 591) and GluR6 (residue 590) occurs in TM2 at the Q/R site, in which unedited subunits contain a Glu residue whereas the edited subunits contain an Arg residue (Bettler and Mülle, 1995).

The **splice site 1** for the alternative splicing of GluR5 is located in the N-terminal domain. GluR5-1 possesses additional 15 amino acids compared to the originally described subunit (GluR5-2b) which has 875 amino acids (Bettler et al., 1990). **Splice site 2**: alternative splicing gives rise to various C-terminal splice variants. GluR5-2a possesses a truncated C-terminus (826 amino acids), GluR5-2b which is the originally described subunit, contains a sequence of 875 amino acids, while GluR5-2c has additional 29 amino acids (904 amino acids) (Sommer et al., 1992). GluR6a and GluR6b receptors have been identified, and they differ by a stretch of 54 and 15 amino acids, respectively (Gregor et al., 1993), and co-assemble in different brain regions (Coussen et al., 2005). GluR7 also undergoes alternative splicing giving rise to C-terminal variants. GluR7a has 888 amino acids, GluR7b contains additional 55 amino acids (943 amino acids), and the frame shift results in a low homology between GluR7a and GluR7b (Schiffer et al., 1997).



### 2.3.2 Distribution of KA receptors

The distribution of KA receptors by *in situ* hybridization has revealed that they are widely expressed throughout the nervous system. However, the expression patterns of the different subunits are very heterogeneous. For example, GluR6 is heavily expressed by hippocampal pyramidal neurons and dentate granule cells (Wisden and Seeburg, 1993; Bahn et al., 1994). Similarly, it has been shown that a population of interneurons in the hippocampus, mainly in the stratum oriens, expresses the GluR5 subunits (Bahn et al., 1994).

In a more recent study, Paternain and colleagues (Paternain et al., 2000) used double *in situ* hybridization of GluR5 and GluR6 together with glutamate decarboxylase 65 (GAD<sub>65</sub>) in the hippocampus and obtained detailed information of KA receptors in hippocampal interneurons. After confirming the expression patterns of the KA receptor subunits obtained previously, they found in a very detailed work that in the CA1 region, ~85% of the neurons expressing GluR5 also express GAD. GluR6 was abundantly expressed by principal cells in the hippocampus, but only few interneurons in CA1 expressed GluR6 transcripts. Furthermore, it was described that in a certain population of GABAergic interneurons (~25%) GluR5 and GluR6 subunits may coexist. A schematic representation of the cellular distribution of KA receptors in the hippocampus is shown in Fig. 5B.

### 2.3.3 Functional properties of KA receptors

The studies of functional properties of KA receptors in the hippocampus have been very fruitful. It has been shown that stimulation in the mossy fiber pathway generates slow excitatory postsynaptic currents (EPSCs) in CA3 pyramidal neurons which were mediated by KA receptors, but not AMPA receptors. This was studied by using the highly selective AMPA receptor antagonist GYKI53655, which blocked any AMPA receptor induced currents in CA3, but did not abolish the EPSCs (Castillo et al., 1997; Vignes and Collingridge, 1997).

Granule cells and CA3 pyramidal cells are enriched with GluR6-containing KA receptors, and the synaptic currents generated by the stimulation of mossy fibers, after blockade of AMPA receptors, are eliminated in GluR6 knockouts (Mulle et al., 1998). Furthermore, in the GluR6 knockouts, higher KA concentrations are also required to

generate seizures. These observations provide direct evidence that GluR6 subunits mediate the epileptogenic actions of KA in CA3.

Presynaptic action of KA has also been shown to be of importance for the release of glutamate. In the mossy fiber-CA3 synapse, KA receptor activation in the mossy fiber terminals has been demonstrated to have a dual role. At low KA concentrations (20-50 nM), an increase of glutamate release from mossy fibers occurs (Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001; Rodriguez-Moreno and Sihra, 2004), while at higher concentrations (>50 nM), presynaptic KA receptors can reduce glutamate release from mossy fibers (Contractor et al., 2000; Kamiya and Ozawa, 2000; Schmitz et al., 2000). The inhibition of glutamate release at the mossy fibers by KA receptor activation has recently been shown to occur through Gi/o protein activation (Negrete-Diaz et al., 2006).

## **2.4 Epilepsy**

Epilepsy is a brain disease that affects about 0.8% of the population having the highest incidence during the early childhood and in the elderly (Pitkänen and Sutula, 2002). This disorder manifests by recurrent unprovoked seizures that result from an electrical imbalance in the brain (Fisher et al., 2005). Several factors have been described as triggers for some of the epilepsies (i.e structural abnormality in the brain, brain injury, infection, genetic mutations), but most of the epilepsy cases remain of unknown etiology. Epilepsy manifests in very different ways, from a single seizure to status epilepticus (SE) lasting for more than 30 minutes (Lowenstein, 1999).

Multiple types of seizures and epileptic syndromes have been described through the years posing a great degree of difficulty for its classification. The international league against epilepsy (ILAE) provides a complete and comprehensive classification of seizure types (<http://www.ilae-epilepsy.org/Visitors/Centre/ctf/seizuretypes.cfm>) and epileptic syndromes (<http://www.ilae-epilepsy.org/Visitors/Centre/ctf/syndromes.cfm>) to identify and help the diagnosis.

TLE is one of the most prevalent types of human focal epilepsies. However, the processes leading to spontaneous seizures involving the hippocampus have not been yet fully determined (Engel, 1996).

### 2.4.1 Animal models of epilepsy

Animal models constitute one of the most valuable tools to better understand the pathophysiology of epilepsy. They allow determining the nature of injuries that might contribute to the subsequent development of epilepsy, to observe and intervene in the disease process subsequent to an injury but prior to the onset of spontaneous seizures, and also to study the chronically epileptic brain in detail, using state of the art physiological, pharmacological, molecular, and anatomical techniques.

The Racine's scale is a common way for researchers to describe seizure behaviour in rodent models of epilepsy. The Racine's scale describes five cumulative stages (classes) of seizure development usually observed in animal models: 1) mouth and facial movements, 2) head nodding, 3) forelimb clonus, 4) rearing, and 5) rearing and falling.

Table 2 shows the spectrum of the most important animal models in use, mainly developed in rodents, according to the method by which epilepsy is induced. These animal models fall in 4 main groups:

|  |   |                   |
|--|---|-------------------|
| <b>1) Electrical stimulation</b>   |   |                   |
| Kindling   |   |                   |
| Perforant-path stimulation (PPS)   |   |                   |
| Maximal electroconvulsive seizures (MECS)                                |   |                   |
| <b>2) Chemoconvulsants (Systemic or intracerebral)</b>                   |   |                   |
| Kainate  | Picrotoxin                                | Pentylenetetrazol |
| Pilocarpine  | Bicuculline                               | Tetanus toxin     |
| <b>3) Physical models</b>  |   |                   |
| Hyperthermia   | Photic stimulation ( <i>Papio papio</i> ) |                   |
| Freeze lesions   | Auditory stimulation (Swiss DBA2 mice)    |                   |
| <b>4) Genetic models (Spontaneous, mutant, transgenics and knockout)</b> |   |                   |
| Genetically epilepsy-prone rat strain (GEPRS)                            | Strasbourg rats (Absence)                 |                   |
| Epileptic beagles (dogs)   | Stargazer                                 |                   |
| Lurcher  | Totterer                                  |                   |

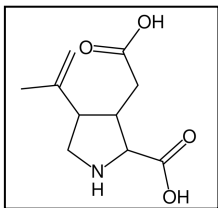
**Table 2.** Classification of the main animal models of epilepsy according to the induction method or cause of seizures.

**1) Electrical stimulation** is one of the approaches used to induce seizures. Kindling, the most studied of them.

**Kindling** is induced by repeated, mild, electrical stimulation of the amygdala, olfactory regions, hippocampus, or other brain regions to induce a progressive and permanent seizure response. The number of stimulations required to kindle an animal, so that, for example, class-4 or class-5 seizures are evoked consistently, depends on several parameters, including the species used. A wide variety of species has been kindled, including frogs, lizards, rodents, cats, dogs, macaques, and baboons, and different species kindle at different rates. Rodents kindle quickly, primates slowly, and carnivores at intermediate rate. For example, self-sustained status epilepticus (SSSE) lasting for 6-20 h was induced by a 20-30 min repeated stimulation of the lateral nucleus of the amygdala in the amygdala kindling model in rats (Nissinen et al., 2000). There are also differences within species (Loscher et al., 1998).

## 2) Chemoconvulsant models

Together with the electrical stimulation, chemoconvulsant models are the most widely used and investigated. A single dose by systemic (intraperitoneal, intravenous or subcutaneous) or intracerebral (intra-cerebroventricular [i.c.v.] or intrahippocampal) administration, is injected to the animals. This provokes SE in minutes-hours depending on the chemoconvulsant, its dose, and the strain and age of the animals. After a few hours, the provoked seizures disappear and a latency period lasting for 3 to 6 weeks leads to the appearance of spontaneous and unprovoked seizures. Chemical convulsants such as KA, pilocarpine, picrotoxin and pentylenetetrazol can induce SE.



**Figure 6.** Kainic acid

**KA** is a glutamate receptor agonist that excites neurons to induce seizures. It is structurally related to domoic acid, which is the toxin responsible for shellfish poisoning. Ingestion of domoic acid causes seizures and hippocampal sclerosis in humans, and survivors develop memory impairment and TLE (Teitelbaum et al., 1990; Cendes et al., 1995). After KA injection, the toxin binds to KA receptors (reviewed above), and to a lesser extent to AMPA receptors inducing neuronal overexcitation with massive release of glutamate, finally leading to seizures.

**Pilocarpine** is a muscarinic acetylcholine receptor agonist. Activation of these receptors has many effects in the brain, and blocking them is likely to contribute to increased neuronal excitability and seizures. The pilocarpine-induced SE rat model may be the most widely used model of TLE. One variation of pilocarpine use is the pre-treatment with lithium which is administered 24 h earlier. After that only a small dose of

pilocarpine is needed to evoke SE. Behavioral seizure activity typically begins in 10 to 30 min. This model has been used both in adult and immature animals (Kubova et al., 2001; Buckmaster et al., 2002; Dudek et al., 2002; Fisahn and Loscher, 2005; Nairismägi et al., 2006; Parent et al., 2006).

**Picrotoxin (PTX), pentylenetetrazol (PTZ), and bicuculline** are antagonists of the ionotropic GABA-A receptors. Therefore, infusion of PTX, PTZ or bicuculline prevents Cl<sup>-</sup> permeability through the GABA-A receptors, and thus restrains the inhibitory effect on the target neuron leading to excitation, and finally seizures (Ben-Ari et al., 1989; Vohora et al., 2000; Hansen et al., 2004).

**3) Physical and 4) genetic models** have also been developed to study epilepsy although they are in less use than electrical stimulation and chemoconvulsants models.

**Hyperthermia model** is used as an approach to febrile seizures, which are relatively common in the childhood. Animals in a cage are heated with hot air until seizures display in the animals. Using this model extensive studies have been performed to verify mossy fiber sprouting, cell death, and long-term consequences of seizures (Toth et al., 1998; Baram et al., 2002; Bender et al., 2003).

**Genetic models of epilepsy** have enormously increased by the discovery of mutations and the use of transgenic animals. More than 70 genes have been identified in humans and mice to be linked to epilepsy. From all mutations, 1/3 of the genes are for ion channels. The rest of the genes control various cellular functions, including ion transport, transmitter synthesis, metabotropic receptors, intracellular signaling, vesicle proteins, and migration (Noebels, 2003). The genetic studies have shown that some epileptic syndromes are linked to more than one gene, and some genes are linked to more than one syndrome (Noebels, 2003).

Many, but not all animal models of experimental epilepsy, exhibit spontaneous seizures of limbic origin. Some exhibit primarily evoked seizures, like the kindling model. Other models induce spontaneous seizures, like the KA and pilocarpine models, but rarely have partial complex seizures as seen in many patients. These models tend to have secondarily generalized tonic-clonic seizures, which are more visible, and therefore, more amenable to laboratory study. Other variants of these models, like the intrahippocampal KA model, have many partial seizures and few generalized seizures. Important advances have

already been obtained from studying epilepsy in the mature brain, but many unique questions and complex issues of epileptogenesis are not fully understood in the immature nervous system.

## 2.5 Neuronal death

Neurons may die as a normal physiological process during development or as a pathological process in diseases (Buss et al., 2006). The best understood mechanism of neuronal cell death is apoptosis, which is regulated by an evolutionarily conserved cellular pathway that consists of the caspase family, the Bcl-2 family, and the adaptor protein Apaf-1. Apoptosis, however, is not the only cellular mechanism that regulates neuronal cell death. Neuronal cell death may exhibit morphological features of autophagy or necrosis, which differ from that of the canonical apoptosis. These types of cell death have classically been defined based on the differences in the ultrastructural morphological features (Rami, 2003).

### 2.5.1 Apoptosis

During embryonic development, excess neurons are removed to ensure proper and precise pre- and postsynaptic connection. The major responsible cell mechanism for this developmental process is apoptosis. **Apoptosis**, also named type 1 cell death, is morphologically characterized by membrane blebbing, cell rounding, cytoplasmic condensation, cytoskeletal collapse, nuclear pyknosis, chromatin condensation, DNA fragmentation, and the formation of membrane bound apoptotic bodies that are rapidly phagocytised and digested by macrophages or neighbouring cells (Kerr et al., 1972; Cohen et al., 1994; Cohen, 1997).

Apoptosis is an active and highly orderly process displaying the characteristic morphologic changes previously mentioned. Hallmarks of apoptosis have been observed during neuronal development (Blomgren et al., 2007) and neuronal cell death caused by acute and chronic injuries (Yuan and Yankner, 2000). The Bcl-2, caspase, and Apaf-1/ced-4 families constitute the core apoptotic machinery in neurons, and it is tightly controlled by the balance of survival and death signals.

In mammalian cells, anti-apoptotic Bcl-2 family proteins and anti-apoptotic kinase Akt and ERK protect the mitochondrial integrity by inhibiting pro-apoptotic Bcl-2 family members. In dying cells, several pro-apoptotic members of Bcl-2 family (tBid, Dp-5, Bim, Bax, Bak, and BAD) may antagonize the anti-apoptotic Bcl-2 family proteins to

induce mitochondrial damage. The subsequent release of cytochrome c from damaged mitochondria induces the formation of apoptosome by recruiting caspase-9 and Apaf-1. Active caspase-9 cleaves and activates caspase-3, which in turn cleaves a variety of cellular substrates such as spectrin, lamins, poly-(ADP-ribose) polymerase (PARP), and inhibitor of caspase-activated DNase (ICAD), and allows caspase-activated DNase (CAD) to induce DNA laddering (Blomgren et al., 2007).

More recently, another mechanism for apoptosis has been uncovered that functions in a caspase independent fashion. **AIF** (apoptosis inducing factor), the protein that triggers this process, is localized to the mitochondrial intermembrane space in living cells and translocates to the cytoplasm and nucleus under certain apoptotic conditions. AIF induces nuclear condensation and large-scale DNA fragmentation (Yu et al., 2002; Zhu et al., 2003; Polster et al., 2005).

### **2.5.2 Autophagy**

A most recently described and less known cell death mechanism is **autophagy**. The autophagy, the type 2 cell death, is characterized at the ultrastructural level by the appearance of intracellular double membrane vacuoles containing cytoplasmic components such as fragments of endoplasmic reticulum or mitochondria and lysosomal hydrolases. Autophagic cell death has been described in neurons during neuronal development and in association with neurodegenerative diseases (Borsello et al., 2003; Yuan et al., 2003; Degtarev et al., 2005). This cell death mechanism has been extensively studied (Klionsky, 2007; Maiuri et al., 2007) and it is naturally occurring in yeasts (Klionsky and Emr, 2000; Ohsumi, 2001) and insects (Lee and Baehrecke, 2001; Gorski et al., 2003).

### **2.5.3 Necrosis**

Necrosis has been traditionally referred as unregulated pathological cell death when compared with the well-structured and stepwise apoptosis. Increasing knowledge has shown common features and pathways in necrosis, which suggests that it is not as “chaotic” as previously thought.

Necrosis, also called type 3 cell death, displays distinct ultrastructural features. The necrosis begins with swelling of intracellular organelles (i.e. mitochondria), followed by the formation of empty spaces in the cytoplasm, which eventually fuse with each other. Later the nucleus and cytoplasmic membrane are destroyed by fragmentation. Although

necrosis has frequently been associated with pathological neuronal cell death, certain developmental neuronal cell death has also been found to exhibit features of necrosis (Clarke, 1990).

Swelling of the mitochondria is accompanied by the loss of the mitochondrial transmembrane potential, but not the release of cytochrome c, indicating that mitochondrial damage inflicted by necrosis may be different from that caused by apoptosis. Also, early loss of ATP synthesis, which might be the result of a failure in homeostatic mechanism, is another feature of necrosis. This is in contrast to the initiation of an energy-dependent suicide mechanism such as apoptosis, in which a certain level of ATP synthesis is maintained until late in the process. Since necrosis is characterized by swelling of intracellular organelles, it is possible that the loss of ATP synthesis is a direct consequence of mitochondrial swelling (Kerr et al., 1972).

Elevated cytoplasmic  $\text{Ca}^{2+}$  has long been implicated in the induction of the mammalian neuronal cell death. However, its significance and mechanism has been controversial and confusing due to the multiple roles of  $\text{Ca}^{2+}$  in the regulation of different cellular processes. It is clear that only when intracellular  $\text{Ca}^{2+}$  concentrations exceed the normal physiological levels does cell death occur. A good example is the abnormal accumulation of glutamate in the synaptic cleft as a result of ischemic brain injury, provokes overstimulation of postsynaptic GluRs, initiating a subsequent cascade, which leads to massive influx of  $\text{Ca}^{2+}$  into neurons (Ben-Ari, 2001; Jourdi et al., 2005b; Volbracht et al., 2005; Zhang and Bhavnani, 2006).

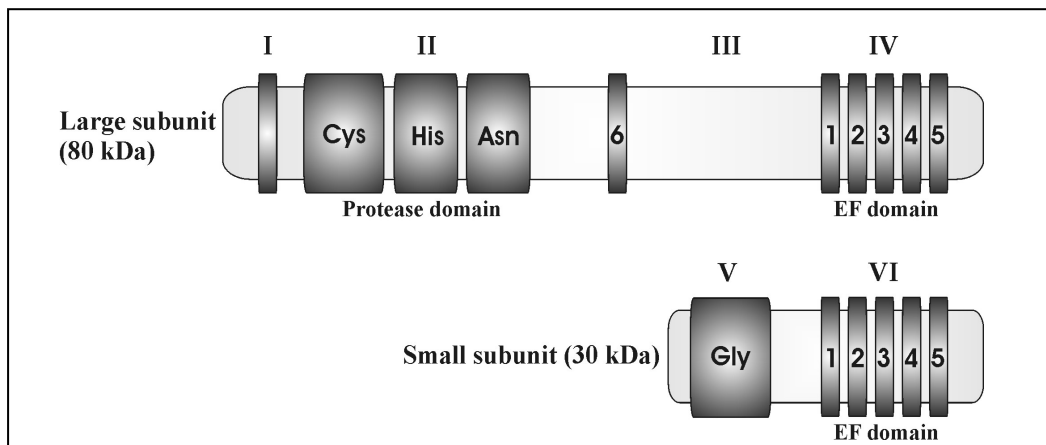
Excessive increases in intracellular  $\text{Ca}^{2+}$  has also been proposed to trigger activation of calpains, the cytosolic  $\text{Ca}^{2+}$ -activated cysteine proteases, which may in turn degrade cytoplasmic proteins. The activation of calpains and aspartyl proteases may be a common downstream step activated by the elevated intracellular  $\text{Ca}^{2+}$  from disruptions of the intracellular endoplasmic reticulum  $\text{Ca}^{2+}$  homeostasis or abnormal membrane  $\text{Ca}^{2+}$  channel activity. Calpains and cathepsins have been proposed to mediate neuronal cell death under a variety of neurotoxic conditions. For example, the “calpain-cathepsin” hypothesis was proposed to explain necrotic cell death after ischemic brain injury (Yamashima et al., 2004).



## 2.5.4 Calpains

Calpains have a cysteine-proteinase domain combined with a calmodulin-like  $\text{Ca}^{2+}$ -binding domain. In fact, the activities of the most representative mammalian calpains,  $\mu$ - and  $m$ -calpains (also called calpains I and II, respectively), are regulated by  $\text{Ca}^{2+}$  concentration. Thus calpain is considered to participate in various intracellular signalling pathways mediated by  $\text{Ca}^{2+}$ . The ubiquitous and constitutive expression of mammalian  $\mu$ - and  $m$ -calpains strongly suggests that they are involved in basic and essential cellular functions. This may be one of the reasons why the physiological functions of calpains are still poorly known. There are two classes of calpains: one (comprising calpains 1, 2, 5, 7, 10, 13, and 15) is ubiquitous in cytosol; the other (comprising calpains 3, 6, 8, 9, 11, and 12) occurs only or mainly in certain tissues (Suzuki et al., 2004).

The calpains consist of two subunits: a distinct larger (about 80 kDa) subunit and a common smaller (about 30 kDa) subunit (Fig. 7). The 80 kDa subunit has four domains: domain I is the N-terminal anchoring  $\alpha$ -helix domain and it is important for regulating



**Figure 7.** Schematic picture of the large and small subunits of the calpain protease showing the different structural and functional domains. Schematic drawing modified from (Zatz and Starling, 2005).

the activity and dissociation of the subunit (Nakagawa et al., 2001) ; domain II, the catalytic domain, has two subdomains in the absence of  $\text{Ca}^{2+}$  (Hosfield et al., 1999; Strobl et al., 2000); domain III binds  $\text{Ca}^{2+}$  and phospholipids (Fernandez-Montalvan et al., 2006), and domain IV, also called the penta-EF-hand domain (an EF-hand unit, consists of two peptide helices connected by a  $\text{Ca}^{2+}$ -binding loop), is important for the dimer formation (Maki et al., 2002). The 30 kDa subunit is composed of two domains, an N-

terminal glycine-clustering hydrophobic region (domain V) and a C-terminal  $\text{Ca}^{2+}$ -binding domain (domain VI) similar to domain IV of the large subunit. This is thought to regulate calpain activity. The subunits are associated through their  $\text{Ca}^{2+}$ -binding domains (domains IV and VI), at least in the absence of  $\text{Ca}^{2+}$  (Crawford et al., 1993; Goll et al., 2003).

#### **2.5.4.1 Calpain activation**

The mechanisms by which calpains are activated and identify their protein targets are complex and poorly understood. Calpain activity is regulated by a ubiquitous specific inhibitor, calpastatin. The calpain–calpastatin interaction is important in regulating the activity of  $\mu$ -calpain and m-calpain (Wendt et al., 2004) but the nature of this regulation in living cells is not understood. Studies using calpastatin have shown that calpains are clearly involved in some types of apoptosis in specific cell types, and are activated in response to certain apoptotic signals (Goll et al., 2003). An intricate strategy for the regulation of calpain activity seems necessary because calpain is an abundant cytoplasmic protease that can cleave many intracellular signalling and structural proteins. Membrane localization of calpains is an important mechanism for regulating their activity. In the early work, it was thought that when calpain binds to the plasma membrane, it is transformed from an inactive, proenzyme into an active, proteolytic enzyme by autolysis. However, other findings indicate that both  $\mu$ -calpain and m-calpain are active proteolytic enzymes before autolysis, and that the interaction with a membrane may bind calpains to their substrates, rather than promote autolysis (Cong et al., 1993; Glading et al., 2001; Zalewska et al., 2004).

Once activated on the membrane, calpain presumably diffuses into the cytosol and becomes resistant to the inhibitory action of calpastatin (Ray et al., 2003). Substrate proteins are digested by the activated calpain on the membrane or in the cytosol. According to Gil-Parrado et al., (2003), calpain activity is regulated not only by calpastatin but also by the differential intracellular localization. In contrast, dissociation of the subunits that constitute calpain appears to be less critical to its regulation.  $\text{Ca}^{2+}$  levels required to initiate autolysis are as high as or even slightly higher than the levels required for proteolytic activity, and are much higher than the free  $\text{Ca}^{2+}$  levels in living cells. A solution to this paradox appeared when it was discovered that the presence of phospholipids, such as phosphatidylinositol, lowered the  $\text{Ca}^{2+}$  levels required for autolysis of  $\mu$ -calpain and m-calpain (Saido et al., 1992). Other studies have shown that

autolysis of these two calpains is an intermolecular process (Inomata et al., 1988), rather than an intramolecular process, as previously thought (Zalewska et al., 2004).

#### **2.5.4.2 Calpain activity in neuronal death**

Calpain proteases, ubiquitously expressed in the brain, are activated by increased intracellular  $\text{Ca}^{2+}$  levels, and are suggested to contribute to excitotoxic neuronal death by targeting components essential for cell survival, e.g. enzymes, transcription factors, and cytoskeletal proteins, including NF proteins (Pant, 1988; Siman et al., 1989; Greenwood et al., 1993; Rami, 2003). Moreover, calpain inhibition has been shown to protect NF proteins from degradation resulting in nerve cell rescue from ischemic (Li et al., 1998; Kunz et al., 2004), and excitotoxic damage (Rami et al., 1997; Lankiewicz et al., 2000; Wu et al., 2004).

Calpains are known to be directly involved in glutamate-induced hippocampal damage, and the activation of these proteases is suggested to be an early, requisite step in the cascade of events initiated by excitotoxic and hypoxic injuries which, through various intracellular signalling pathways, contributes to cytoskeletal degradation, and finally leads to nerve cell death both *in vivo* and *in vitro* conditions (Siman et al., 1989; Minger et al., 1998; Lankiewicz et al., 2000; Stys and Jiang, 2002; Rami, 2003; Araujo et al., 2004; Wu et al., 2004). Moreover, spectrin breakdown products, indicators of the  $\text{Ca}^{2+}$ -activated calpain activity, have been detected in the adult rat hippocampus after the i.c.v. injection of KA (Siman et al., 1989), and soon after KA-induced seizures in the DG of the adult rat hippocampus (Bi et al., 1996). In addition, increased calpain activity has occurred fast after the ischemic insult in the CA1 region (Rami, 2003).

#### **2.5.5 Pathophysiology and neuronal death in epilepsy**

Given the big variety of the pathological hallmarks found in epilepsy in humans, animal models are the most valuable tools to better understand the pathophysiology of epilepsy. They allow us to determine the nature of injuries that might contribute to the subsequent development of epilepsy, to observe and intervene in the disease process subsequent to an injury but prior to the onset of spontaneous seizures (epileptogenesis), and also to study the chronically epileptic brain in detail. Furthermore, also *in vitro* studies using cell and organotypic cultures allow us to study all the mechanisms, which are not possible in humans.

Pathological studies have been conducted in most of the SE animal models, including the KA and pilocarpine models, as well as electrical models. These studies reported hippocampal pathology resembling typical patterns of mesial temporal sclerosis evident in many patients with TLE (Ben-Ari, 1985; Engel, 1996; Buckmaster, 2004; Sloviter, 2005). Accompanying these specific patterns of neuronal loss within the hippocampus are circuit rearrangements, which are evident in both humans with TLE and experimental SE models of TLE (Covolan and Mello, 2000; Buckmaster et al., 2002) .

In experimental models of epilepsy in adult rats, the extension of neuronal damage is shown to be correlated with the severity and duration of the seizures (Ben-Ari, 1985; Covolan and Mello, 2000). Neuronal damage after seizures has been described in several brain structures such as hippocampus, thalamus, amygdaloid region, and different cortical areas.

Hippocampal neuronal death, in particular in the CA3 and CA1 regions, together with the synaptic reorganization occurring by the sprouting of the DG granule cells mossy fibers, have frequently been detected in adult epileptic rats after seizures (Fujikawa et al., 2000; Lynch et al., 2000; Ekdahl et al., 2003; Parent et al., 2006). The occurrence of similar abnormalities in epileptic immature rats is still controversial, and variable results have been obtained depending on the age of the animal, epilepsy model, and route of drug administration (Toth et al., 1998; Haas et al., 2001; Humphrey et al., 2002; Dong et al., 2003). The divergence in the results of neuronal damage and sprouting in the immature rats, and the difference with adult animals, will be more extensively reviewed in the discussion.

### 3. AIMS OF THE STUDY

This work was focused on several questions about the expression and role of NF proteins in the developing rat brain, specifically in the hippocampus. The main aims were:

- I. To study the normal developmental expression of the NF proteins in the early postnatal rat hippocampus.
  
- II. To elucidate the seizure-induced changes in levels and cellular localization of NF proteins using both *in vivo* and *in vitro* models of epilepsy in the developing rat hippocampus.
  
- III. To unfold the importance of NF proteins and calpain proteases in KA-induced neuronal death using *in vitro* models of experimental epilepsy.

## **4. MATERIALS AND METHODS**

### **4.1 Antibodies**

For the NF work, five different monoclonal antibodies were used for the Western blotting and immunocytochemical studies. Clones N52, NN18, and NR4 detect a phosphorylation-independent epitope of NF-H, NF-M, and NF-L, respectively. All three antibodies were purchased from Sigma (St. Louis, MO, USA). Clone SMI 31 detects an extensively phosphorylated epitope of NF-H and, to a lesser extent of NF-M, and clone SMI 32 detects a non-phosphorylated epitope of NF-H. The antibodies of the SMI series were purchased from Sternberger Monoclonals Inc. (Lutherville, MD, USA). All five antibodies are mouse IgG isotypes.

To detect the primary antibodies, an HRP conjugated goat anti-mouse IgG antibody (Sigma) was used in the Western blots, and a biotin SP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in the immunocytochemical studies.

### **4.2 *In vivo* studies**

#### **4.2.1 Use of laboratory animals (I-IV)**

A total of 150-160 rats were used for the *in vivo* studies, and approximately 55-60 animals were used for the studies with OHCs.

All animal procedures were conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC, and had the approval of the Animal Use and Care Committee of the University of Turku. All efforts were made to minimize the pain, discomfort, and number of the experimental animals.

#### **4.2.2 Developmental studies (II)**

The studies of NF expression and subregional and cellular localization in the developing hippocampus were performed using Sprague-Dawley rats of postnatal (P) ages P1, P3, P6, P8, P11, P14, P17, and P21.

#### **4.2.3 Status epilepticus in 9-days-old rats (III)**

To induce SE in 9-day-old Sprague-Dawley rats, a single dose of KA (Tocris Cookson Ltd, Avomouth, UK) (2mg/kg), was injected intraperitoneally (i.p.), and thereafter the pups were carefully followed up to detect the signs of seizures. Within 5 min after the

injection, pups first showed deep breathing and wet mouth followed by scratching with further progression to generalised tonic/clonic seizures within 12-15 min. A subset of rats was sacrificed 30 and 60 min after the injection, while in the remaining rats SE was stopped 1 h after the KA treatment by injecting a single dose (i.p.) of paraldehyde (0.3 ml/kg) (Fluka Chemie AG, Buchs, Switzerland). Shortly after paraldehyde, 0.9% NaCl was injected (i.p.) to the pups at the dose of 2.5% of the body weight to help the recovery. After a careful follow-up of 2-3 h after the paraldehyde injection, pups were taken back to the cages with the dams.

For further studies, rats were sacrificed at different time points after the KA injection (3 and 6 h; 1, 3 and 7 days; and 2 and 4 weeks). All rats used after 30 min (one study group) and 60 min (all other rats) exhibited seizure behaviour. Control rats received the same volume of 0.9% NaCl as those of KA-treated, but in order to minimize the discomfort of the experimental animals, did not receive any further injections, but were otherwise treated as the KA-injected rats described above.

#### **4.2.4 Status epilepticus in adult rats (III)**

Adult rats (250-350 g) received an injection of 12 mg/kg of KA (i.p.), and after that, were carefully followed up to detect the signs of seizures. About 20% of the rats did not exhibit any behavioural signs of seizures, and were not included in the study. All rats included in this study had the entire epilepsy behavioural spectrum including the sustained limbic motor seizures (Ben-Ari, 1985). After recurrent limbic motor seizures for 50 min, a single dose of paraldehyde (0.6 mg/ml, i.p.) was given to stop the seizures. Rats were then taken back to their cages after the follow-up of 2-3 h. At that time, they did not exhibit any signs of seizures. Control adult rats received the same volume of 0.9% NaCl as those of KA-treated, but were otherwise treated as the KA-injected rats described above.

#### **4.3 Organotypic hippocampal cultures (I, IV)**

Hippocampal slice cultures were prepared from P 6-7 Sprague-Dawley rats using the method of Stoppini et al., (1991). Rats were decapitated, the brains were placed in cold Gey's balanced salt solution (Gibco, UK) supplemented with glucose (6.5 mg/ml). Hippocampal slices (400 µm) were cut perpendicular to the septotemporal axis using a McIlwain tissue chopper, and placed on top of semipermeable membrane inserts (Millipore Corporation, Bedford, MA, USA) in a six-well plate containing culture

medium [50% of minimum essential medium, 25% Hanks's balanced salt solution, 25% heat-inactivated horse serum, 25 mM HEPES, supplemented with GlutaMaxII (Gibco) and 6.5 mg/ml glucose, pH adjusted to 7.2]. Slices were cultured in an incubator (37 °C, 5% CO<sub>2</sub>) for 7 days *in vitro* (DIV) with medium change twice a week. No antibiotics were used.

#### **4.4 Pharmacological treatments in the *in vitro* studies (IV)**

Hippocampal slices (7 DIV) were incubated for 3, 6, 12, 24 and 48 h together with KA (5 μM), and thereafter prepared for Western blotting. In addition, the 7-DIV cultures were treated with various pharmacological compounds for 24 h with the following concentrations; KA (5 μM), the AMPA/KA selective antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) (Tocris Cookson Ltd, Avomouth, UK), the L-type Ca<sup>2+</sup>-channel blocker nifedipine (10 μM) (Tocris), the calpain protease inhibitor MDL-28170 (0.5, 5, 25 and 50 μM) (Biomol, Plymouth Meeting, PA, USA), and the NMDA-receptor antagonist MK-801 (0.5 μM) (Tocris). Treated and control slices were then used for Western blotting, immunocytochemistry, and neuronal death studies. In all experiments, 7-DIV cultures were used, since KA treatment at this *in vitro* age induces selective CA3 nerve cell damage while sparing the other main hippocampal nerve cell types (Holopainen et al., 2004).

#### **4.5 SDS-PAGE and Western blotting (I-IV)**

##### **4.5.1 Harvesting and processing of the hippocampal tissue**

In the *in vivo* studies, rats were decapitated, hippocampi quickly removed, placed in ice-cold homogenization buffer (50 mM Tris-HCl [pH 7.4], 1% SDS, 2 mM EDTA, 1 mM PMSF, and 0.7 mM DTT), homogenized using Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany) Later samples were immediately boiled, centrifuged at 12 000 rpm for 30 minutes at 4°C, and supernatants collected, frozen and stored at -80°C until used. Total protein concentration of the samples was measured using Lowry based Biorad DC Protein assay (Bio-Rad, CA, USA).

##### **4.5.2 SDS-PAGE and Western blotting**

Equal amounts of proteins were applied to each lane of gels for SDS-PAGE, separated by electrophoresis with a 7.5% acrylamide minigel using Mini-protean II (Bio-Rad), and transferred to a polyvinylidene fluoride (PVDF) Immobilon-P (Millipore Corp., Bedford, MA, USA) membrane using semi-dry system Transblot SD (Bio-Rad). Membranes were



incubated overnight at 4°C with the primary antibodies, washed, and then incubated with the HRP-conjugated secondary antibody) for 1 h at room temperature. The signal was obtained using chemiluminescence ECL system (Amersham, Buckinghamshire, UK) and Hyperfilm ECL (Amersham). The film was developed and the optical signals were quantified with Image J 1.20s (NIH, USA).

#### **4.6 Immunocytochemistry and light microscopy (I-IV)**

For immunocytochemistry in the *in vivo* experiments, the rats were deeply anaesthetized with 50 mg/kg of pentobarbital, transcardially rinsed with 0.9% NaCl, and thereafter perfused with 4% paraformaldehyde in PBS (pH 7.4). Brains were rapidly removed, postfixed, cryoprotected, rapidly frozen and thereafter kept at -80°C until used. For the immunostaining, the brains were cryosectioned in 40 µm slices, collected in PBS (pH 7.4) + 0.1% Triton X-100 and processed in a free-floating system.

In the *in vitro* studies, after different treatments, the OHCs were first washed with 0.1 M PBS (pH 7.4), fixed with 4% paraformaldehyde (PFA) for 1h at room temperature, and then processed with antigen retrieval method. After that, slices were mechanically detached from the insert, and staining steps were carried out free floating. All the further steps in the immunocytochemistry were common for the brain slices and OHCs.

Before the incubation with antibodies, endogenous peroxidase activity was quenched, slices washed and then incubated in a blocking solution containing 2% bovine serum albumin, 2% horse serum, and 0.1% Triton X-100 in PBS (pH 7.4) for 1 h, and thereafter with the primary antibodies for 24 or 48 h at 4°C in blocking solution. The slices were incubated with the biotin conjugated secondary antibody, rinsed, and incubated with avidin-peroxidase conjugate (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) in blocking solution at room temperature. The staining was detected using 3,3'-diaminobenzidine (DAB) as a chromogen. Finally, the brain slices were transferred to gelatin-coated slides, cleared in xylene and mounted. In each experiment, three to four slices in which the primary antibody was omitted but were otherwise treated as indicated above, served as negative control. Preparations were examined in a Leica DM R microscope (Heerbrugg, Switzerland).

The immunoreactivity in the hippocampal development study was scored using the following scheme: -, negative ; -/+, weakly positive ; +, positive ; ++, moderately positive ; +++, strongly positive. Digital camera Olympus U-TV1 X (Olympus Optical

Co., Ltd., Tokyo, Japan) was used to capture pictures using Olympus BX60 microscope (Olympus) and pictures were further processed using Adobe Photoshop (version 6) and Corel Draw (version 10.0).

#### **4.7 Timm's staining (III)**

Timm's stain was used to detect whether mossy fiber sprouting occurred in the hippocampus of the KA-treated P9 and P21 rats. Rats were transcardially perfused with sodium sulphide solution (2.925 g Na<sub>2</sub>S, 2.975 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O in 500 ml of water), and then with 4% PFA in PBS (pH 7.4) at room temperature. After that, brains were rapidly removed, postfixed, cryoprotected, and frozen. Later brains were sliced (20 μm), mounted on glass slides and immediately processed for Timm's staining. Sections were developed in the dark for 20-30 minutes in a 12:6:2 mixture of 20% arabic gum, 5.6% hydroxiquinone, citric acid-sodium citrate buffer, and finally 0.5% of the total volume from the 17% AgNO<sub>3</sub> stock solution. After that, slides were washed, dehydrated in alcohol, cleared in xylene, mounted, and examined with a Leica DM R microscope (Heerbrugg, Switzerland) under bright field optics.

#### **4.8 Fluoro-Jade B and thionin stainings (III-IV)**

**Fluoro-Jade B**, an anionic fluorescein with excitation peaks at 362 and 390 nm and the emission peak at 550 nm, is an effective marker of neuronal degeneration regardless of the nature of the injury (Schmued and Hopkins, 2000).

For the FJB staining, brain slices were rehydrated with alcohol series, transferred for 2-5 min to 0.06% potassium permanganate (KMnO<sub>4</sub>) prepared in distilled water, washed twice with distilled water, and transferred to 0.001% FJB solution for 30 min. After the staining, slides were washed with distilled water, dried, cleared in xylene, and coverslipped with mounting medium.

**Thionin staining** was used to study the integrity of the hippocampal neuronal layers *in vivo* and *in vitro*. For the staining *in vitro*, hippocampal slices were removed from semi-permeable membranes to gelatin-coated glass slides, and briefly stained in 0.1% thionin, dehydrated in alcohol series, cleared in xylene, and coverslipped. The amount of neurons in the CA3 regions was scored using the following scheme: 0 = no stained neurons in the cell layer (all neurons dead in terms of loss of Nissl staining); 1 = some stained neurons; 2 = sparse number of stained neurons; 3 = many stained neurons but with slightly

disturbed cell layer integrity; 4 = numerous stained neurons with good cell layer integrity (regarded as normal).

## 4.9 Confocal microscopy (IV)

FJB-stained specimens were examined with a Leica TCS SP confocal microscopy system (Leica, Heidelberg, Germany) equipped with an Argon-Krypton laser (Omnichrome, Melles Griot, Carlsbad, CA). The laser wavelength used for excitation of FJB was 488 nm, and the emission detection window was 500-600 nm. Confocal image stacks were acquired at 2  $\mu\text{m}$  steps by using a 10x objective (HC PL APO 10x/0.40), and processed with the Leica TCS NT/SP Scanware (version 1.6.587) software. Reconstruction of the images of the whole hippocampal slice was accomplished afterwards from 1  $\text{mm}^2$  maximum projections using Adobe Photoshop. The focus of the study was on the CA3 region. For the scoring analyses, the area of stained neurons (i.e. degenerating neurons) was measured from maximum projections of the FJB-stained hippocampi (n=10-15 in each experiment group) using ImageJ software (NIH, USA) as recently described in detail (Kukko-Lukjanov et al., 2006). The following scoring system, in which the scoring numbers indicate the area (in  $\text{mm}^2$ ) with FJB-stained CA3a/b neurons was used to evaluate the extent of damage; 0 = no FJB-stained neurons (regarded as normal), 1 = up to 0.100  $\text{mm}^2$ ; 2 = 0.101-0.200  $\text{mm}^2$ ; 3 = 0.201-0.300  $\text{mm}^2$ ; and 4 = larger than 0.300  $\text{mm}^2$ .

## 4.10 Statistical analysis (I-IV)

The following statistical analyses were used in the original articles.

**I.** In the control groups, the statistical significance of differences in the immunoblots between the experimental groups was analyzed with the nonparametric one-way analysis of variance (ANOVA) Kruskal-Wallis Test. The unpaired Student's t-test with Welch correction was used to analyze the differences between the controls and the KA-treated cultures of the same age, and between the two age groups in the KA-treated cultures. The overall group differences of the score numbers after thionin staining were assessed by one-way ANOVA. If a significant effect was found, Tukey-Kramer Multiple Comparison Test was used to define the statistical significance of differences between the experimental groups and between the two age groups in KA-treated slices.

**II.** The statistical significance of the overall change in the signal intensity in the Western blots of NF-L, NF-M, and NF-H through P1–P21 was calculated with the two-way ANOVA, and Tukey’s multiple comparison test was used as a post-hoc test.

**III.** The statistical significance of differences between the control and KA-treated rats in the signal intensity in the Western blots of the phosphorylation-independent epitopes of NF-L, NF-M, and NF-H, the phosphorylated epitopes of NF-M and NF-H, and the nonphosphorylated epitope of NF-H was assessed by one-way ANOVA.

**IV.** The overall differences in the signal intensity in Western blots of NF-L, NF-M, NF-H after KA treatment of different durations and treatments, and the overall group differences in the score numbers after the FJB and thionin stainings were assessed with one-way ANOVA with the Tukey-Kramer multiple comparison test as a post hoc test. The difference between two experimental groups was assessed with the Student’s two-tailed t-test.

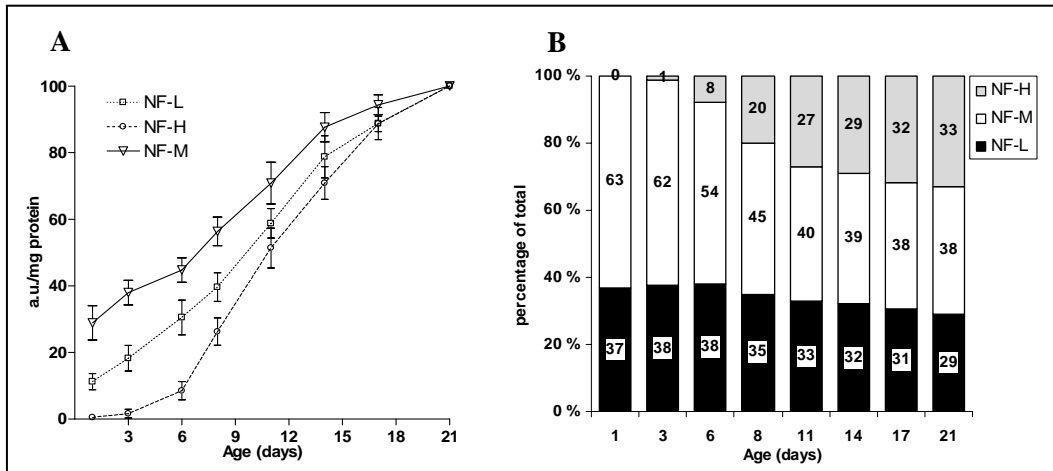
The level of significance was set at  $P < 0.05$  in all original publications. The statistical analyses were performed using GraphPad Prism software versions 3.0 and 4.0 (GraphPad Software, San Diego, CA).

## 5. RESULTS

### 5.1 Neurofilaments in the *in vivo* studies

#### 5.1.1 Developmental expression of neurofilaments in the rat hippocampus (II)

The developmental expression pattern of the three NF proteins during the first three weeks of postnatal development is shown in Fig. 8. Already at P1, the signal of NF-L was about 10% of that observed at P21 and it constantly increased during the whole period studied, although its expression increased at a slower rate during the third week. At P1, the expression of NF-M was already about 30% of that at P21, it markedly further increased during the second week accounting about 85% of that observed at P21, and started to plateau during the third week. A completely different developmental time course was detected for NF-H. At P3 it was hardly detectable, during the first week its expression was less than 20% of that observed at P21, but through P7 to P14 it dramatically increased up to about 70% of that at P21. After that its increase was less pronounced.



**Figure 8.** **A)** Developmental expression profile of NF-L, NF-M and NF-H during the early postnatal development (P1- P21) of the rat hippocampus. **B)** Percentage of each of the NFs of the total at the time points studied. Abbreviations: a.u.; arbitrary units.

At P1 and P3, NF-M was by far the NF protein most abundantly expressed providing more than 60% of the total signal and the contribution of NF-H to the total NF pool was almost negligible. The percentage of NF-L was not significantly changed ( $p=0.07$ ) through P1 to P21 remaining about 30-35% of the total signal, whereas the percentage of

NF-M was significantly ( $p < 0.001$ ) decreased, and the percentage of NF-H significantly ( $p < 0.001$ ) increased. At P14, the contribution of NF-L, NF-M, and NF-H proteins to the total NF signal was stabilized, and during the third postnatal week no significant changes were observed.

### 5.1.2 Cellular distribution of neurofilaments in the developing rat hippocampus (II)

The detailed distribution of NF-L, NF-M and NF-M in the rat hippocampus during P1-P21 as visualized by immunostaining is presented in Table 3. A common feature for all three NF proteins is their increased expression during development, and the regionally different expression pattern.

| Region               | NF-L |     |     |     | NF-M |     |     |     | NF-H |     |     |     |
|----------------------|------|-----|-----|-----|------|-----|-----|-----|------|-----|-----|-----|
|                      | P1   | P7  | P14 | P21 | P1   | P7  | P14 | P21 | P1   | P7  | P14 | P21 |
| <i>CA 1 field</i>    |      |     |     |     |      |     |     |     |      |     |     |     |
| Sp                   | -    | -   | -   | -   | -    | -   | -   | -   | -    | -   | -   | -   |
| So                   | -    | -/+ | -/+ | -/+ | -/+  | +   | +   | +   | -    | +   | +   | +   |
| Sr                   | -    | -/+ | -/+ | +   | -/+  | +   | +   | +   | -    | +   | ++  | ++  |
| Slm                  | -    | -/+ | +   | +   | +    | -/+ | ++  | ++  | -    | -   | +   | +   |
| Interneurons         | -    | -   | -   | -   | -    | -   | -   | -   | -    | -/+ | +   | +   |
| <i>CA 3 field</i>    |      |     |     |     |      |     |     |     |      |     |     |     |
| Sp                   | -    | +   | ++  | ++  | -    | -   | -   | -   | -    | ++  | ++  | ++  |
| So                   | -    | -/+ | -/+ | -/+ | -/+  | -/+ | +   | ++  | -    | +   | +   | +   |
| Sr                   | -    | -/+ | +   | +   | +    | +   | ++  | ++  | -    | ++  | ++  | +++ |
| Sl                   | -    | +   | ++  | +++ | -/+  | ++  | +++ | +++ | -    | +   | +   | +   |
| Interneurons         | -    | -   | +   | +   | -    | -   | -   | -   | -    | +   | +   | +   |
| <i>Dentate gyrus</i> |      |     |     |     |      |     |     |     |      |     |     |     |
| Sg                   | -    | -   | -   | -   | -    | -   | -   | -   | -    | -   | -   | -   |
| Mf                   | -    | +   | ++  | +++ | -    | ++  | +++ | +++ | -    | -   | -   | -   |
| Mi                   | -    | -/+ | +   | +   | -    | -/+ | ++  | ++  | -    | -/+ | ++  | ++  |
| Mm                   | -    | -   | -/+ | -/+ | -    | -/+ | +   | +   | -    | -   | +   | +   |
| Mo                   | -    | -   | -/+ | -/+ | -    | -/+ | -/+ | +   | -    | -   | +   | +   |
| Hilus                | -    | -/+ | +   | +   | +    | +   | +   | +   | -    | +   | ++  | ++  |
| Interneurons         | -    | -/+ | +   | +   | -    | -   | -   | -   | -    | +   | +   | +   |

**Table 3.** Developmental changes in the distribution of NF-L, NF-M and NF-H immunoreactivity in the various hippocampal areas during the postnatal development.

The lack of immunoreactivity in the cell bodies of CA1 pyramidal cells and granule cells at all ages was the only common feature for the three NFs. **NF-L** immunoreactivity was detectable both in cell processes in all hippocampal subregions, in the CA3 pyramidal cells bodies, and interneurons. **NF-M** immunoreactivity was most prominent in the younger animals. Furthermore, NF-M staining was only present in processes, more prominently in axonal areas, lacking from all types of neuronal cell bodies. Interestingly,

the most prominent staining was in the mossy fibers at all ages studied. In accordance with the Western blotting results, no **NF-H** immunoreactivity was observed at P1. At older ages, three main differences in NF-H immunoreactivity were detected when compared to NF-M staining. 1) The CA3 neuronal cell bodies were moderately immunopositive at P7, and remained so up to P21. 2) The MOSSY FIBERS remained immunonegative at all ages, but the granule cell dendrites became immunopositive at P14, and 3) immunopositive interneurons were detected in the CA1, CA3, and DG granule cell layers at P7 throughout P21.

### **5.1.3 Neurofilament expression after SE in 9-day-old rats (III)**

Western blotting was used to detect changes in the expression of NF proteins after the KA- induced SE in P9. The analysis of Western blots showed that the response of NF-H, NF-M, and NF-L to seizures was surprisingly similar. Already 30 min after KA injection, the signal of all three NFs significantly increased about 40% from the control levels, and remained elevated up to 6 h. However, their expression returned to the control levels 1 day after the treatment, and remained thereafter unchanged up to 7 days.

## **5.2 Expression and localization of neurofilament proteins in organotypic hippocampal cultures**

### **5.2.1 Neurofilament expression during the *in vitro* maturation of OHCs (I)**

The distribution of NF-immunoreactivity after 11, 18, and 25 DIV in various hippocampal regions in slices cultured under normal conditions was studied. In general, the changes in the immunoreactivity during the *in vitro* maturation of the slices were not pronounced. In all *in vitro* age groups, both the cell body and the proximal dendrites of the CA3a/b and CA3c pyramidal neurons were strongly immunoreactive to NF-H and also strongly to NF-M and NF-L. The staining in the cell bodies was restricted to the rim of cytoplasm surrounding the unstained nucleus.

No NF-H, NF-M, and NF-L immunoreactivities were observed in the CA1 pyramidal neurons at any age. A few weakly NF-L positive granule cells were detected in each age group, whereas some NF-H and NF-M positive granule cells only in the older cultures. In the molecular layers of the DG, the mean staining intensity with NF-H, NF-M and NF-L was weak through 11 to 25 DIV, and the amount of positively stained fibers remained stable between the different age groups.

## 5.2.2 Neurofilament expression after KA treatment in the OHCs (I, IV)

**In the first set of studies,** Western blotting was used to detect the changes of NF-L, NF-M, and NF-H proteins in cultured slices at 11 and 18 DIV after KA treatment. The treatment with KA (5  $\mu$ M) decreased ( $p < 0.001$ ) the signal of all three NF proteins in both age groups, the reduction being significantly ( $p < 0.05$ ) more pronounced in the older than in the younger slices. The reduction in the NF-H signal was 75% at 11 DIV, and 83% at 18 DIV, whereas the reduction of NF-M signal was only 27% and 44% at 11 DIV and 18 DIV, respectively. The corresponding values for NF-L were 48% and 68% respectively.

| Treatment    | NF-L |     |     | NF-M |     |     | NF-H |     |     |
|--------------|------|-----|-----|------|-----|-----|------|-----|-----|
|              | CA3  | CA1 | DG  | CA3  | CA1 | DG  | CA3  | CA1 | DG  |
| Control      | ++   | +   | +   | ++   | +   | +   | ++   | ++  | +   |
| KA           | -/+  | -/+ | -/+ | -/+  | -/+ | -/+ | +    | +   | ++  |
| KA+CNQX      | +++  | +   | ++  | ++   | +   | ++  | +++  | ++  | ++  |
| KA+MDL-28170 | +++  | -/+ | ++  | ++   | -/+ | ++  | +++  | ++  | ++  |
| KA+NIF       | -/+  | +   | -/+ | -/+  | +   | -/+ | +    | +   | -/+ |
| KA+MK-801    | -/+  | -/+ | -/+ | -/+  | -/+ | -/+ | -/+  | +   | -/+ |

**Table 4.** Immunocytochemical staining of NF-L, NF-M and NF-H in control slices, and in slices treated with different pharmacological compounds.

**In the second set of studies,** Western blotting was used to assess the time course of KA-induced NF protein degradation in 7-DIV hippocampal slices after 3, 6, 12, 24 and 48 h treatment. As early as 3 h after the treatment, the signal of all NF proteins started to decrease, and this decline was significant ( $p < 0.01$ ) after 6 h. The signals further significantly ( $p < 0.001$ ) decreased with the treatment time, and after the 24-48 h KA treatment, the values were about 40% of those detected in control cultures.

**Western blotting and immunocytochemistry** were also used to detect changes in the expression of NF-L, NF-M and NF-H in 7-DIV slices treated for 24 h either with KA (5  $\mu$ M) alone or combined with CNQX (10  $\mu$ M), MDL-28170 (0.5-50  $\mu$ M), nifedipine (10  $\mu$ M), and MK-801 (0.5  $\mu$ M). In KA-treated cultures analysed by Western blotting, the signal of all NF proteins decreased by about 60%, while CNQX and the calpain inhibitor MDL-28170 effectively blocked the KA-induced degradation resulting in the NF levels



comparable to those of control slices. Nifedipine and MK-801 did not significantly block the KA-induced NF protein degradation. Immunocytochemistry confirmed the results. The visual scoring of NF-L, NF-M and NF-H immunoreactivity in control and treated slices are compiled in Table 4.

### **5.3 KA-induced neuronal damage *in vivo* and *in vitro* (I, III, IV)**

In order to detect whether KA-induced SE resulted in neuronal death *in vivo* in P9 rats, FJB staining together with the conventional thionin staining were carried out. No FJB positive cells were detected at any time point (30 min; 1, 3, 6 h; 1, 3 days, and 1, 2, and 4 weeks) in any hippocampal region indicating that KA treatment did not result in neuronal death. In adult rats having SE for less than 60 min, numerous FJB-stained fluorescent cells were detected 1d after SE in the CA3a/b region indicating neuronal degeneration.

In the OHCs, no FJB-stained neurons were detected in any hippocampal subregion in control slices indicating good survival of neurons in culture conditions. In contrast, the CA3a/b region was heavily stained with FJB in KA-treated slices. FJB staining was also carried out in 7-DIV cultures treated for 24 h with KA (5  $\mu$ M) alone, or combined with CNQX (10  $\mu$ M), and MDL-28170 (0.5, 5, 25 and 50  $\mu$ M). The area of FJB-stained neurons was significantly decreased in the presence of KA+CNQX, and concentration-dependently in slices treated with KA+MDL-28170 (50  $\mu$ M), while nifedipine and MK-801 had no effect. The conventional thionin staining was carried out after the 48-h culture period in normal medium. The staining confirmed that the main hippocampal cell layers were well-preserved in control cultures, whereas KA treatment resulted in massive loss of CA3 neurons, which was significantly alleviated by CNQX, and dose-dependently by MDL-28170.

### **5.4 Reorganization of hippocampal circuitry after KA-induced SE in P9 rats (III)**

Timm's staining was used to detect the mossy fiber sprouting in the inner molecular layer of the DG 1, 2 and 4 weeks after SE in KA-treated and in their age-matched control rats. In P9 rats, despite induction of seizures, no staining could be detected in the inner molecular layer of the DG either in the control or in the KA-treated animals at any time points studied.

## **6. DISCUSSION**

### **6.1 *In vivo* animal models of epilepsy**

The experimental animal models of SE in adult rats show several pathological features, which resemble the neuropathology of human TLE, making the experimental models useful for epilepsy research. Those features include a development of hippocampal sclerosis and granule cell synaptic reorganization, and chronic epilepsy. The “silent” period, defined as epileptogenesis provides a window of opportunity to test antiepileptogenic treatments, and it also represents a pre-epileptic state that can help to distinguish between causes and effects of chronic epileptic seizures (Pitkänen and Sutula, 2002; Pitkänen et al., 2007).

In the KA model of experimental epilepsy in adult rats, SE is followed by the epileptogenetic period which lasts for 4-8 weeks. Later on, spontaneous seizures appear in the adult rats, but not in the immature rats which had SE before P21 (Wasterlain et al., 2002). Thus, in the model we have used (seizures are induced by KA in P9 rats) we have studied the short and long term effects of SE.

It is not known why SE in immature animals does not lead to spontaneous seizures since they are more prone to seizures. Lower doses of KA (2mg/kg in this study) are used in immature animals to induce the SE compare to adults (10-12mg/kg) (Buckmaster and Dudek, 1997; Pitkänen et al., 1999; Fujikawa et al., 2000; Araujo et al., 2008). Furthermore, higher mortality occurs in the immature animals due to the severity of the seizures. Thus, in order to reduce the mortality, we limited the duration of the SE with a single dose of paraldehyde 1 h after the injection, as published recently (Kubova et al., 2001). The paraldehyde suppressed SE but occasional seizures still appeared in the following hours. In accordance to this, the continuation of the ictal activity has been shown by EEG recording in immature rats even without any behavioral signs (Kubova et al., 2004; Druga et al., 2005).

### **6.2 Neuronal damage after seizures in the developing brain**

In our study, P9 rats exhibited a remarkable resistance to neuronal death even when exposed to severe, continuous seizures. This is in keeping with earlier studies, which have shown resistance of the immature hippocampus to KA-induced seizures (Sperber et al., 1991; Haas et al., 2001; Rizzi et al., 2003). In contrast, neuronal damage in the hippocampus has been detected in some studies using the KA model (Montgomery et al.,

1999; Humphrey et al., 2002; Dong et al., 2003), and also in the thalamic region in P12 rats 12 h after the pilocarpine application (Kubova et al., 2001).

It should be noted that although all immature, the exact postnatal age of animals used in these studies is variable, which may be of major importance in determining neuronal vulnerability to seizures. Moreover, the local (i.c.v or i.h.) KA application used in some studies in developing rats (Leite et al., 1996; Montgomery et al., 1999; Humphrey et al., 2002; Dong et al., 2003), could lead to neuronal death by the direct excitotoxicity of KA, not due to the induced seizures. In keeping with this idea, one study has shown that the ipsilateral intrahippocampal KA injection induced severe seizures, but neuronal death was only detected in the lesion side, not in the contralateral part (Leite et al., 1996). These results suggest the resistance of the immature brain to seizure-induced neuronal damage in the KA model as also shown in our studies, although when given locally, KA leads to strong cytotoxic insults.

In contrast to immature animals, neuronal death has frequently been detected in adult epileptic rats, particularly in CA3 and CA1, irrespective of the treatment protocol, i.e. i.p, intraventricular, subcutaneous, and intrahippocampal (Pitkänen et al., 1999; Bengzon et al., 2002; Scharfman et al., 2002; Araujo et al., 2008). The resistance of neurons to seizure-induced death in the developing hippocampus has also been shown in a rat model of febrile seizures (Toth et al., 1998; Baram et al., 2002; Bender et al., 2003). Moreover, it has been shown with a silver staining method that febrile seizures lead to injury of hippocampal neurons within 24 h, but within 4 weeks after seizures, no significant neuronal loss was detected (Toth et al., 1998).

In other studies in which KA has been used to induced SE in P9 rats, in spite of no obvious neuropathological hallmarks, changes in the expression of some GABA-A receptor units and their pharmacological properties have been detected (Laurén et al., 2005; Laurén et al., 2007). KA has also been shown to induce a long term changes of hippocampal plasticity, thus affecting to long term potentiation (LTP), and impairing spatial learning (Lynch et al., 2000).

Also long-term motor development, and behavioural and cognitive impairments have been shown in P12 rats after pilocarpine-induced SE (Kubova et al., 2000; Kubova et al., 2004). Moreover, based on MRI and histological methods, neurodegeneration and volume reduction have been detected in the same model (Nairismägi et al., 2006).

### **6.3 Mossy fiber sprouting in developing rats after seizures**

Collateral sprouting of mossy fibers in the inner molecular layer of the DG has frequently been detected in adult epileptic humans and experimental animals (Wenzel et al., 2000; Buckmaster et al., 2002; Epsztein et al., 2005; Kang et al., 2006). Similar reorganization of mossy fibers has also been detected 2 weeks after the KA treatment in cultured organotypic hippocampal slices (Routbort et al., 1999; Bausch and McNamara, 2004).

Our results indicate that neither neuronal death nor mossy fiber sprouting occurred after KA-induced SE in P9 rats. These results are in accordance with earlier studies in developing hippocampus, in which the lack of cell death with the absence of mossy fiber sprouting are common features (Haas et al., 2001; Rizzi et al., 2003). Only in the hyperthermia model of febrile seizures developed by Baram et al., mossy fiber sprouting has been detected in the developing brain (Baram et al., 2002; Bender et al., 2003). Earlier studies in which flurothyl was used to induce seizures in immature rats, increase in mossy fibers was detected despite lack of neuronal damage (Liu et al., 1999).

### **6.4 Organotypic hippocampal cultures as a research tool**

Organotypic hippocampal slice cultures can be used as an *in vitro* model to study the cellular and molecular changes induced by enhanced excitatory activity. Hippocampal slice cultures are prepared from postnatal rat (usually P6-7), and they can be cultured from one week up to 1-2 months (Stoppini et al., 1991; Routbort et al., 1999; Bausch and McNamara, 2000; Holopainen, 2005). It has been shown in several studies that the hippocampal structure and the morphology of the hippocampus are well preserved in OHCs. Furthermore, the neuronal maturation, receptor expression, dendritic and axonal formation together with receptor expression, resembles that seen *in vivo* (Dailey et al., 1994; Frotscher and Heimrich, 1995; Gahwiler et al., 1997; Holopainen and Lauren, 2003). All those morphological features have been assessed during the first 2-3 weeks after the preparation of the OHCs. The cytoskeletal maturation has also been shown to be similar between the *in vitro* OHCs and their *in vivo* counterparts (Hartel and Matus, 1997). The preparation of slices results in loss of normal afferent input to dentate granule cells, which upon culturing favours the growth of aberrant, excitatory connections of mossy fibers into the molecular layers of the DG (Caeser and Aertsen, 1991; Frotscher et al., 1995; Bausch and McNamara, 2000, 2004).

### 6.4.1 Study of excitotoxic neuronal damage using OHCs

Excitotoxic neuronal damage caused by pathological overactivation of glutamate receptors, is a major factor in a number of CNS insults and disorders such as epileptic seizures, hypoxia and ischemia (Lee et al., 1999; Bengzon et al., 2002; Rami, 2003).

Diverse studies have been carried out with OHCs to verify the vulnerability of the specific hippocampal subfields to glutamate and GluR agonists such as *N*-methyl-NMDA and KA (Bruce et al., 1995; Patrylo and Dudek, 1998; Lahtinen et al., 2001; Borsello et al., 2003). In contrast, GluR antagonists have been reported to protect *in vitro* against hypoglycaemia (Tasker et al., 1992), hypoxia (Newell et al., 1995) or a combination of these conditions mimicking ischemia (Strasser and Fischer, 1995). Also oxygen-glucose deprivation has been a common *in vitro* model for ischemia in studies clarifying the potential neuroprotective effects and mechanisms of compounds such as GluR antagonists (Rytter et al., 2003), caspase inhibitors (Ray et al., 2000), and anticonvulsants (Rekling, 2003).

The neuronal damage reported in our *in vitro* studies using KA in OHCs, is at variance with the lack of neuronal damage reported in the *in vivo* studies in P9 rats. However, the result obtained using the OHCs are in keeping with the models of epilepsy in developing rats in which local injection of KA is given (i.h. o i.c.v), as discussed in the chapter 6.2. In contrast, in our *in vivo* model, the developing rats receive a systemic i.p. injection of KA which induce SE.

The local administration of KA in developing rats induces a more intense activation of not only KA receptors, but other glutamatergic receptors such as AMPA. As the result, severe neuronal damage is found due to the direct toxic effect of KA in parallel to the possible damage provoked by the induced seizures.

Hippocampal slice cultures have several advantages. They can survive for long periods *in vitro*, experimental conditions can be controlled, and confounding variables when performing *in vivo* pharmacological experiments can be reduced. Furthermore, they mimic the *in vivo* situation better than neuronal cell cultures, since the hippocampal connectivity, and the neuronal diversity are well-preserved as discussed above.

## **6.5 Neurofilaments in the hippocampal development**

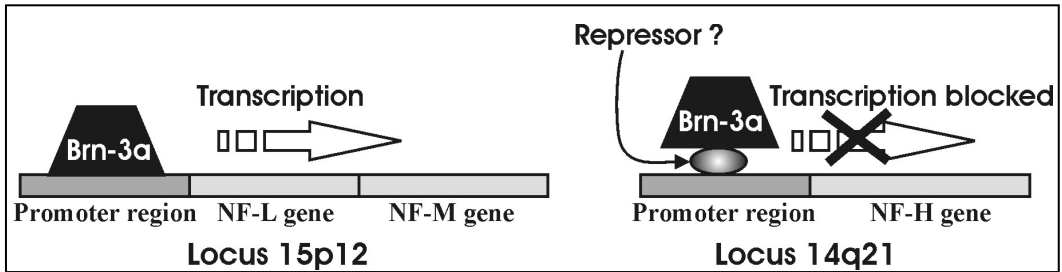
### **6.5.1 Expression of neurofilaments in the developing rat hippocampus**

We performed a detailed study on the expression profiles and localization of the different NF proteins; NF-L, NF-M, and NF-H, phosphorylated epitopes of NF-M and NF-H, and a non-phosphorylated epitope of NF-H, during the early postnatal development of the rat hippocampus.

NF-L and NF-M had a very different expression pattern than that of NF-H. While the expression of NF-L and NF-M clearly started during the prenatal period, the expression of NF-H seemed to be exclusively postnatal, and it was highly increased during the second postnatal week. This is in keeping with a recent mRNA study in hamster forebrain (Kost et al., 1992), which showed that NF-L and NF-M mRNAs were expressed at very low concentrations in the embryonic brain and their expression increased postnatally up to P28, and then declined. Also the NF-L mRNA expression in mouse hippocampus was greatly increased during the postnatal period (Kure and Brown, 1995). In contrast, NF-H mRNA has not been detected at embryonic stages, and its expression has been low prior to P9, then increased 2.5-fold between P9 and P11, and the levels have further increased up to P28 (Kure and Brown, 1995).

The late expression of NF-H compared to NF-L and NF-M has been regarded as an indication of neuronal differentiation (Lee and Cleveland, 1996). Thus NF-L and NF-M might serve as structural elements in small immature axons while mature NFs containing NF-H are more stable, space-filling proteins, which might be involved in the regulation of interaction between filaments and other cellular structures (Lee et al., 1993, Lee and Cleveland, 1996).

It is of interest to speculate about the functional importance of differential developmental expression of the three NFs. The transcription factor Brn-3a is responsible for inducing and controlling the expression of the three NF genes (Smith et al., 1997). Furthermore, Brn-3a can induce neuronal differentiation by regulating the expression of genes such as those encoding synaptosome-associated protein 25 (SNAP-25) (Lakin et al., 1995) , alpha-internexin (Budhram-Mahadeo et al., 1995), and it can also inhibit neuronal apoptosis *in vitro* via the activation of Bcl-2 and Bcl-x (Smith et al., 1998a; Smith et al., 1998b; Sugars et al., 2001).



**Figure 9.** Suggested mechanism of differential expression of NF proteins in early neuronal development. Transcription of NF-H might be delayed due to its different chromosomal location and restricted access of the Brn-3a transcription factor to the promoter.

Despite the *in vitro* coordinated induction of NFs as shown by Smith et al., (1997), the *in vivo* developmental induction seems quite different as shown in our study. The different chromosomal location of NF-L and NF-M, both contiguous in the same locus (15p12), with respect to NF-H (14q21), could be a contributing factor for the difference in the expression. The access of the Brn-3a transcription factor to the promoter region of NF-H could be blocked or regulated, while the access to NF-L and NF-M coding region is “open” and expression of these two proteins starts earlier (See Fig. 9). The biological significance of this developmental regulation remains, however, unexplained.

### 6.5.2 Cell type specificity of neurofilament immunoreactivity

At the cellular and subcellular level, in our *in vivo* developmental study, we detected variability in the staining pattern of NF proteins and their phosphorylation in the main neuronal cell types in the developing hippocampus. Three other main observations are of importance. 1) The complete lack of NF protein immunoreactivity in the cell bodies of the DG granule cell and CA1 pyramidal neurons at all developmental stages, 2) positive but differential immunostaining in axons and dendrites of granular cells, and 3) the absence of NF-M staining in the CA3 neuronal perikaryon.

The immunonegativity of DG granule cell bodies is in keeping with earlier studies with SMI 311, a mixture of monoclonal antibodies against non-phosphorylated epitopes of NF-M and NF-H in developing rats (Shetty and Turner, 1995b). However, variable staining patterns ranging from immunonegativity to intense staining have been observed in adult rats (Yang et al., 1995), guinea-pigs (Vickers and Costa, 1992) and rabbits (Van der Zee et al., 1997) using various antibodies against NF-H, NF-M and NF-L. The immunonegativity of granule cell bodies at P14 and even at P21 is remarkable, since at that time the majority of granule cells are postmitotic, more or less differentiated neurons

(Bayer, 1985). One possible mechanism explaining the lack of staining in the cell bodies could be that the mRNA transcripts of the different NF proteins are transported to the axons and dendrites, and translated *in situ*. The presence of mRNA in processes has earlier been shown for NF-L mRNA in axons of the rat hypothalamo-hypophyseal tract (Mohr et al., 1995), NF-M mRNA in the goldfish Mauthner axon (Weiner et al., 1996) as well as all three NF mRNAs in the rat sciatic nerve (Sotelo-Silveira et al., 2000). This mechanism could also explain the immunonegativity of CA1 pyramidal cell bodies, which has earlier been reported also by other groups in both young (Shetty and Turner, 1995b) and adult rats (Yang et al., 1995).

### **6.5.3 The importance of neurofilament phosphorylation**

In our studies we observed that the expression profiles of the phosphorylation-dependent and the phosphorylation-independent epitopes of NF-M and NF-H had a very similar temporal pattern in keeping with the idea that the phosphorylated and nonphosphorylated NFs can exist side by side within the axons (Brown, 1998). It has been suggested that the phosphorylation of NFs might be responsible for the cytoskeletal reorganization in neurons. In particular, the phosphorylation of NF-M and NF-H has been suggested to play a critical role in organizing the NFs and their ability to mediate the radial growth of axons (Lee and Cleveland, 1996). Furthermore, the phosphorylation/dephosphorylation of NFs is suggested to be a dynamic process continuing during the lifetime in the axons (Nixon and Lewis, 1986; Julien and Mushynski, 1998).

Selective phosphorylation of NF-L may play an important role in the structural plasticity of NF proteins as has been shown in the visual cortex of adult rats. In these naïve rats kept in the dark, no immunoreactivity was detected for the phosphorylated epitope of NF-L, whereas light exposure induced marked and selective phosphorylation of NF-L (Hashimoto et al., 2001). Furthermore, in hippocampal slices of adult rats the phosphorylation of NF-L has been shown in apical dendrites of pyramidal cells (Hashimoto et al., 2000). Consequently, it has been suggested that the phosphorylation of NF-L could be associated with the induction and/or maintenance of neuronal plasticity during the LTP. In ischemia (Kaku et al., 1993), KA-induced seizures (Wang et al., 1994; Yang et al., 1995) and traumatic brain injury (Posmantur et al., 2000), phosphorylation / dephosphorylation is thought to be one early sign of cellular damage, the changes in the dendrites preceding those in the axons (Posmantur et al., 2000).



In the *in vivo* study of experimental epilepsy, our results show neither dephosphorylation nor degradation of NF proteins in response to SE in the immature rats. The possible protective role of phosphorylation in this context may partly be explained by some earlier *in vitro* studies, which indicate that the dephosphorylation of NF proteins enhances their susceptibility to degradation by calpains, the main group of proteases responsible for NF degradation (Goldstein et al., 1987; Pant, 1988; Greenwood et al., 1993).

#### **6.5.4 The significance of neurofilaments in the developing hippocampus**

The results showed a highly regulated expression of the NFs and modifications of their composition and distribution within cell bodies, axons and to some extent also in dendrites during the development of the rat hippocampus. This together with earlier studies by other groups (Fliegner et al., 1994; Baas, 1997) suggest the importance of NFs for the normal neural development. Recent studies on NF knockout mouse lines further suggest that NF proteins are required for the radial growth and elongation of axons (Lee and Cleveland, 1996; Walker et al., 2001), but their more detailed role in the plasticity of dendrites and spines remains to be elucidated.

Our results also show a direct link between the NF expression and maturation of hippocampal connectivity. **In the DG molecular layers, the inner molecular layer receives commissural/associational afferents.** This area at P1, when DG granule cells are still actively proliferating, is virtually devoid of staining, and only a weak NF-M immunoreactivity was detected. At P7, increased maturation of the afferent fibers occurs, and prominent NF-L and NF-M staining was observed, while the NF-H immunostaining was almost absent. At P14, together with the increased expression of NF-L and NF-M in the inner molecular layer, a rapid increase of NF-H immunoreactivity was detected suggesting advanced maturation of the afferents to the inner molecular layer. The medium and outer molecular layers of the DG receive preferentially afferent inputs from the EC. At P1, only clear NF-M immunoreactivity was detected, and at P7 immunoreactivities of both NF-M and NF-H were present, suggesting an early maturation of the entorhinal afferents to the hippocampus.

In the CA1 region, the staining of NFs in the str. radiatum and str. lacunosum-moleculare was diverse. Since pyramidal cells are of prenatal origin, the connectivity at the studied ages was more mature compared to the DG.

Further studies focusing on dynamic properties of NF proteins in the developing brain, in particular in the hippocampus, are needed to better understand their functional role during the normal development and in pathological situations. The use of the existing NFs knockout mouse lines would be useful to reveal the different roles of each of the NFs in the physiological and functional maturation of the hippocampus.

## 6.6 Neurofilaments in the *in vivo* and *in vitro* experimental epilepsy

The main findings of our study show novel data indicating fast but transient reactivity of NF proteins together with resistance to degradation in response to KA-induced SE in the developing hippocampus. There are only a few studies in adult rats elucidating the response of NF proteins to seizures (Wang et al., 1994; Shetty and Turner, 1995a; Yang et al., 1995; Yang et al., 1996), and no earlier studies focusing on the reactivity of the NF proteins in the developing hippocampus.

In adult animals, degradation of NF proteins in the hippocampus after KA-induced seizures has been reported. The decreased expression of NF-H has been detected 1 day after KA treatment followed by a decrease in NF-L within 3 days (Wang et al., 1994). In immunocytochemical studies, decreased NF-L, NF-M, and NF-H immunoreactivity has been detected 3 days after the KA injection (Wang et al., 1994; Shetty and Turner, 1995b; Yang et al., 1996). In our work we also demonstrate that seizures lasting less than 1 h are enough to cause NF degradation in the hippocampus of adult rats. These findings in the adult brain after KA treatment are strikingly different from our results in the developing hippocampus, in which no degradation was detected but instead a fast and transient increase in the expression of all three NF proteins.

In contrast to the *in vivo* results, in cultured organotypic hippocampal slices, KA treatment resulted in a fast degradation of NF proteins. The early degradation (within 1 h) of NF proteins after an excitotoxic insult has also been detected after the hypoxic insult in the optic nerve (Stys and Jiang, 2002), and shortly after oxygen and glucose deprivation in acute brain slices prepared from adult rats (Tekkok et al., 2005). Moreover, NF-H degradation occurs within 1–2 h of traumatic spinal cord injury (Schumacher et al., 1999), and within hours of a glutamate-induced insult in cultured cortical neurons (Chung et al., 2005). The NF degradation also seemed to be age-dependant, being more pronounced in the older than in younger cultures, somehow mimicking the *in vivo* results during maturation.

The fast reactivity of NF proteins detected now, is in keeping with studies which show that KA induces a rapid transcriptional activation and translation of immediate early genes (IEG) such as c-fos and c-jun together with fast increase in the level of NF-L mRNA (Willoughby et al., 1997; Woldbye et al., 1997; Ressler et al., 2002). In the future, it would be of importance to elucidate the cascade that activates Brn-3a transcription factor and in turn, the transcription of NF genes.

## **6.7 Calpains, neurofilaments and KA-induced neuronal damage**

By treating OHC with KA we have studied the contribution of NF proteins to neuronal survival by Western blotting and immunocytochemistry. This treatment results in excitotoxic nerve cell damage in these cultures (Holopainen et al., 2004). We hypothesized that the highly permeable, novel calpain inhibitor MDL-28170, glutamate receptor antagonists (CNQX and MK-801), and the L-type  $\text{Ca}^{2+}$ -channel blocker nifedipine could protect neurons from degeneration.

Calpains are known to be directly involved in glutamate-induced hippocampal damage, and their activation is suggested to be an early, requisite step in the cascade of events initiated by excitotoxic injury which, through various intracellular signalling pathways, contributes to cytoskeletal degradation, and finally leads to nerve cell death both *in vivo* and *in vitro* conditions (Lankiewicz et al., 2000; Stys and Jiang, 2002; Rami, 2003; Araujo et al., 2004; Wu et al., 2004). Moreover, spectrin breakdown products, indicators of the  $\text{Ca}^{2+}$ -activated calpain activity, have been detected in the adult rat hippocampus after the intracerebroventricular injection of KA (Siman et al., 1989), and shortly after KA-induced seizures in the DG of the adult rat hippocampus (Bi et al., 1996). The fast NF degradation shown here suggests that the degradation of cytoskeleton may be an early step in the process of neuronal death. Moreover, calpain activation could be of major importance in many neuropathological conditions such as excitotoxic insults.

The marked KA-induced degradation of all three NF proteins in our OHCs was effectively inhibited with the calpain inhibitor MDL-28170, and with the potent AMPA/KA receptor antagonist CNQX, whereas the blockade of  $\text{Ca}^{2+}$  entrance with the L-type  $\text{Ca}^{2+}$ -channel blocker nifedipine, and the use of the NMDA receptor antagonist MK-801 had no protective effect. The observed beneficial effect of calpain inhibitors is in accordance with earlier studies, which have shown that calpain inhibitors are effective in reducing excitotoxic and hypoxic injuries, and decreasing spectrin, calcineurin, and

NF breakdown (Li et al., 1998; Kunz et al., 2004; Wu et al., 2004). Moreover, calpain inhibition has been effective in protecting neurons from excitotoxic insults both *in vitro* (Lankiewicz et al., 2000; Araujo et al., 2004; Wu et al., 2004) and *in vivo* conditions (Wu et al., 2004; Higuchi et al., 2005). A recent publication has confirmed *in vivo* our *in vitro* results. The injection of the calpain inhibitor MDL-28170 to adult rats, has dramatically reduced neuronal damage caused by KA- induced seizures (Araujo et al., 2008).

The fact that in our study the calpain inhibitor was added simultaneously with KA, and not prior to KA, mimics in a more relevant way the clinical situation, and further corroborates the idea that calpain activation at the early stage of the insult may contribute to NF degradation. This renders neurons more vulnerable to insults, which was effectively inhibited by the calpain inhibitor.

The low KA concentration (5  $\mu\text{M}$ ) used in our study is within the range (3-8  $\mu\text{M}$ ), which activates mainly KA receptors, although also AMPA receptors are to some extent activated (Kristensen et al., 2001). In the presence of CNQX,  $\text{Ca}^{2+}$  entry via AMPA/KA receptor activation is reduced, and results in lower calpain activation. This may explain why MDL-28170 prevented NF degradation to the same extent as CNQX. This is in keeping with the recent study by Araujo et al. (2004), in which the specific AMPA receptor antagonist NBQX inhibited the cleavage of a calpain substrate in cultured hippocampal neurons comparable to that of the calpain inhibitor MDL-28170. Our hypothesis of the CNQX mechanism of action is also in agreement with a recent study, in which AMPA receptor positive modulators have activated calpain proteases leading to breakdown of spectrin, and this was effectively inhibited by a calpain inhibitor (Jourdi et al., 2005a; Jourdi et al., 2005b).

On the other hand, our results also suggest that NMDA receptors and L-type  $\text{Ca}^{2+}$  do not play any major role in KA-induced NF degradation and neuronal damage. These results have been confirmed in a recent work by Araujo et al., (2007). In addition they suggest that  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchangers (NCX) might play an important role in the KA-induced neuronal damage.

## 7. CONCLUSIONS

The NF proteins are important neuronal intermediate filaments and known hallmarks in several important pathologies. The hippocampus, located in the inner temporal lobe of the brain, is involved in many important brain functions such as learning and memory. Moreover, the hippocampus is a region of special relevance in epilepsy. Epilepsy is a brain pathology characterized by spontaneous seizures, but its pathology is poorly understood in the developing brain.

In this work we have studied the role of NF proteins in the developing hippocampus and experimental epilepsy. Thus, we have used developing rats (1-up to 21-day-old) and organotypic hippocampal cultures for developmental, experimental epilepsy and pharmacology studies.

Our results show that NF-L and NF-M proteins are expressed already at postnatal day 1, while NF-H delays its expression mainly to the second postnatal week. Furthermore, the localization of the NFs is variable in the different cell types and subcellular locations. Cell bodies of DG and CA1 pyramidal cells were not stained at any age while CA3 pyramidal cells were immunopositive. Moreover, NF-M was only detectable in processes. The mossy fibers were immunopositive only to NF-L and NF-M during the studied period, indicating that during the early postnatal growth NF-H might not be essential.

KA-induced seizures in P9 rats resulted only in a transient increase of NFs during the first few hours after SE, but no NF degradation, which typically occurs in adult animals, was detected. Together with the lack of NF degradation, the most remarkable results were the absence of neuronal death and mossy fiber sprouting at any time point studied.

The *in vitro* studies indicate that NF proteins are rapidly degraded after KA incubation. Furthermore, in older cultures there is higher NF degradation and neuronal damage in response to excitotoxic insults. Moreover, the pharmacological studies indicated that AMPA receptor antagonist CNQX, and calpain inhibitor MDL-28170 are effective protective compounds against KA-induced NF degradation and neuronal damage. In addition, the results show that NMDA receptors, and L-type  $\text{Ca}^{2+}$  channels do not have any important role in the KA-induced damage.

In conclusion:

Our results indicate that the developmental expression of NF in the rat hippocampus is differentially regulated and targeted in the different hippocampal cell types. Furthermore, despite the severe seizures, the mechanism leading to NF degradation and cell death are not activated in P9 rats unlike in adults. The reason for this remains unknown.

The results using organotypic hippocampal cultures confirm the validity of this *in vitro* model to study development processes and to perform pharmacological studies. Results also point towards calpain proteases as interesting pharmacological targets to reduce neuronal damage after acute excitotoxic insults.

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## ACKNOWLEDGEMENTS

This thesis was carried out at the Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku. I am grateful to professors Risto Huupponen, Mika Scheinin and Esa Korpi for allowing me to use the facilities of the department. I also greatly appreciate the Finnish Graduate School of Neuroscience for educational and financial support.

To my supervisor Docent Irma Holopainen. I want to express my gratitude for your guidance to complete this thesis work. I am sincerely grateful for your support and the lengthy, and sometimes tense, discussions. I want to also thank you also for allowing me to conduct the work, and learn from my own mistakes.

I gratefully acknowledge the official reviewers of this thesis, Professor John Eriksson and Docent Claudio Rivera for their constructive criticism and encouraging comments.

I also want to thank the co-authors of my articles: Docent Mikko Uusi-Oukari, MSc Tiina Kaisa Kukko-Lukjanov, and MSc Nina Jalava. I thank to all the present and former members of the Department of Pharmacology, Drug Development and Therapeutics for their warm and professional work environment.

To the other members of the Hippo-group, MSc Tiina Kaisa Kukko-Lukjanov, PhD Hanna Laurén, MSc Niina Jalava, and MSc Juha Järvelä. I want to thank you all for the wonderful personal and professional environment in the group. It has been a real pleasure to share all this time with all of you.

I want to give my greatest thanks to my whole family, my parents Anunciación and Gaspar, my sister Jessica, and particularly to my beloved wife Kaili, and my son Daniel, who gave the love, inspiration and support necessary to reach this goal.

The financial support of the Finnish Graduate School of Neurosciences, the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Special Grant for Clinical Research (EVO), the Oskar Öflund Foundation, the Finnish Epilepsy Association and the Turku University Foundation is gratefully acknowledge.

Turku, April 2008

Francisco R. López Picón

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