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KAINIC ACID-INDUCED SEIZURES: INFLAMMATION AND EXCITOTOXIC NEURONAL DAMAGE IN THE DEVELOPING RAT HIPPOCAMPUS

by

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To my family

ABSTRACT

Juha Järvelä: Kainic acid-induced seizures: inflammation and excitotoxic neuronal damage in the developing rat hippocampus. Department of Pharmacology, Drug Development and Therapeutics, University of Turku and Turku Graduate School of Biomedical Sciences.

Epileptic seizures are harmful to the developing brain. During epileptic seizures, overactivation of glutamate receptors (GluR) leads to neuronal degeneration, defined as excitotoxicity. The hippocampus is especially vulnerable to excitotoxic neuronal death, but its mechanism has remained incompletely known in the developing brain. Recently, signs of activation of inflammatory processes after epileptic seizures have been detected in the hippocampus. The purpose of this thesis was to study the inflammatory reaction and death mechanisms in excitoxic neurodegeneration induced by the glutamate analogue kainic acid (KA) in the developing hippocampus. Organotypic hippocampal slice cultures (OHCs), prepared from 6-7-day-old rats (P6-7) and treated with KA, served as an *in vitro* model. KA-induced status epilepticus in P9 and P21 rats was used as an *in vivo* model. The results showed that the pyramidal cell layers of the hippocampus were the most susceptible to irreversible and age-specific neurodegeneration, which occurred in the juvenile (P21), but not in the immature (P9), rat hippocampus. The primary death mechanism was necrosis as there were no significant changes in the expression of selected apoptosis markers and morphological cellular features of necrosis were found. Inflammatory response was similarly age-dependent after KA treatment as a rapid, fulminant and wide response was detected in the juvenile, but not in the immature, rat brain. An anti-inflammatory drug treatment, given before KA, was not neuroprotective in OHCs, possibly because of the timing of the treatment. In summary, the results suggest that KA induces an age-dependent inflammatory response and necrotic neurodegeneration, which may cause disturbances in hippocampal connectivity and promote epileptogenesis.

Keywords: epilepsy, developing brain, hippocampus, neurodegeneration, apoptosis, inflammation

TIIVISTELMÄ

Juha Järvelä: Kainiinihapon aiheuttamat kouristukset: tulehdusreaktio ja eksitotoksinen hermosoluvaurio kehittyvän rotan hippokampuksessa. Farmakologia, lääkekehitys ja lääkehoito, Turun yliopisto ja Turun biolääketieteellinen tutkijakoulu.

Epileptiset kohtaukset ovat haitallisia kehittyville aivoille. Glutamaattireseptorien yliaktivaatio kohtausten aikana aiheuttaa hermosolukuolemaa, mikä määritellään eksitotoksisuudeksi. Hermosolukuolemaa tapahtuu erityisesti hippokampuksen alueella, mutta sen mekanismi on jäänyt epäselväksi kehittyvissä aivoissa. Tulehdusreaktion on äskettäin havaittu aktivoituvan pian kohtausten jälkeen. Tässä työssä tutkittiin glutamaattianalogi kainiinihapon aiheuttaman hermosolukuoleman mekanismeja, tulehdusreaktiota ja sen merkitystä kehittyvän rotan hippokampuksen vauriossa. Organotyyppisten hippokampusleikeviljelmien käsittely kainiinihapolla ja sillä aiheutettu status epilepticus (SE) 9 ja 21 päivän ikäisille rotille mallinsivat eksitotoksisuutta ja pitkittynyttä epileptistä kohtausta. Tulokset osoittivat, että hippokampuksen pyramidaalialueiden hermosolut olivat altteimpia vauriolle, joka oli peruuttamaton ja ikäriippuvainen, koska sitä havaittiin vain 21 päivän mutta ei 9 päivän ikäisillä rotilla. Pääasiallinen solukuolemamekansmi oli nekroosi, koska apoptoosiin liittyvien proteiinien ilmentyminen ei muuttunut merkittävästi ja soluissa havaittiin nekroosin piirteitä. Tulehdusreaktio aktivoitui ikäriippuvaisesti kainiinihappokäsittelyn jälkeen: 21 päivän ikäisillä rotilla aktivaatio tapahtui nopeasti, voimakkaasti ja kokonaisvaltaisesti, mutta 9 päivän ikäisillä rotilla aktivaatio oli erittäin heikko. Tulehduskipulääkekäsittely ennen kainiinihapon lisäystä ei estänyt hermosoluvauriota hippokampusleikeviljelmissä, mikä voi johtua lääkkeenannon ajoituksesta. Yhteenvetona todetaan, että kainiinihappo johti koe-eläimillä tulehdusreaktion ikäriippuvaiseen aktivaatioon, joka voi edesauttaa nekroottista hermosoluvauriota ja häiritä normaalien hermosoluyhteyksien muodostumista altistaen epilepsian kehittymiselle.

Avainsanat: epilepsia, kehittyvät aivot, hippokampus, hermosolukuolema, apoptoosi, tulehdus

CONTENTS

ABBREVIATIONS

5LOX	5-lipoxygenase
AA	arachidonic acid
AED	antiepileptic drug
AIF	apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
apaf-1	apoptotic protease activating factor-1
ATP	adenosine triphosphate
BBB	blood-brain barrier
BH	bcl-2 homology domain
BSA	bovine serum albumin
CA	*cornu ammonis* (lat. Ammon's horn)
cDNA	complementary DNA
CNS	central nervous system
COX	cyclooxygenase
DAB	3,3'-diaminobenzidinetetrahydrochloride
DG	dentate gyrus
DIV	days *in vitro*
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EC	entorhinal cortex
EP	prostaglandin E receptor
ER	endoplasmic reticulum
FJB	Fluoro-Jade B
GABA	gamma-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
GLT-1	glial glutamate transporter-1
GluR	glutamate receptor
HI	hypoxia-ischemia
HRP	horseradish peroxidise
IFN	interferon
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
i.p.	intraperitoneal
IP_3	inositol triphosphate
KA	kainic acid
KAR	kainic acid receptor

MCP	monocyte chemotactic protein
MMP	matrix metalloproteinase
mRNA	messenger RNA
NMDA	N-methyl-*D*-aspartate
NMDAR	N-methyl-*D*-aspartate receptor
NS-398	N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide
OHC	organotypic hippocampal slice culture
P	postnatal day
PARP	poly(ADP ribose)polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PG	prostaglandin
ROS	reactive oxygen species
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SE	status epilepticus
TBS	Tris-buffered saline
TGF	transforming growth factor
TLE	temporal lobe epilepsy
TNF	tumor necrosis factor

LIST OF ORIGINAL COMMUNICATIONS

I Holopainen IE, Järvelä J, Lopez-Picon FR, Pelliniemi LJ, Kukko-Lukjanov TK (2004) Mechanisms of kainate-induced region-specific neuronal death in immature organotypic hippocampal slice cultures. Neurochem Int 45:1-10.

II Järvelä JT, Lopez-Picon FR, Holopainen IE (2008) Age-dependent cyclooxygenase-2 induction and neuronal damage after status epilepticus in the postnatal rat hippocampus. Epilepsia 49:832-841.

III Järvelä JT, Ruohonen S, Kukko-Lukjanov TK, Plysjuk A, Lopez-Picon FR, Holopainen IE (2011) Kainic acid-induced neurodegeneration and activation of inflammatory processes in organotypic hippocampal slice cultures: Treatment with cyclooxygenase-2 inhibitor does not prevent neuronal death. Neuropharmacology 60:1116-1125.

IV Järvelä JT, Lopez-Picon FR, Plysjuk A, Ruohonen S, Holopainen IE (2011) Temporal profiles of age-dependent changes in cytokine mRNA expression and glial cell activation after status epilepticus in the postnatal rat hippocampus. J Neuroinflammation.8:29.

1 INTRODUCTION

Epilepsy is a chronic neurological disease, which affects roughly 1% of the human population (Pitkänen and Sutula, 2002). Epilepsy is defined as as a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures (Fisher et al., 2005). Status epilepticus (SE) is defined as a condition in which epileptic activity (e.g. seizure) persists for more than 30 minutes or recurrent seizures over period of 30 minutes without full recovery of consciousness between seizures (Commission on Classification and Terminology of the International League Against Epilepsy, 1981; Lowenstein et al., 1999). Epileptic seizures are a consequence of electrical disturbance in the brain, characterized by an imbalance between excitation and inhibition (Fisher et al., 2005). The developing brain is more prone to seizures, as excitation is physiologically favored over inhibition during maturation (Holmes and Ben-Ari, 2001).

Epileptic seizures are harmful to the developing brain. Harmful effects include neuronal degeneration and disturbances in developmental processes which may in turn predispose to recurrence of seizures (Ben-Ari and Holmes, 2006). These effects are highly age- and seizure model-dependent. For example, when SE is induced by an intraperitoneal injection of the glutamatae analogue kainic acid (KA), neurodegeneration occurs in the more mature postnatal brain, but not the immature brain (Haas et al., 2001; Ravizza et al., 2005). The acute cellular harmful effects are later evident in the child as cognitive impairment, e. g. learning disability and developmental delay (Holmes and Lenck-Santini, 2006). Among the most vulnerable brain regions to seizure-induced damage is the hippocampus, a part of the limbic system in the brain. The hippocampus has a central physiological role in learning and memory.

Seizure-induced neurodegeneration is caused by overactivation of glutamate receptors by glutamate, the major excitatory neurotransmitter of the brain, leading to excessive calcium ion (Ca^{2+}) influx inside the neuronal cell resulting in neuronal damage, which is defined excitotoxicity (Olney, 1969). However, the exact molecular mechanisms of excitotoxic neuronal death in the developing hippocampus are incompletely known. It has remained unelucidated, whether this death occurs via the harmful form of cell death, called necrosis, or the less harmful programmed cell death, called apoptosis (Kerr et al., 1972). Recently, also activation of inflammatory processes has been proposed to contribute to excitotoxic neuronal damage in the adult brain, and some signs of inflammatory responses after this have been detected in the developing brain as well (Rizzi et al., 2003; Jung et al., 2006).

The main objectives of this thesis were to study the neuronal death mechanism in excitoxic neurodegeneration and to investigate the contribution of inflammatory processes to this by using *in vitro* and *in vivo* rat models of epileptic seizures and excitotoxicity of the developing hippocampus.

The goal was to clarify these molecular mechanisms, which could contribute to the development of drugs to counter the harmful effects of epileptic seizures in pediatric patients.

2 REVIEW OF THE LITERATURE

2.1 Glutamate

2.1.1 Glutamate as a neurotransmitter

Glutamate, or glutamic acid, is an amino acid and the major excitatory neurotransmitter in the central nervous system (CNS). At the synapse, glutamate is stored in vesicles. Nerve impulses trigger its release from the pre-synaptic nerve terminals and glutamate binds to and activates glutamate receptors (GluR) in the post-synaptic neuron. It has been known for over four decades that glutamate can cause neuronal destruction in the mammalian brain (Olney, 1969), which subsequently was named excitotoxicity, due to neuronal death resulting from excessive depolarization produced by the excitatory substance (Olney, 1971; Olney et al., 1972). Excitotoxicity, in the form of overactivation of GluRs, has since been found to be an important contributing factor in different brain insults and lesions, such as hypoxia-ischemia (HI) and epilepsy (Whetsell 1996; Mattson et al., 2000; Bengzon et al., 2002).

2.1.2 Glutamate receptors

GluRs are integral membrane proteins. There are two kinds of GluRs; ionotropic and metabotropic. Furthermore, the ionotropic GluRs can be divided in three families according to the activating agonists: N-methyl-*D*-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainic acid (or kainate, KA).

2.1.2.1 NMDA receptors

Three subunit families have been identified for NMDA receptors (NMDAR); NR1, NR2 and NR3, which have different isoforms and splice variants (for references, see Cull-Candy et al., 2001). Receptors function as heteromeric assemblies composed of multiple NR1 subunits in combination with at least one member of the NR2 family. The NR3 subunit does not form functional receptors alone, but can co-assemble with NR1/NR2 complex. Each subunit confers distinct properties to the receptor assembly and functional attributes of the receptors are largely determined by the expression of the various subunits and isoforms (Cull-Candy et al., 2001). For example, the NR3A subunit suppresses receptor activity and thus decreases NMDAR-mediated neurotransmission (Henson et al., 2010). This diversity is also evident in pharmacology, as NMDAR antagonists and channel blockers show selectivity for certain subunit compositions (Chazot et al., 1994). The central functional attributes of NMDARs are voltage-sensitive block of the cation channel by extracellular Mg^{2+}, a high permeability to Ca^{2+} and unusually slow "activation/deactivation" kinetics (Cull-Candy et al., 2001). Excessive calcium influx through NMDARs can cause excitotoxic neuronal death, and

blockade of NMDARs has been neuroprotective in animal models of both stroke and seizure (Lee et al., 1999). However, the potential usefulness of NMDAR antagonists as drugs is limited by side effects. For example, using NMDAR antagonists has resulted in dopaminergic hyperactivity and behavioural changes characteristic of schizophrenia (for references, see Olney et al., 1999). Furthermore, mice with reduced NMDAR expression have been proposed as models of schizophrenia (Mohn et al., 1999). Interistingly, NMDA preconditioning (i.e. treatment with a sub-lethal dose of NMDA) has conveyed neuroprotection against glutamate and KA-induced neurotoxicity, which suggests the idea that neurons have the ability to generate tolerance against excitotoxins (Mohammadi et al., 2009; Navon et al., 2011).

2.1.2.2 Kainate / AMPA type glutamate receptors

In the past, AMPA and KA receptors have been considered as only one functional unit because of difficulties in differentiating them, and they were called AMPA/KA receptors (Catarzi et al., 2007). Classification of these receptors according to their preferred agonist has been replaced by classification based on the proteins which form the receptor. AMPA receptors are heteromers and consist of four different subunits, GluR1, GluR2, GluR3, and GluR4 (Catarzi et al., 2007).

KA receptors are comprised of a tetramer of subunits of five different types: GluR5, GluR6, GluR7, KA1 and KA2 (for review, see Pinheiro and Mulle, 2006). GluR5, GluR6 and GluR7 subunits can form functional homomeric receptor-channels activated by KA and show low affinity for glutamate (Egjeberg et al., 1991; Schiffer et al., 1997). KA1 and KA2 subunits mediate high affinity for the ligands, but cannot form functional channels without the other subunits (Werner et al., 1991; Herb et al., 1992). Kainate and AMPA receptors act mainly as cation-selective ion channels (Dingledine et al., 1999). Activation of postsynaptic KA receptors by e.g. glutamate generates an excitatory postsynaptic current that leads to the cumulative depolarization of the postsynaptic membrane (for references, see Pinheiro and Mulle, 2006). There are also presynaptic KA receptors, which act as autoreceptors and can regulate neurotransmitter release (for references, see Lerma et al., 2001; Pinheiro and Mulle, 2006).

2.1.2.3 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluR), in contrast to ionotropic glutamate receptors, mediate slow synaptic responses due to their coupling to intracellular G proteins and second messengers (Nakanishi, 1994). They are divided into three groups. Group I includes mGluR1 and mGluR5 subtypes and is coupled to inositol triphosphate (IP_3)/Ca^{2+} signal transduction. Group II includes mGluR2 and mGluR3 subtypes, and group III mGluR4 and mGluR6–mGluR8 subtypes. Both Group II and III subtypes are negatively coupled to adenylate cyclase (Nakanishi 1994). These receptors play a role in synaptic plasticity, modulation of neural excitability, and neurotransmitter release (Conn and Pin, 1997). The mGluR1 subtype has been proposed to play an important role in regulating hippocampal network activity after early-life seizures (Avallone et al., 2006).

2.1.2.4 Developmental changes in glutamate receptors

Glutamate has a critical role during development, as activation of GluRs is involved in synaptogenesis, synaptic plasticity and neuronal migration (Collard et al., 1993; Yen et al., 1993; Behar et al., 1999). The subunit expression of different GluRs is variable through development, as e. g. during the first postnatal month in the rat hippocampus the expression profile of most of the subunits of the ionotropic glutamate receptor types (NMDAR, AMPAR and KAR) changes (Moyner et al., 1994; Pickard et al., 2000; Ritter et al., 2002).

In general, the expression of NMDAR subunits NR2B, NR2D, and NR3A decreases during development, whereas the expression of NR2A, NR2C, and NR3B subunits increases and peaks during the third postnatal week in the rat (Monyer et al., 1994; Ritter et al., 2002; Henson et al., 2010). The high levels of NR2B, NR2D, and NR3A in the immature brain contribute to increased NMDAR-mediated Ca^{2+} influx and thus lower the threshold for seizures (Lau and Zukin, 2007). There is an increase in AMPARs containing the GluR2 subunit in the hippocampus during the first 2 postnatal weeks of rat brain development, reaching its pinnacle at P14 and then declining (Pickard et al., 2000). The GluR4 subunit is expressed mainly early in the hippocampus while, in contrast, the GluR1 to GluR3 subunit expression increases with development (Zhu et al., 2000). In the CA1 region of the hippocampus, the GluR2 receptor subunit replaces the GluR1 subunit (Ritter et al., 2002), which could lead to a reduced Ca^{2+} influx. Of the mGluR subunits, mGluR1, mGluR2, and mGluR4 messenger RNA (mRNA) expression is low at birth in the rat brain, but increases during development. In contrast, mGluR3 and mGluR5 are highly expressed at birth, but decrease during maturation (Catania et al., 1994). It has been suggested that mGluR3 and mGluR5 may play a role in synaptogenesis, and mGluR2, and mGluR4 in mature synaptic transmission (Catania et al., 1994; Avallone et al., 2006).

2.1.3 Kainic acid

KA was first isolated from a type of seaweed in Japan (Murakami and Takemoto, 1953). Subsequently, it was discovered that local application of KA on neurons has excitatory action and, furthermore, that KA causes neuronal destruction, especially in the pyramidal cells of the hippocampus (Nadler et al., 1978). KA activates KARs and, on higher concentrations, AMPARs (Lerma etal., 1993). KA-induced cell death has been confirmed to occur through activation of KARs, as, for example, GluR6-knock down protects from KA-induced cell death (Mulle et al., 1998). KARs are widely expressed throughout the developing rat brain (e.g. in the hippocampus, cortex, thalamus and cerebellum) (Bahn et al., 1994). However, expression patterns of different subunits in different brain areas are variable (Bahn et al., 1994). In the hippocampus, KAR subunit expression is detectable in the hippocampus at the same time as the formation of hippocampal structures begins (at around embryonic day 14 in the rat) (Bahn et al., 1994). There is variation in the KAR subunit expression also between different regions in the developing rat hippocampus. For example, the pyramidal and dentate

granule cells express GluR6 in abundance, a population of the interneurons express GluR5, while the KA1 subunit expression is mostly restricted to CA3 (Wisden and Seeburg, 1993; Bahn et al, 1994). There are many transient peaks in KAR (as well as in AMPAR and NMDAR) subunit expression during the postnatal development (from P0 to P35), which possibly denote important time-points in the development of the rat hippocampus (e.g. P3 and P7) (Ritter et al., 2002). In general, KAR subunit expression decreases in the rat hippocampus from the levels observed at the time of birth during postnatal development (Ritter et al., 2002).

Extent and location of KA-induced neurodgeneration seems to depend on the concentration KA used and the route of its administration. For example, an intraperitoneal (i.p.) injection of KA leads in most cases to damage in the CA1 region while an intracerebroventricular or intramygdaloid injection of KA damages more prominently the CA3 region (Nadler et al., 1978; Sperk, 1994). A systemic or an intracerebral injection of KA induces epileptic seizures and thus KA administration is widely used as a model of temporal lobe epilepsy (TLE) (Nadler 1981; Ben-Ari, 1985; Vincent and Mulle, 2009).

2.2 Hippocampus

2.2.1 Structure

The hippocampus is a structure located in the temporal lobe of the brain. It consists of the following subregions; dentate gyrus (DG), and *cornu ammonis* (CA) regions CA3, CA2 and CA1 and the subiculum (Fig. 1) (Amaral and Lavenex, 2007). The DG has two layers; the granular cell layer housing the cell bodies of the granular cell type of neurons, and the molecular cell layer, which consists mostly of dendrites of the granular cells and axons projected towards them from the entorhinal cortex (EC) (known as the perforant path) (Amaral and Lavenex, 2007). The axons of the granular cells (known as mossy fibers) project towards the CA3 region. The CA regions have the pyramidal cell layer at their core, consisting of the cell bodies of the pyramidal cell type of neurons, and next to it *stratum oriens* (on the outside curve of the pyramidal cell layer) and *stratum radiatum* (on the inside curve) (Amaral and Lavenex, 2007). *Stratum oriens* houses the basal dendrites of the pyramidal cells, while *stratum radiatum* contains the apical dendrites. The axons of the CA3 region (known as Schaeffer collaterals) project first into the *stratum oriens* (of the same region) and then to the *stratum radiatum* of the CA1 region. Axons from the CA1 region are extended towards the subiculum and from there axons extend back to the EC (Amaral and Lavenex, 2007).

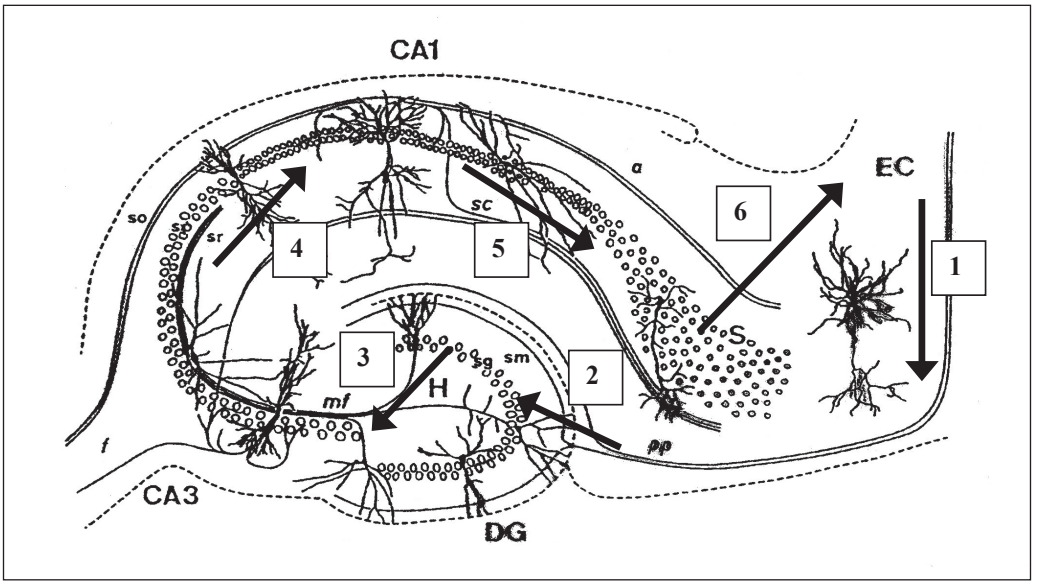

Figure 1. Schematic representation of a cross-sectioned hippocampus and a simplified illustration of the order of the main excitatory signal transduction from the entorhinal cortex through the hippocampal circuitry (arrows and numbers). The signal arrives from the entorhinal cortex via the perforant pathway (1), is received by dentate granule cells in the DG (2), and transmitted to the pyramidal cells in CA3 (3). From CA3 the signal is relayed to the CA1 region (4) before exiting the hippocampus via the subiculum (5) and heading back to the entorhinal cortex (6). Modified from Caeser and Aertsen, 1991. Abbreviations: a, alveus; DG, dentate gyrus; EC, entorhinal cortex; f, fimbria; H, hilus; mf, mossy fiber; pp, perforant pathway; S, subiculum; sc, Schaeffer collaterals; sg, stratum granulare; sm, stratum moleculare; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum.

2.2.2 Function

The hippocampus is a part of the limbic system and is involved in many functions, for example learning and memory. Roughly, the main excitatory pathway begins with input from the entorhinal cortex at the DG and continues through the CA regions (from CA3 to CA1) to the subiculum and from there back to the entorhinal cortex (Amaral and Lavenex, 2007).

The vast majority of the neuronal connections in the hippocampus are excitatory with glutamate as neurotransmitter, but a small portion (about 10%) of the neurons are inhibitory interneurons, found in all regions of the hippocampus, with gamma-aminobutyric acid (GABA) acting as the transmitter (Freund and Buzsaki, 1996). The interneurons have an important role in balancing and synchronizing the primarily excitatory activity (Freund and Buzsaki, 1996).

The major extrinsic input into the hippocampus comes from the EC via the perforant pathway (Witter et al., 1988). The fibers terminate in the outer two-thirds of the molecular layer of the DG (Van Hoesen and Pandya, 1975). Additionally, the DG receives some input from hypothalamus, septal nuclei and monoaminergic brain stem nuclei. The CA3

and CA1 regions receive extrahippocampal input from cortical and subcortical areas and especially abundantly from the amygdala (Pikkarainen et al., 1999; Pitkänen et al., 2000). The DG does not project extrahippocampally, but the CA3 has some extrinsic output, most importantly to the lateral septal nucleus (Swanson and Cowan, 1977). The CA1 has more extrahippocampal projections than CA3. In addition to the major output route back to the EC through the subiculum, the CA1 projects to the lateral septal area, and a number of other subcortical and cortical areas.

Among the brain structures, the hippocampus is especially vulnerable to damage from disturbances, such as epileptic seizures. The subregions of the hippocampus differ in susceptibility to neuronal damage, the CA3 and CA1 regions regarded as more vulnerable than the DG (Faherty *et al*., 1999; Liu *et al*., 2001).

2.2.3 Development

Morphologically, the primate (including human) hippocampus develops earlier than in rodents, as the majority of developmental processes take place *in utero* (Bayer, 1980; Lavenex et al., 2007). Soon (3-4 days) after birth, human babies have recognitional memory, which is based on hippocampal function (Pascalis & Schoenen, 1994). In the rat brain in the first 2 postnatal weeks, a so-called brain growth spurt takes place, during which there is an intense neuronal growth and synaptogenesis, and brain size grows rapidly (Dobbing and Sands, 1979). Matching these developmental stages in rodents and humans is complicated, due to variation in parameters. Roughly, the developmental stage of a rat at the age of 7-10 days is similar to a full-term newborn baby, and a 3-week-old rat is comparable to a young child (reviewed by Avishai-Eliner et al., 2002). In the rat hippocampus, neurons in the Ammon's horn are generated prenatally, whereas granule cells in the DG are mostly generated postnatally (Bayer, 1980). Furthermore, interneurons are generated prenatally in rats, ahead of the glutamatergic pyramidal neurons (Bayer, 1980).

2.3 Epilepsy

Epilepsy is a common neurological disease affecting roughly 1% of humans the highest incidence being in infants and the elderly (Pitkänen and Sutula, 2002). Epilepsy is defined as a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition (Fisher et al., 2005). In epilepsy, electrical imbalance in the brain results in recurrent seizures (Fisher et al., 2005). Status epilepticus (SE) is defined as a condition in which epileptic activity (e.g. seizure) persists for more than 30 minutes or recurrent seizures over period of 30 minutes without full recovery of consciousness between seizures (Commission on Classification and Terminology of the International League Against Epilepsy, 1981; Lowenstein et al., 1999). The possible causes of epilepsy are diverse, e.g. brain injury, stroke, brain infection, genetic alterations, but its etiology remains unknown in most cases.

In acquired epilepsy, an initial brain insult and/or seizure is followed by a silent, seizure-free period, which is defined epileptogenesis, during which epilepsy develops (for references, see Pitkänen and Lukasiuk, 2011). It is a dynamic and diverse process characterized by alteration of neuronal connections, neurodegeneration, neurogenesis, gliosis, axonal sprouting, dendritic plasticity, blood–brain barrier (BBB) damage, recruitment of inflammatory cells into brain tissue, reorganisation of the extracellular matrix, and reorganisation of the molecular architecture of individual neuronal cells (Pitkänen and Lukasiuk, 2011). Epileptogenesis culminates in development of chronic spontaneous seizures, i.e. epilepsy.

2.3.1 Epilepsy in the developing brain

There is a higher incidence of seizures in the immature brain than the mature brain, due to the imbalance favouring excitation over inhibition during maturation (Holmes and Ben-Ari, 2001). For example, GABA is the major inhibitory neurotransmitter in the adult brain, but it is depolarizing during early neuronal development before maturation of the K^+/Cl^- co-transporter (Rivera et al., 1999). However, the immature brain is more resistant to seizure-induced damage in the form of neuronal degeneration compared to the mature adult brain (Baram et al., 2002; Ben-Ari and Holmes, 2006). In contrast, the immature brain is susceptible to disturbances in developmental processes such as displacement in cell migration and aberrant maturation of synapses, which may in turn predispose to recurrence of seizures (Ben-Ari and Holmes, 2006). The susceptibility to neuronal degeneration caused by seizures or SE is dependent on age and maturational stage. Results vary according to the seizure and SE model used, but in general either no nerve cell death or minor damage has been detected in the hippocampus, amygdala, and temporal cortical regions of animals younger than two-weeks of age (Nitecka et al., 1984; Cavalheiro et al., 1987; Sperber et al., 1991; Haas et al., 2001; Cilio et al., 2003), whereas more severe damage has been observed in the brain of rats older than 2 weeks (Sankar et al., 1998; Druga et al., 2005; Holopainen, 2008).

In addition to neuronal loss, harmful effects of early life SE in rodents also manifest as an increased risk of later seizures or even developing TLE in adulthood (Holmes and Ben-Ari, 1998; Dube et al., 2000; Cilio et al.,2003; Kubova et al., 2004), and cognitive impairment such as memory deficits and spatial learning (Lynch et al., 2000; Kubova et al., 2004). Similar consequences are seen in humans: although the majority of children with epilepsy do relatively well, a part of them suffer from developmental delay, learning disabilities, and behavioural and psychosocial problems (for references, see Holmes and Lenck-Santini, 2006).

Antiepileptic drugs (AEDs) are used continuously to prevent seizures. However, in the developing brain, AEDs trigger apoptotic neurodegeneration, which may be the cause of side effects observed in pediatric patients taking AEDs such as developmental delay, cognitive impairment and microcephaly (Bittigau et al., 2002). Phenytoin and valproic acid can trigger region-specific apoptotic neuronal death in P7-P8 rats at plasma concentrations relevant for seizure suppression in humans (Bittigau et al., 2002; Kim et al., 2007b). At

this age neurons are rapidly growing and establishing synaptic connections (Bittigau et al., 2002; Kim et al., 2007b). Furthermore, neurotoxicity of AEDs is suggested to be age-dependent, and the molecular mechanism responsible for this is proposed to be the impairment of neurotrophin-mediated, survival-promoting signals in the brain (Bittigau et al., 2002). In humans, the period of synaptogenesis extends from the last 3 months of pregnancy into the third year of life (Dobbing and Sands, 1979), and both phenytoin and valproic acid are currently used at this age. Moreover, combining AEDs to reduce seizure frequency may be even more deleterious for the immature brain than the use of a single drug alone according to experimental data. For example, the combined treatment with phenytoin and carbamazepine significantly exacerbated phenytoin-induced apoptotic neuronal death in several brain regions of P7-P8 rats, while topiramate, given alone, caused no damage, but when combined with phenytoin, enhanced phenytoin-induced neurodegeneration (Kim et al., 2007a). However, topiramate can induce neuroprotection in experimental HI models, as it has prevented hypomyelinating injury caused by HI insult in P7 rats, and decreased the subsequent neuromotor deficits (Follett et al., 2004). It also suppressed seizures and ameliorated long-term neurobehavioral deficits after HI-induced seizures in P10 rats (Koh et al., 2004). Furthermore, gabapentin enhanced neuronal survival and attenuated seizures in an ischemic experimental model, in which the right common artery was ligated in P12 rats, and immediately after the ligation, rats received an i.p. injection of gabapentin (Traa et al., 2008). These experimental data suggest that newer AEDs can, at least when used as monotherapy, have beneficial effects after brain insults in the immature brain.

2.3.2 Animal models of epilepsy

Epilepsy, a many-faceted disease, can also be modelled in different ways, depending on the aims of the study. For example, the chronic disease condition can be induced or, in contrast, acute seizures may be elicited. Seizures are induced by chemoconvulsants or electrical stimulation. Most common chemoconvulsants, i.e. compounds used to induce SE, are KA and pilocarpine, which are administered systemically (intaperitoneally, intravenously or subcutaneously) or injected intracerebrally. KA acts on KARs and, at higher concentrations, also AMPARs and causes the depolarization of the neurons which have these receptors (reviewed above). In the hippocampus, in particular the pyramidal neurons in the CA3 area (Bahn et al., 1994), in which the KA receptor density is the highest in the brain, are vulnerable to generation of epileptiform activity after depolarization with KA due to their network-like organization and tendency to generate bursts (Ben-Ari, 1985; Lothman et al., 1991; Strowbridge 1999; Hemond et al., 2008; Vincent and Mulle, 2009). Pilocarpine is a muscarinic acetylcholine receptor agonist. Commonly, pilocarpine administration is preceded by pre-treatment with lithium, which sentitizes the brain to pilocarpine allowing the usage of lower doses of pilocarpine (Jope et al., 1986). Pilocarpine has been used to generate seizures both in immature and adult rodents (Kubova et al., 2001; Dudek et al., 2002). In contrast to the KA model, neuronal damage in the hippocampus has also been detected in postnatal day (P) 12-14 rats using

pilocarpine (Sankar et al., 1998). Following the administration of the chemoconvulsant, SE with generalized tonic-clonic seizures is provoked in minutes to hours depending on the drug used, its concentration, and the strain and age of the animals. After a few hours, the seizures disappear, and may or may not recur spontaneously later.

Kindling is a commonly used method, which was conceived already decades ago (Goddard et al., 1969). In kindling, excitatory stimulation is mechanically applied into the brain to create chronic TLE (Goddard et al., 1969). The targeted brain area, for example the hippocampus or the amygdala, is stimulated with implanted electrodes mildly and repeatedly, which eventually leads to spontaneous seizures (Sayin et al., 2003). In electrically induced SE, a single and stronger electrical stimulation is applied to specific sites of the brain, which leads to SE. The most sensitive area for SE induction is basolateral amygdala (Goddard et al., 1969; Nissinen et al., 2000).

2.3.3 In vitro model: Organotypic hippocampal slice cultures

As the hippocampus is organized in a lamellar fashion, isolated secions cut perpendicularly to the septotemporal axis retain the majority of the intrinsic connections intact and thus may operate as independent functional units (Caeser and Aertsen, 1991). Organotypic hippocampal slice cultures (OHC) are tissue cultures cut in such fashion from the hippocampi of 6 to 7 day-old animals, and model the developing hippocampus. In these cultures, cellular maturation, synaptic organization and functional connections develop similarly to *in situ* conditions (Caeser and Aertsen, 1991; Gähwiler et al., 1997; Holopainen, 2005). Furthermore, glutamate analogue treatment of these cultures induces synaptic reorganization and functional changes compatible to the *in vivo* models of excitotoxicity, such as epileptic animals (Routbort et al., 1999). Conversely, treatment with for example non-NMDA antagonists blocks the increased activity (Routbort et al., 1999; Bausch and McNamara, 2000). The severity and location of neuronal damage caused by glutamate and its analogues (KA, AMPA, NMDA) in OHCs depends on the agent used, its concentration and length of treatment time (Vornov et al., 1991; Bruce et al., 1995; Kristensen et al., 2001).

2.4 Cell death mechanisms

Excitotoxicity is a process, in which excessive glutamate overactivates Ca^{2+} permeable, GluR-operated, channels causing Ca^{2+} influx into the neuron, and the resulting imbalance in intracellular Ca^{2+} causes the activation of lethal signalling pathways ending in neuronal death (Orrenius et al., 2003). However, the type of cell death may vary (Orrenius et al., 2003). Early effects of Ca^{2+} influx include the activation of Ca^{2+}-dependent enzymes, and generation of reactive oxygen species (ROS) (Wang et al., 2005). The mechanism of neuronal death in excitotoxicity has been extensively investigated in models of different CNS disturbances (e.g. hypoxia, ischemia, traumatic brain injury, epilepsy) in the adult brain with somewhat conflicting results, but in the developing brain, the death mechanism has remained incompletely studied.

Cell death has classically been divided in two main categories: necrosis and apoptosis (Kroemer et al., 2005). See Table 1 for comparison of morphological features between apoptosis and necrosis. This view has been challenged and evidence has been presented of other types of cell death and also cell death with mixed features. Furthermore, new cell death mechanisms, e.g. autophagic cell death and excitotoxic cell death as a cell death entity of its own, have been proposed (Kroemer et al., 2005).

Table 1. Comparison of morphological features of apoptosis and necrosis

Apoptosis	Necrosis
Cell shrinkage	Cell swelling
Chromatin condensation, DNA fragmentation	Partial chromatin digestion
Cell membrane integrity maintained	Cell membrane rupture, integrity lost
Cell organelles remain intact	Cell organelles destroyed

2.4.1 Apoptosis

Apoptosis is a form of programmed cell death, which is usually a physiological mechanism, although it can occur as a response to an insult. In the embryonic brain, in its physiological role, it ensures controlled development in removing excess neurons. Apoptosis is characterized by shrinkage of the nucleus with chromatin condensation and cleavage, while cell membranes and organelles remain initially intact (Kerr etal., 1972; Kroemer et al., 2005). Eventually the apoptotic cell is vacuolized into apoptotic bodies, which are ingested by phagocytes. In this way, cell death does not cause disturbances to the surrounding area.

2.4.1.1 Caspases

Apoptosis is commonly dependent on cysteine protease enzymes, known as caspases. Apoptotic caspases can be divided into initiator caspases (caspase-2, -8, -9, -10 and -12) and effector caspases (caspase-3, -6 and -7) (Slee et al., 1999). Caspase-3 is the most prevalent caspase in the cell and responsible for the majority of apoptotic functions (Zimmermann et al., 2001; Maiuri et al., 2007). Caspases are produced in inactive forms called pro-caspases, and effector caspases require cleavage to become activated (Wilson et al., 1994). During apoptosis, activated effector caspases cleave and inactivate vital proteins for cellular survival, such as the DNA-repairing enzyme poly(ADP ribose) polymerase (PARP) (Lazbenik et al., 1994). Also inactive caspases are substrates for active caspases. Gene disruption experiments have established that caspases are essential for normal brain development, as for example deletion of caspase-3 or caspase-9 leads to developmental defects in the CNS and forebrain malformation (Kuida et al., 1996; Hakem et al., 1998). There is some variance in the expression levels of caspases during rat brain development. For example, caspase-2 expression rises 4 weeks after birth, while caspase-3 expression is high from embryonic day 19 to 2 weeks after birth, and decreasing thereafter (Shimohama et al., 1999).

2.4.1.2 Bcl-2 family proteins

The bcl-2 family proteins are important for the intrinsic apoptotic pathway. They share one or more of four domains of homology (called bcl-2 homology domains, BH), which are important in dimeriztion and in functioning as regulators of apoptosis (Gross et al., 1998; Cory and Adams, 2002). Bcl-2 family proteins can be divided into pro-apoptotic (e.g. bax, bad, bak, bim) and anti-apoptotic (e.g. bcl-2, bcl-x, bcl-w), based on their function. The actions of the pro-apoptotic bcl-2 family proteins (detailed below) are countered by the anti-apoptotic bcl-2 family proteins under stable conditions, usually by forming a heterodimer (Zimmermann et al., 2001; Maiuri et al., 2007). Bax is the key pro-apoptotic bcl-2 family protein during brain development, as its disruption results in increased numbers of neurons in selected neuronal populations (for references, see Kuan et al., 2002). Of the anti-apoptotic bcl-2 family proteins, only bcl-x disruption produces a dramatically changed neurodevelopmental phenotype, while disruption of bcl-2 affects only programmed cell death in specific neuronal subpopulations during embryogenesis and after the period of naturally occurring cell death (for references, see Kuan et al., 2002). In general, the expression of bcl-2 family proteins seems to be downregulated during the rat brain development. For example, bcl-2 protein level was at its highest at embryonic day 19 and decreased after birth, while bax levels were high from embryonic day 19 to 2 weeks after birth, and decreased from 4 weeks after birth onwards (Shimohama et al., 1998).

2.4.1.3 Extrinsic and intrinsic apoptotic pathways

Apoptosic pathways can be divided into extrinsic and intrinsic (Zimmermann et al., 2001; Maiuri et al., 2007). In the extrinsic apoptosic pathway, a death signal comes from outside the cell and binds to a death receptor on the cell membrane (e.g. Fas). This starts a cascade during which caspase-8 is activated, which in turn activates other caspases, such as caspase-3. Upon activation by cleavage, caspase-3 cleaves proteins vital for cell survival, for example PARP.

In the intrinsic pathway (Fig. 2), members of the pro-apoptotic bcl-2 family, e.g. bax, undergo a conformational change and translocate from the cytosol onto the mitochondrial outer membrane after withdrawal of survival signals or induction by cellular stress (Zimmermann et al., 2001; Maiuri et al., 2007). On the mitochondrial membrane, they participate in the formation of pores on the membrane, which leads to release of cytochrome c from the mitochondria into the cytosol (Zimmermann et al., 2001; Maiuri et al., 2007). Cytochrome c together with caspase-9 and apoptotic protease activating factor-1 (apaf-1) form a complex called apoptosome, which activates executor caspases, including caspase-3 (Zou et al., 1997). Extrinsic and intrinsic pathways share the last steps of the pathway, i.e. the activation of caspases, but the pathways may also interconnect earlier; for example caspase-8 is able to recruit pro-apoptotic bcl-2 family proteins (Zimmermann et al., 2001).

Additionally, alternative caspase-independent apoptosis pathways have been identified. An example of this is a pathway in which apoptosis is induced by the apoptosis-inducing factor (AIF) (Yu et al., 2002; Zhu et al., 2003). AIF is a mitochondrial protein,

which translocates to the nucleus, where it induces large scale DNA fragmentation and apoptosis (Henshall and Simon, 2005). In addition, the role of PARP in cell death has been found to be more complicated than merely contributing to apoptosis by loss-of-function (for references, see Virag and Szabo, 2002). PARP activation in DNA damage has been hypothesized to have three different outcome possibilities (Virag and Szabo, 2002). If the DNA damage is mild, PARP functions normally and depending on the sufficiency of DNA repair, the cell either survives or goes to apoptosis. However, if DNA damage is excessive, PARP is overactivated resulting in depletion of cellular energy stores, i.e. nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate (ATP), which leads to necrosis.

Besides mitochondria, apoptosis can be initiated by other cell organelles as well. For example, unrepairable DNA damage in the cell nucleus causes the p53 protein to increase the expression of pro-apoptotic bcl-2 family proteins (Ferri and Kroemer, 2001). Also endoplasmic reticulum (ER) stress can initiate apoptosis, for example by calcium release from the ER into the cytosol. Additionally, apoptosis may be locally regulated by the bcl-2 family proteins and caspase-12 located at the ER (Ferri and Kroemer, 2001).

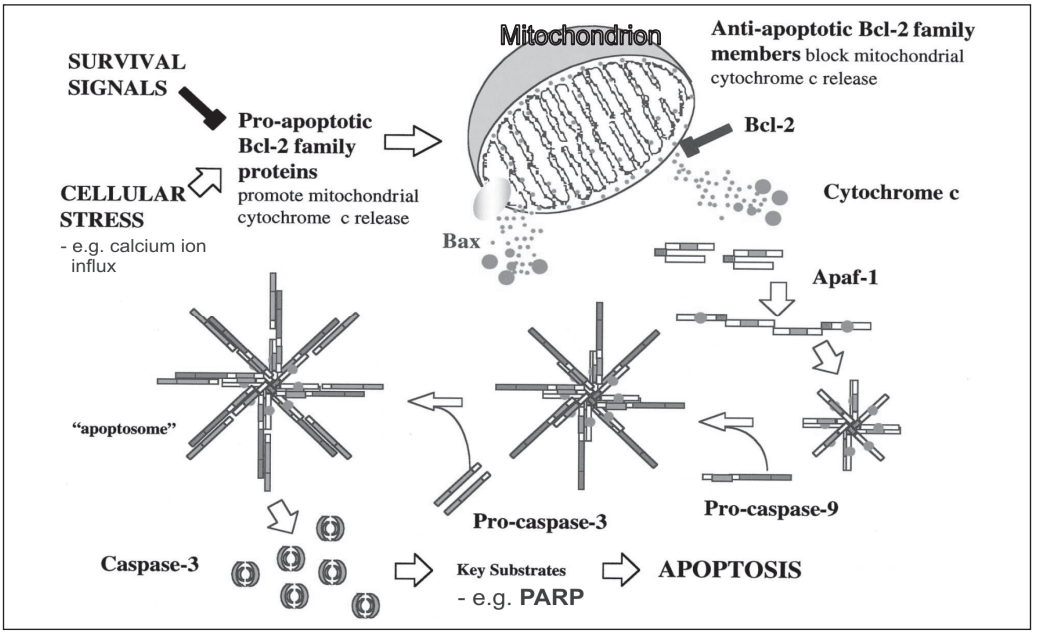

Figure 2. A simplified illustration of the intrinsic apoptosic pathway. Modified from Zimmermann et al., 2001. Abbreviations: Apaf-1, apoptotic protease activating factor-1; PARP, poly(ADP ribose)polymerase

2.4.2 Necrosis

Necrosis is traditionally regarded as a pathologic and unregulated form of cell death and is characterized by swelling of cell organelles and cell membrane breakdown, while the nucleus and chromatin remain intact in the early phase but are fragmented later

(Kroemer et al., 2005). Necrosis often leads to harmful effects on the surrounding area as toxic cell debris is released from the dying cell on other cells and, as a consequence, also the inflammatory reaction is activated. However, evidence is accumulating that necrosis might also have some elements of regulation and programming. For example, Fas receptor stimulation has been shown to elicit a caspase-independent pathway resulting in necrosis (Holler et al., 2000; Matsumura et al., 2000). Necrosis is still largely identified by absence of markers of apoptosis or features of other cell death types (described below).

2.4.3 Other types of cell death

Autophagic cell death is a relatively recently discovered form of cell death and is described as a type of cell death that occurs in the absence of chromatin condensation but accompanied by massive autophagic vacuolization of the cytoplasm. (Maiuri et al., 2007; Kroemer et al., 2009). Autophagic cell death has been detected during neuronal development and neurodegenerative diseases (Borsello et al., 2003: Yuan et al., 2003). Also a protective role has been suggested for autophagy, since its loss in the CNS seems to cause neurodegeneration (Komatsu et al., 2006). It has been suggested that autophagy and apoptosis have abundant molecular crosstalk and may be triggered by common upstream signals, such as the bcl-2 family proteins (Maiuri et al., 2007). *In vivo* studies of autophagic cell death are still limited, but are expected due to considerable interest in this cell death mechanism.

Excitotoxic cell death has been presented as a separate entity, but so far has not been fully endorsed as an independent death mechanism due to common regulators and overlap with other death types (Kroemer et al., 2005; Kroemer et al., 2009). Signs of mixed features of different types of cell death have also been described, such as death receptor mediated autophagic cell death with necrotic morphology (sometimes called necroptosis) in ischemic neuronal injury (Degterev et al., 2005).

2.4.4 Death mechanism in excitotoxic neuronal death in the brain

In the mature brain, parts of the apoptotic pathways have been shown to be activated after excitotoxic insult or seizures. At the mitochondrial level in the intrinsic pathway, members of the bcl-2 family of proteins are triggered after seizures; e.g. bcl-2 expression has been observed to be decreased compared to the pro-apoptotic bax, which also translocated from the cytosol to the mitochondria (Lopez et al., 1999; Henshall et al., 2002; Korhonen et al., 2003). AIF has been found to be involved in both bax-dependent and bax-independent neuronal apoptosis in NMDA- and KA-mediated excitotoxicity (Cheung et al., 2005). Additionally, gene disruption studies in mice have implicated the pro-apoptotic bcl-2 family proteins Bim and Puma as mediators of hippocampal neuronal death caused by KA-induced seizures, while bcl-w has been found to have seizure- suppressing and neuroprotective functions (Murphy et al., 2007; Engel et al., 2010; Murphy et al., 2010). Signs of extrinsic pathway involvement in excitotoxiciy have been noticed in the form of caspase-8 processing and attenuation of neuronal death by caspase-8 inhibition (Henshall et al., 2001; Li et al., 2006). However, results concerning

caspase-3 activation after KA have been somewhat contradictory, as caspase-3 has been detected to be either activated (Faherty et al., 1999), inactive (Fujikawa et al., 2002) or only marginally activatied (Narkilahti et al., 2003). Increased levels of bax, cytochrome c and caspase-3 have been detected in relatively mature 18-25 days *in vitro* (DIV) OHCs after 50 μM KA treatment (Sakhi et al., 1997; Liu et al., 2001). However, inhibition of caspases only partially protects neurons from KA toxicity, implying involvement of multiple mechanisms (Liu et al., 2001). The role of PARP in excitotoxic neuronal death has been suggested to be linked to AIF. PARP activation stimulated by glutamate analogues (e.g. NMDA) or ischemia is required for translocation of AIF from the mitochondria to the nucleus, which implies the existence of PARP-dependent but caspase-independent form of programmed cell death (Yu et al., 2002; Culmsee et al., 2005).

Excitotoxic cell death mechanisms have been far less studied in the developing brain. In P14-28 rats, SE-induced CA1 nerve cell death might involve both early necrosis and apoptosis within days of the insult (Sankar et al., 1998; Wasterlain et al, 2002). It has also been suggested that the mechanism of excitotoxic cell death in the developing hippocampus has a temporal dependence with necrosis being the dominant cell death mechanism acutely (a few days) after the excitotoxic insult, and apoptosis appearing later, in the delayed phase of neuronal death (i.e. 1-2 months after the insult) (Humphrey et al., 2002). More specifically, it has been proposed that in the perinatal brain, the loss of connections from the neurons that die by non-apoptotic mechanisms in the first wave cause the apoptotic death of the neurons, which received these connections prior to the insult, in the second wave (Young et al., 2004).

2.5 Inflammation as a mediator of neuronal damage in epilepsy

Activation of the innate immunity, i.e. inflammation, after seizures or an excitotoxic insult has been proposed to contribute to nerve cell damage in adult rats (for references, see Vezzani and Granata, 2005). Some signs of induction of inflammatory mediators after seizures have also been detected in the developing brain, (Rizzi et al., 2003). Inflammatory processes are activated within hours after seizures or an excitotoxic insult and include upregulation of cyclooxygenase (COX) enzymes, increased production of prostaglandins and cytokines and activation of microglia and astrocytes (Tocco et al., 1997; Jankowsky et al., 2001; Vezzani and Granata, 2005). Also, the potential of anti-inflammatory drugs to protect from seizure-induced / excitoxoic damage has been investigated, albeit almost exclusively in the adult brain.

2.5.1 Inflammatory processes in the brain

2.5.1.1 COX-enzymes and prostaglandins

There are two isoforms of the COX-enzyme: COX-1 and COX-2. In the brain, COX-1 is constitutively expressed mainly in the microglia (Yermakova et al., 1999). In contrast, COX-2 is expressed at low levels in physiological conditions in neurons associated with glutamatergic neurotransmission, and is inducible by a variety of stimuli (Kaufmann et

al., 1996). The main function of the cyclooxygenase enzymes is to catalyze the first step on the transformation of arachidonic acid (AA) into prostaglandins (PG), which in the brain bind to G-protein coupled receptors located in the hypothalamus, thalamus, and limbic system (Watanabe et al., 1989; Breyer et al., 2001). Specifically, AA is catalyzed by COX into PGH_2, of which PGE_2, $PGF_2\alpha$, PGD_2, PGI_2, and thromboxane A_2 are then derived.

PGs have highly diverse and sometimes even antagonistic functions, possibly depending on the receptor subtype to which they bind (for references, see Breyer et al., 2001). COX-2 expression is upregulated and PG production and/or release is increased within hours after seizures or excitotoxic insult in the brain, while neuronal degeneration is detected later, which enables the hypothesis that these inflammatory mediators might contribute to neuronal damage (Baran et al., 1987; Yamagata et al., 1993; Kaufmann et al., 1996; Tocco et al., 1997; Yoshikawa et al., 2006). Of the PGs, particularly PGE_2 has been studied in this context, and it has been suggested that its increase after KA-induced seizures is dual-phased: an early (within 30 min) KA receptor-mediated phase, and a later (several hours to 24 h) phase involving COX-2 induction (Yoshikawa et al., 2006). Furthermore, it has been proposed that binding of PGE_2 to prostaglandin E receptor-1 (EP1) contributes to neuronal death, while its binding to prostaglandin E receptor-2 (EP2) promotes neuroprotection (McCullough et al., 2004; Carlson et al., 2009).

2.5.1.2 Glial cells

Microglia and astrocytes belong to the glial cell family, which includes also oligodendrocytes. Glial cells are non-neuronal cells in the CNS that form myelin (oligodendrocytes) and maintain homeostasis in the CNS and thus support neurons. However, disturbances in normal glial cell function during pathological conditions in the brain might lead to harmful effects. Recently, the role of glial cells, in particular microglia and astrocytes in pathological brain conditions has come under intense scrutiny. At the birth of the rat, the vast majority of brain cells are neurons, with only about 6% being non-neuronal cells (Bandeira et al., 2009). During the second postnatal week, massive gliogenesis takes place (Bandeira et al., 2009). Astrocytes and oligodendrocytes are generated locally, while microglia are monocytes that have infiltrated into the brain (Prinz and Mildner, 2011).

2.5.1.2.1 Microglia

Microglia are the resident immunocompetent cells in the CNS and form the main immunological defence system in the brain (Streit and Kincaid-Colton, 1995; Kreutzberg 1996; Hanisch, 2002). Microglia infiltrate the brain during early embryonic development, before the maturation of the BBB (Kreutzberg, 1996). In physiological conditions, microglia have an appereance, which has been termed “resting”, characterized by a small soma and processes which are thin and branched (Kreutzberg, 1996; Shapiro et al., 2008). Despite the resting appereance, microglia are constantly in action, surveying the neurons and their microenvironment biochemically with their highly mobile processes and aborizations (Nimmerjahn et al., 2005; Hanisch and Kettenmann, 2007). Following

certain pathological events which threaten the structural or functional integrity of the CNS, for example seizures, microglia go through a morphological and functional change (Giaume et al., 2007; Hanisch and Kettenmann, 2007; Shapiro et al., 2008). Activated microglia are characterized by an enlarged cell body and thickened and more ramified processes (Giaume et al., 2007; Hanisch and Kettenmann, 2007; Shapiro et al., 2008). Microglia also migrate towards the damaged location, attracted by stimuli from degenerating neurons (Hailer et al., 1997; Heppner et al., 1998). In addition to having receptors for several activity modulators, microglia themselves can produce and release a variety of signal molecules, such as cytokines (pro-inflammatory or anti-inflammatory) and growth factors (Hanisch, 2002; Giaume et al., 2007; Hanisch and Kettenmann, 2007). The functional response of microglia is therefore diverse and can lead to neuroprotection or neuronal death depending on the activating conditions and severity of insult (Giaume et al., 2007; Hanisch and Kettenmann, 2007).

Microglial activation has been detected after KA-induced seizures in the developing (P15, P21) rat hippocampus as well as in hippocampal slice cultures after KA-treatment (Rizzi et al., 2003; Somera-Molina et al., 2007; Cho et al., 2008). Microglial morphology changes from resting to an active appereance and expression of microglial markers, such as the Iba1 protein, increases (Rizzi et al., 2003; Somera-Molina et al., 2007; Cho et al., 2008).

2.5.1.2.2 Astrocytes

Astrocytes are star-shaped cells in the brain, with processes that form sheet-like extensions that enwrap neurons and blood vessels. They have traditionally been viewed as mostly passive supporting cells for the neurons. However, new discoveries have been made of late, which highlight the importance of astrocytes and the diversity in their organization and functions in physiological as well as in pathological settings (for review, see Kimelberg, 2010). Astrocytes are linked to each other and communicate via gap junctions to form activity groups (Giaume and McCarthy, 1996). Connexins are the molecular constituents of gap junctions and their expression is robust in astrocytes (Giaume and Mccarthy, 1996). In pathological conditions, connexins and gap junctions have been shown to have both augmenting and attenuating effects on neuronal degeneration (Giaume et al., 2007). Upon activation in certain conditions, e.g. seizures, astrocytes, like microglia, go through a morphological change, becoming hypertrophied with thickened processes (Shapiro et al., 2008).

On the molecular level, astrocytes respond to activating stimuli with a rise in intracellular Ca^{2+}, which can in turn cause the release of chemical mediators from astrocytes, such as glutamate and ATP (for references, see Haydon and Carmignoto, 2006). Astrocytes have the ability to limit neuronal excitation in at least two ways: maintaining low concentrations of extracellular potassium (K^{+}) and by glutamate uptake from the synaptic cleft, for which purpose they express two types of glutamate transporters in rodents: glial glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST) (Rothstein et al., 1994: Lehre et al., 1995). During seizures, astrocytic glutamate uptake may become impaired or they may even release glutamate

which may contribute to excitotoxicity and development of later seizures (Wetherington et al., 2008). Astrocytes, like microglia, can also produce and release cytokines, both pro- and anti-inflammatory, and growth factors (Vezzani et al., 2008; Kim et al., 2010). Thus, in summary, the response of astrocytes in pathological conditions, such as epileptic seizures, can promote either neuroprotection or neurodegeneration.

Like microglia, astrocytes are activated after KA-induced seizures in the developing (P15, P21) rat hippocampus as well as in hippocampal slice cultures after KA-treatment (Rizzi et al., 2003; Somera-Molina et al., 2007; Cho et al., 2008). Astrocyte morphology changes from resting to active appereance and the expression of the astrocyte marker glial fibrillary acidic protein (GFAP) increases (Rizzi et al., 2003; Somera-Molina et al., 2007; Cho et al., 2008).

2.5.1.3 Cytokines and growth factors

Cytokines are soluble polypeptides mediating cell-signalling in inflammation and are secreted in the CNS by microglia and astrocytes (reviewed above). Cytokines can be divided into pro-inflammatory, e.g. tumor necrosis factor- α (TNF-α) and interleukin-1 (IL-1), or anti-inflammatory, e.g. interleukin-10 (IL-10), based on their function, i.e. they either promote or suppress inflammation, respectively. Cytokines such as IL-1β, TNF-α, and IL-6 have been found to be induced rapidly in seizure models (including KA-induced seizures) in the developing and the mature brain, i.e. within a few hours after injection of chemoconvulsant (Jankowsky and Patterson, 2001; Ravizza et al., 2005). Their role in seizure-induced neuronal degeneration has recently come under increasing scrutiny. Pro-inflammatory cytokines, such as IL-1, have been implicated in neurodegeneration (Rothwell, 1998). Suggested mechanisms of cytokine-mediated neurotoxicity have been autocrine or paracrine production of neurotoxic compounds and enhancement of NMDA- and AMPA-mediated Ca^{2+} influx into neurons (Allan et al., 2005; Vezzani and Baram, 2007). However, pro-inflammatory cytokines have been reported to have neuroprotective functions as well, such as stimulation of antioxidant pathways and induction of calbindin to counter the rise in intracellular Ca^{2+} (Allan et al., 2005). Therefore, classical pro-inflammatory cytokines such as IL-1 and TNF can have either toxic or trophic effects, possibly depending on their extracellular concentrations, duration of their induction, and receptor types activated (Vezzani et al., 2008). In addition to neurotoxicity, pro-inflammatory cytokines have also been implicated in epileptogenesis, i.e. increasing susceptibility for the development of spontaneous seizures after the initial seizure. For example, transgenic mice overexpressing IL-6 have shown increased sensitivity to seizures (Samland et al., 2003), and chronic IL-1β expression has recently been associated with the development of spontaneous limbic seizures after prolonged febrile seizures in P11 rats (Dube et al., 2010). Conversely, IL-1 receptor antagonist (IL-1Ra) has mediated anticonvulsant effects in adult rodents in a number of limbic seizure models, such as intrahippcompal KA and bicuculline (a $GABA_A$ receptor antagonist) injection, and kindling (Vezzani et al., 2002).

The anti-inflammatory cytokine IL-10 suppresses inflammation by inhibiting pro-inflammatory cytokine production (Moore et al., 1993). Its role in seizures and

excitotoxicity has remained incompletely studied. IL-10 and its receptor were found to be upregulated after a topical excitotoxic lesion by NMDA (Gonzalez et al., 2009). Furthermore, administration of IL-10 has been shown to increase the threshold for febrile seizures (Ishizaki et al., 2009).

The members of the matrix metalloproteinase (MMP) family are involved in the metabolism in the pericellular environment and extracellular matrix breakdown and remodelling (for references, see Sternlicht and Werb, 2001). MMPs have been implicated in the pathogenesis of neurological disorders such as cerebral ischemia, Alzheimer's disease and brain trauma (for references, see Yong, 2005). MMP-9, one member of the MMP family, is suggested to have an important role in synaptic plasticity and learning (Meighan et al., 2006). Furthermore, its expression and activity have been found to be increased after KA-induced SE (Zhang et al., 1998; Jourquin et al., 2003). Interferon gamma (IFN-γ) has immunostimulatory and immunomodulatory capabilities. Its mRNA expression was detected to be enhanced after lithium-pilocarpine induced SE in adult rats while the expression of its receptor was upregulated in neurons, and, furthermore, neutralization of the IFN-γ receptor aggravated injury, suggesting a protective role for IFN-γ in epilepsy (Ryu, 2010).

Growth factors, also produced by microglia and astrocytes, stimulate cellular growth, proliferation and differentiation. The expression of some growth factors, such as glial cell line- derived neurotrophic factor (GDNF), and transforming growth factor-β (TGF-β) has been found to be induced after KA administration (Morgan et al., 1993; Humpel et al., 1994). Both have been reported to protect neurons from KA-induced degeneration (Martin et al., 1995; Prehn and Miller, 1996; Kim et al., 2002). On the other hand, while seizure-induced changes in GDNF signalling seem to be important for neuroprotective responses, they might also contribute to abnormal excitability and development of epilepsy via effects on synaptic plasticity (Kokaia et al., 1999).

2.5.1.4 Developmental differences in activation of inflammatory processes

Activation of the innate immunity after seizures in the developing brain has been suggested to be age-specific. Tocco et al. (1997) found that constitutive COX-2 mRNA expression increases with maturation and is enhanced after KA-induced seizures in the hippocampus of juvenile P14 and P21 rats, but not in immature P7 rats. Also COX-2 protein expression has been detected to be increased with development and induced in the developing hippocampus after repeated fluorothyl-induced seizures (Kim and Jang, 2006). Microglial and astrocyte activation seems to be age-dependent as well, as there was little glial activation acutely (4 and 24 h) after KA-induced SE in P9 rats, whereas pronounced activation was observed in P15 and P21 rats (Rizzi et al., 2003). Cytokine mRNA expression also showed age-specifity in the same study. Expression of IL-1β, TNF-α and IL-6 was studied, and all were induced 4 h after SE in P21 rats, but only IL-1β in P15 rats, and none in P9 rats. Similar pattern of age-dependent inflammatory response has been detected in other excitotoxicity models. For example, after hypoxic-ischemic insult, expression of monocyte chemotactic protein-1 (MCP-1) and IL-18 was

more prominently increased in hippocampal endothelial cells and perivascular areas of P21 than P9 mice (Qiu et al., 2007). Taken together, it seems that inflammatory processes are activated to a greater extent in the more mature juvenile brain than in the immature brain in excitotoxicity.

2.5.2 Anti-inflammatory drugs as neuroprotectants

The observation that activation of inflammatory processes after seizures precedes and might contribute to neuronal damage in the brain has lead to experiments testing the hypothetic neuroprotective potential of anti-inflammatory drugs. These experiments, carried out almost exclusively in the mature brain, have yielded mixed and sometimes even contradictory results.

COX-2 selective inhibitors are commonly and widely used as pain-killers and, despite recent setbacks stemming from their harmful cardiovascular side effects in adults, therefore present the most straightforward candidate. Both *in vitro* and *in vivo* studies have been carried out. In primary cortical cultures, COX-2 selective inhibitors flubriprofen and N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) afforded protection in NMDA- but not in KA-induced neurotoxicity (Hewett et al., 2000). In another cell culture study, NS-398 prevented KA-induced death in hippocampal neurons but not in cortical neurons (Kim et al., 2001). More recently, *in vivo* studies with e.g. celecoxib and rofecoxib claim to have resulted in neuroprotection from seizure-induced damage (Kunz and Oliw, 2001b; Kawaguchi et al., 2005; Hewett et al., 2006; Jung et al., 2006). However, in other studies COX-2 selective inhibitors even exacerbated KA-induced neurotoxicity (Baik et al., 1999; Kunz and Oliw 2001a). It has been suggested that timing of COX-2 inhibition is the determing factor for the outcome. Specifically, pre-treatment does not lead to neuroprotection because it does not block the late-phase PGE_2 increase, whereas post-treatment (a few hours after KA) is able to do so (Gobbo and O'Mara, 2004; Takemiya et al., 2006). Furthermore, the transient increase in PGE_2 production immediately following insult might even be neuroprotective (Gobbo and O'Mara, 2004) and pre-treatment with COX-2 inhibitors could be deleterious because it prevents increased PGE_2 production.

In models of ischemic brain injury, another setting of excitotoxicity, both non-selective and COX-2 selective inhibitors have protected against neuronal injury (Candelario-Jalil and Fiebich, 2008). In the adult rat brain, indomethacin and ibuprofen treatments have reduced infarct size in focal brain ischemia and protected CA1 neurons from global ischemia (Patel et al., 1993; Buccellati et al., 1998). Also COX-2 selective inhibitors NS-398 and nimesulide reduced infarct size in focal and global ischemia in rats (Nogawa et al., 1997; Candelario-Jalil et al., 2004). However, indomethacin, ibuprofen and COX-2 selective inhibitors have not afforded neuroprotection in all ischemia studies (for refrences, see Candelario-Jalial and Fiebich, 2008). Clinical potential of COX-inhibitors in treatment of ischemic brain injury is hampered by the increased cardiovascular risk and the possible existence of also a cerebrovascular risk, which is observed especially with large doses and/or chronic treatment (Andersohn et al., 2006). However, short-term

administration might be a clinically viable treatment option in stroke (Candelario-Jalil and Fiebach, 2008).

Other attempts to alleviate or prevent excitotoxic neurodgeneration by anti-inflammatory drugs have included using an IL-1 receptor antagonist, which inhibited microglial activation and afforded neuroprotection in NMDA (50 μM for 4 h)-mediated neurotoxicity in OHCs (Vogt et al., 2008). Minocycline is an emerging candidate as it is a potent inhibitor of microglial activation and caspase-related apopototic pathways, and has shown promise in alleviating neuronal degeneration in ischemia, KA-induced excitotoxicity *in vitro* and after KA-induced seizures in the hippocampus (Yrjänheikki et al., 1999; Chen et al., 2000; Tikka et al., 2001; Heo et al., 2006).

3 AIMS OF THE STUDY

The purpose of this work was to elucidate the incompletely known mechanisms underlying excitotoxic neuronal death caused by KA in the developing hippocampus by using *in vitro* and *in vivo* models. The main aims were:

I To examine the extent, localisation and temporal profile of KA-induced neurodgeneration in the developing hippocampus.

II To determine the death mechanism of KA-induced neuronal degeneration in the developing hippocampus.

III To investigate the activation of inflammatory processes after KA treatment and its possible contribution to KA-induced neurodegeneration in the developing hippocampus.

IV To detect age-dependent differences in KA-induced activation of inflammatory processes and neuronal death in the developing hippocampus

4 MATERIALS AND METHODS

4.1 Experimental epilepsy models

4.1.1 Animals (I-IV)

For *in vitro* studies in organotypic hippocampal slice cultures, 6- to 7-day old Wistar (I) or Harlan Sprague-Dawley (III) rats were used. For *in vivo* studies (II, IV), 9-day-old and 21-day-old Harlan Sprague-Dawley rats were used. The age-groups for the *in vivo* studies were selected to compare two different developmental stages: the immature (P9) rat and the more mature juvenile (P21) rat. All animal experiments were conducted in accordance with the guidelines of the European Community Council Directives 86/609/EEC, and had the approval of the Animal Use and Care Committee of the University of Turku (I-II) or the Office of the Regional Government of Western Finland (III-IV). All efforts were made to minimize the pain, discomfort, and number of experimental animals.

4.1.2 Status epilepticus induced by kainic acid injection (II, IV)

4.1.2.1 Status epilepticus in 9-day-old rats (II, IV)

Group 1 (II): P9 rat pups were weighted, and an i.p. single dose of KA (2 mg/kg) (Tocris Cookson Ltd., Avomouth, UK) was given. After the injection, the pups first manifested stiffening, scratching, head nodding, and forelimb clonus within 5 min with further progression to generalized tonic-clonic seizures within 12-15 min. In order to ensure the survival of P9 rats, SE was stopped with a single dose (0.3 ml/kg, i.p.) of paraldehyde (Fluka Chemie AG, Buchs, Switzerland) 1 h after the KA injection. Shortly after that, 0.9% NaCl was injected (i.p.) at the dose of 2.5% of the body weight to help the recovery. The mortality of rat pups was about 20%. After the cessation of seizures, pups were taken back to the cages with their mother, and used for studies later. Instead of KA injection, P9 control rats received the comparable volume of 0.9% NaCl as given to the KA-treated rats, but to minimize the discomfort of the experimental animals, they did not receive any further injections although were otherwise treated as their age-matched KA-injected rats. Moreover, in this age group, the KA-treated and their age-matched control rats were separated from their mothers for equivalent time.

Group 2 (IV): P9 rat pups were weighted, and a single dose of KA (1.2 mg/kg) (Tocris) was given i.p. The pups first manifested stiffening, scratching, head nodding, and forelimb clonus within 20-30 min after the injection with further progression to generalized tonic-clonic seizures within 45-60 min, which continued for 3-4 h after the KA injection. The mortality of the rat pups was negligible. After the cessation of seizures, pups were taken back to the cages with their mother. Instead of KA injection, P9 control rats received the comparable volume of 0.9% NaCl as given to the KA-treated rats. The KA-treated and their age-matched control rats were separated from their mothers for equivalent time.

4.1.2.2 Status epilepticus in 21- day-old rats (II, IV)

P21 rats were weighted, and an i.p. single dose of KA (7 mg/kg) (Tocris Cookson Ltd., Avomouth, UK) was given. Rats first showed scratching, chewing, and myoclonic jerks within 20-30 min followed by wet dog shakes, head nodding, forelimb clonus, and rearing and falling. Behavioural signs of generalized tonic-clonic seizures occurred within 45-60 min after the injection, which continued up to 3-4 h after the KA injection. The mortality of animals was negligible. Instead of KA injection, P21 control rats received the comparable volume of 0.9% NaCl as given to the KA-injected rats, but were otherwise treated as their age-matched treated rats. Rats were visually followed-up for about 2 h after the cessation of behavioural seizure signs, and they were thereafter taken back to their cages. All KA-injected rats included in the studies manifested the behavioural seizure signs as described above.

4.1.3 Organotypic hippocampal slice cultures (I, III)

Hippocampal slice cultures were prepared from the hippocampi of P6-7 rat pups using the modfied method of Stoppini et al. (1991). Hippocampi were dissected and placed immediately in cold Gey's balanced salt solution (Gibco, UK) supplemented with glucose (6.5 mg/ml). Slices (400 μm) were cut perpendicular to the septotemporal axis of the hippocampus using a McIlwain tissue chopper. Slices from the middle third of the hippocampus were separated, and placed on top of semipermeable membrane inserts (Millipore) (three to four slices per insert) in a six-well plate containing 1.2 ml of culture medium (50% of minimum essential medium, 25% Hanks' balanced salt solution (Gibco), 25% heat-inactivated horse serum, 25 mM HEPES, supplemented with 0.5 ml Gluta-MaxII (Gibco) and 6.5 mg/ml glucose, pH adjusted to 7.2). Slices were cultured in an incubator (37 °C, 5% CO_2) for 7 DIV with medium changes twice a week. No antibiotics were used. At 7 DIV slices were treated with KA (1, 2.5 or 5 μM) for various times by transferring the inserts into fresh culture medium containing KA. A subset of slices were left untreated with only culture medium change to serve as controls. Additionally, the COX-2 selective inhibitor NS-398 (10, 30, 60 or 90 μM), dissolved in dimethyl sulfoxide (DMSO), was added to the culture medium of a further subset of slices 1 h before adding KA. Slices were then further cultured in the presence of both compounds.

4.2 Antibodies (I-IV)

Primary and secondary antibodies were used in Western blotting and immunocytochemical experiments. The list of primary mouse monoclonal and rabbit polyclonal antibodies used is shown in Table 2. Appropriate (anti-mouse or anti-rabbit) secondary antibodies to detect primary antibodies were horseradish peroxidise (HRP)-conjugated goat IgG in Western blotting experiments, and either biotin SP-conjugated or fluorescent Alexa 488/568 goat IgG in imunocytochemical experiments.

Table 2. Primary antibodies used in Western blotting and immunostainings.

Target protein	Species	Origin (Company)
Actin	Rabbit	Sigma-Aldrich
Bax	Mouse	Sigma-Aldrich
Bcl-2	Rabbit	Santa Cruz Biotechnology
Caspase-3 (cleaved)	Rabbit	Cell Signaling Technology
COX-2	Rabbit	Cayman Chemical
GFAP	Mouse	Sigma-Aldrich
Iba1	Rabbit	Wako Chemicals
PARP	Mouse	Sigma-Aldrich
Tubulin (β)	Mouse	Sigma-Aldrich

Abbreviations; COX-2, cyclooxygenase-2; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1; PARP, poly(ADP ribose)polymerase.

4.3 Histological staining methods

4.3.1 Fluoro-Jade B (I-III)

Fluoro-Jade B (FJB) (Histo-Chem Inc., Jefferson, AR, USA) was used to detect degenerating neurons. This dye is an anionic tribasic fluorescence derivative with excitation peaks of 362 and 390 nm, and an emission peak of 550 nm, and it stains degenerating neurons and their processes regardless of the mechanism by which a nerve cell dies (Schmued and Hopkins, 2000). Tissue slices were first transferred to 0.06% potassium permanganate (KMnO4, Sigma) for 2–5 min to reduce background staining. Thereafter, slices were transferred to 0.001% FJB solution for 30 min. After staining, slices on glasses were dried, immersed in xylene, and coverslipped. The number of stained neurons was scored using the following categories: (0) no FBJ-stained neurons in the cell layer (regarded as normal); (1) some (1–4) stained neurons; (2) very sparse number of stained neurons; (3) many stained neurons; (4) a lot of stained neurons.

4.3.2 Thionin staining (III)

Thionin staining was used to detect loss of damaged neurons. Tissue slices were fixed, transferred to glasses, and briefly stained with 0.1% thionin. Thereafter they were dehydrated in alcohol series, cleared in xylene, and coverslipped. The loss of neurons (i.e. absence of staining) in the CA3a/b pyramidal cell layer was scored blinded as follows: (0) layer intact; (1) few lost neurons; (2) sparse number of lost neurons; (3) majority of neurons lost; (4) entire layer disappeared.

4.4 SDS-PAGE and Western blotting

Tissue slices were collected in ice-cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 1% sodium dodecyl sulphate (SDS), 2 mM ethylenediaminetetraacetic

acid (EDTA), 1 mM phenylmethylsulphonylfluoride, and 0.7 mM dithiothreitol, homogenized (Ultra-Turrax T25, Janke and Kunkel, Staufen, Germany), boiled, and centrifuged at +4 °C. The supernatants were frozen, and stored at –70 °C until used. Total protein concentration of the samples was determined using the Lowry-based assay (Bio-Rad, CA, USA). Equal amount of protein was applied to wells in 7.5-12% acrylamide mini gels, and separated by electrophoresis. Proteins were transferred to polyvinylidene fluoride Immobilon-P (Millipore, Bedford, MA, USA) membranes, which were thereafter incubated overnight at + 4 °C with the primary antibodies. Afterwards, membranes were washed and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. The signal was obtained using the enhanced chemiluminescence ECL system (Amersham, Buckinghamshire, UK). The film (Hyperfilm ECL, Amersham, UK) was developed, and the optical signals were quantified with Image J 1.20s (NIH, Bethesda, MD, USA). A semi-quantitative analysis was calculated as percentage of the untreated control samples, the level of which was set at 100%.

4.5 Immunocytochemistry (I-IV)

For the *in vivo* experiments, rats were deeply anesthetized with 50 mg/kg of pentobarbital, and fixed by transcardiac perfusion with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4). Brains were rapidly removed, postfixed at + 4 °C, processed with antigen retrieval protocol, cryoprotected in 30% sucrose in PBS at + 4 °C, frozen, and kept at -80 °C until used. For immunostaining, brains were cryosectioned in 40 μm slices, collected in Tris buffered-saline (TBS, pH 7.4) containing 0.1% Triton X-100, and immediately processed in a free-floating system.

For the *in vitro* experiments, inserts with cultured hippocampal slices were first rinsed with PBS (pH 7.2), fixed with 4% paraformaldehyde, and washed with PBS. Slices were then carefully mechanically detached from the inserts, collected in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Triton X-100, and immediately processed in the free-floating system. All further steps were common for brain slices and OHCs.

After incubation in a blocking solution containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in TBS (pH 7.4), slices were incubated with the primary antibody overnight at +4 °C in blocking solution. After washings, slices were incubated with the biotin SP-conjugated secondary, followed by incubation with the avidin-peroxidase conjugate (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). The staining was detected using 3,3'-diaminobenzidinetetrahydrochloride (DAB) (Sigma) as a chromogen. Three to four brain slices or OHCs, in which the primary antibody was omitted but which were otherwise treated as indicated above, served as negative controls. The immunoreactivity was examined with microscope under bright field. Alternatively, after primary antibody incubation, a fluorescent secondary antibody Alexa 477 or Alexa 568 (Invitrogen, Carlsbad, CA, USA) was used and the specimen were examined with microscope using an appropriate filter.

4.6 Quantitative real-time PCR (III, IV)

Quantitative real-time polymerase chain reaction (qPCR) was used to investigate expression levels of mRNA of several proteins after KA treatment both *in vivo* (IV) and *in vitro* (III). Hippocampi or cultured hippocampal slices were quickly frozen with liquid nitrogen, and stored at -70 ºC until used. For the experiments, thawed samples were homogenized with Ultra-Turrax, and the total RNA extraction was carried out with Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) using the protocol supplied by the manufacturer. Total RNA was reverse transcribed to complementary DNA (cDNA), performed by the M-MuLV Reverse Transcriptase RNase H+ (including RNase inhibitor) (Finnzymes, Espoo, Finland). Quantitative real-time PCR was performed using TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA, USA). Amplification was carried out with the ABI 7300 Real-Time PCR System (Applied Biosystems) with a two-step PCR protocol (preincubation of 10 min at +95 °C followed by 40 cycles at +95 °C for 15 s, and for 1 min at +60 °C). All cytokine primers and probes were designed using Primer Express software (Applied Biosystems) avoiding contaminating genomic DNA amplification by positioning one of the primers or a probe over the exon/intron boundary. The cytokine cDNA was amplified using primers and probes as described in Table 3. The mRNA quantification results were normalized using the rat ribosomal 18s RNA (rRNA) (Applied Biosystems). Before the study, the stability of rRNA expression in the samples was validated with an experiment in which different concentrations of sample mRNA were used and rRNA expression was measured. The stability of rRNA in stress conditions has also been previously shown (Derks et al., 2008). In all studies, each sample was run in triplicate, and the comparative threshold method (= ΔΔCT method) was used to examine the relative quantification of the samples (Relative Quantitation computer software, Applied Biosystems). Fold-expression changes were calculated using the equation $2^{-\Delta\Delta CT}$.

Table 3. The sequences of primers and probes used to amplify the cytokine cDNA.

Cytokine	5' primer	3' primer	Probe
GDNF	GCCACCATCAAAAGACTGAAAAG	CGGTTCCTCTCTCTTCGAGGA	TCACCAGATAAACAAGCGGCGGCA
IFN-γ	TCGAATCGCACCTGATCACTA	GGGTTGTTCACCTCGAACTTG	CATCCTTTTTTGCTTTACTGTTGCTGAGAAG
IL-1β	GAAAGACGGCACACCCACC	AAACCGCTTTTCCATCTTCTTCT	TGCAGCTGGAGAGTGTGGATCCCAAAC
IL-10	CCCTCTGGATACAGCTGCG	GCTCCACTGCCTTGCTTTTATT	CGCTGTCATCGATTTCTCCCCTGTGA
MMP-9	GTATGGTCGTGGCTCTAAACCTG	TCGGCTGTGGTTCAGCTG	CCCAAGGCCTCCAGCCACCAC
TGF-β1	TGAGTGGCTGTCTTTTGACGTC	CCTGTATTCCGTCTCCTTGGTT	CTGGAGTTGTCCGGCAGTGGCTGA
TNF-α	GACCCTCACACTCAGATCATCTTCT	ACGCTGGCTCAGCCACTC	TAGCCCACGTCGTAGCAAACCACCAA

The probes were labeled with FAM at the 5' end and with TAMRA at the 3' end. Abbreviations: A, adenine; C, cytosine; G, guanine; GDNF, glial cell line-derived neurotrophic factor; IFN-γ, interferon gamma; IL-1β, interleukin-1 beta; IL-10, interleukin-10; MMP-9, matrix metalloproteinase-9; T, thymine; TGF-β1, transforming growth factor- beta 1; TNF-α, tumor necrosis factor alpha.

4.7 ELISA (III, IV)

Two kinds of enzyme-linked immunosorbent assay (ELISA) kits were used. One to measure PGE_2 release into the culture medium from OHCs (III) and another to measure *in vivo* IL-1β protein expression in the hippocampus after SE (IV).

In the *in vitro* study, PGE_2 amount in the culture medium of OHCs was measured 4h after KA (5 μM) treatment alone, and after the pre-treatment of slices with the COX-2 inhibitor (90 μM) for 1 h prior to addition of KA (5 μM). Medium was collected and stored at –70 °C until used. The concentration of PGE_2 released into the culture medium of 8 slices per group was measured with an ELISA kit (Cayman Chemical). The protocol supplied by the manufacturer was followed.

In the *in vivo* study, three control rats and four KA-injected rats (8 h after injection) were used. Rats were decapitated, hippocampi quickly isolated, placed in ice-cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 1% SDS, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride, and 0.7 mM dithiothreitol, homogenized with Ultra-Turrax, boiled, and centrifuged at 4 °C. The supernatants were frozen, and stored at –70 °C until used. Total protein concentration of the samples was measured using the Lowry-based assay (Bio-Rad, CA, USA). A commercial ELISA kit (Raybiotech, Norcross, GA, USA) was used and the protocol supplied by the manufacturer was followed. Results are given as pg of IL-1β per milligram of total protein.

4.8 Microscopy techniques

4.8.1 Light and fluorescent microscopy (I-IV)

Immunostained sections, in which DAB was used as chromogen (II-IV), and thionin stained sections (III) were examined with a Leica DMR microscope (Heerbrugg, Germany) under bright field. Immunostained sections, in which a fluorescent secondary antibody was used (I-IV), and FJB-stained sections (I-III) were examined with the Leica DMR microscope using the appropriate filter. Images were captured using an Olympus U-TV1 X digital camera (Olympus Optical, Tokyo, Japan), and further processed with Adobe Photoshop Elements (version 3.0) and Corel Draw (version 11.0).

4.8.2 Confocal microscopy (I, III)

Some FJB-stained sections (I) and immunostained sections, in which a fluorescent secondary antibody was used (III), were also examined with a Leica TCS SP confocal microscope. The excitation wavelength for FJB and Alexa 488 was 488 nm and emission detection window was 500-550 nm. The excitation wavelength for Alexa 568 was 568 nm and emission detection window was 600-700 nm.

4.8.3 Electron microscopy (I)

Slices were fixed with freshly prepared 5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA), the CA3a/b region was isolated by knife cuts,

and the specimen was further prepared according to standard procedures. For the light microscopy, orientation sections from the CA3a/b region were cut at 1 μm thickness and stained with toluidine blue. The ultrathin sections (70 nm) were stained with 5% uranyl acetate and 5% lead citrate in Ultrostainer (Leica, Wien, Austria), and examined in a JEM–1200EX (JEOL, Tokyo, Japan) transmission electron microscope.

4.9 Statistical analysis (I-IV)

All statistical analyses were performed using the GraphPad Prism software program (versions 3.0 and 4.0, GraphPad Software, San Diego, CA, USA). The level of significance was set at $p<0.05$. The following statistical analyses were used in the original articles.

I. The overall group differences in the score numbers after the FJB staining within the acute and recovery groups were assessed with two-way analysis of variance (ANOVA), and the statistical significance of differences between the experimental groups with one-way ANOVA with Tukey–Kramer Multiple Comparison Test as a *post hoc* test. The nonparametric one-way ANOVA with Kruskal–Wallis Test was used to analyze the group differences (acute and recovery groups) in the Western blots.

II. The statistical significance of the overall changes in the signal intensity in Western blots of the control and KA-treated rats was measured with the Kruskal-Wallis test using Dunn's Multiple Comparison Test as a *post hoc* test.

III. The statistical significance of the differences between control and treatment groups was analyzed with one-way ANOVA using Tukey's test as *post hoc* test, except for Fluoro-Jade B and thionin scoring, and microglial cell counting, in which Student's unpaired *t*-test was used.

IV. Statistical significance of differences between the groups was analyzed with one-way ANOVA with Tukey's test as a *post hoc* test in experiments in which there were three or more groups. Differences between the two age groups at the studied time-points were analyzed with two-way ANOVA. Statistical significance of differences in experiments having two groups was analyzed with Student's unpaired t-test.

5 RESULTS

5.1 Time-course and localization of kainic acid-induced nerve cell damage (I-III)

5.1.1 *In vivo* (II)

In order to investigate the localization and extent of neuronal damage after KA-induced SE, control and treated P9 and P21 rat hippocampal slices were stained with FJB 24 h after SE. KA-induced SE caused a region specific neuronal damage, detectable as positively stained neurons, in the CA1 region of P21 rats 24 h after SE. Additionally, some positive neurons were observed in the CA3 region. No control rats of either age or P9 KA-treated rats exhibited FJB-positive staining in the hippocampus.

5.1.2 *In vitro* (I, III)

In order to reveal the time-course and localization of KA-induced neuronal damage *in vitro*, OHCs were treated with 5 μM KA for various times: 6 h, 12 h, 24 h, and 48 h, and additionally in each time-group a subset of slices was further cultured in normal medium after KA treatment for an additional 48 h recovery period (I). KA treatment caused a region specific and dose-dependent neuronal damage. First FJB-positive neurons were found in the CA3a/b region after 12 h of KA treatment. Positively stained neurons were also observed in CA3c and DG regions after 48 h of treatment. Positive FJB-staining was detected neither in control slices nor in slices treated for 6 h with KA. Significant increases in the amount of FJB-positive neurons, as measured by scoring, were found after the 48 h recovery period compared to the respective acute 6, 12 and 24 h groups in the CA3a/b region, and the 12 and 24 h groups in the CA3c region. In the DG, the difference between acute and recovery groups was not significant.

In order to establish dose-dependency for neuronal damage caused by KA treatment, OHCs were treated with 1, 2.5 and 5 μM KA for 24 h in another experiment (III). Of the slices treated with 1 μM KA, 17.7% (3/17) of slices exhibited FJB-positive neurons, while positive staining was found in 57.1% (16/28), and 88.9% (16/18) of slices treated with 2.5, and 5 μM KA, respectively. The severity of damage was in general mild with 1 μM KA, mild to moderate with 2.5 μM KA, and moderate to severe with 5 μM KA. In all KA concentrations used, neuronal damage was almost exclusively located in the CA3a/b region with occasional staining also in the CA3c region after the 5 μM KA treatment. Disappearance of neurons in the CA3a/b region was also observed when OHCs treated for 24 h KA followed by a 48 h recovery period were stained with thionin.

5.2 Expression of apoptosis markers in excitotoxic nerve cell damage (I, II)

The expression of proteins involved in apoptosis was studied by Western blotting and immunocytochemistry *in vitro* in KA-treated OHCs (I) and *in vivo* in the hippocampus of P21 rats after KA-induced SE (II). Studied proteins included active caspase-3 (I, II), uncleaved and cleaved PARP (I), bax (II) and bcl-2 (II). In OHCs, the expression of neither caspase-3 nor PARP (uncleaved or cleaved) changed significantly after 4, 8, or 24 h (5 μM) KA treatment compared to the control group. *In vivo*, in the P21 rat hippocampus there were no significant differences in the expression of bax or active caspase-3 24 h, 3 d or 7 d after KA injection compared to the control level. Bcl-2 expression significantly decreased 24 h after KA injection, but returned to the control level 3 d after SE, and remained so 7 d after SE. Single caspase-3 positive cells were observed similarly in the CA1 region of both the control rats and KA-treated rats, while the cellular structure of stained cells seemed irregular in the KA-treated group in comparison to the normal structure of the stained cells in the control group.

5.3 Activation of inflammatory processes after kainic acid treatment

5.3.1 COX-2 expression and localization and PGE_2 production (II, III)

COX-2 expression was studied *in vivo* (II) after KA-induced SE in P9 and P21 rat brains and *in vitro* in OHCs (III). The expression of PGE2, produced from arachidonic acid by COX-2, was studied with EIA from the culture medium of OHCs 4 h after the initiation of KA treatment (III).

II. The expression of COX-2 increased almost five-fold 24 h after KA injection in P21 rats, and returned to the control level within 3 days after the injection where it remained also 7 d after SE. In P9 rats, however, COX-2 expression remained unchanged compared to controls at all time-points studied (6 h, 24 h, 7 days). Immunoreactivity of COX-2 was pronouncedly increased compared to controls in the hippocampi of P21 rats 24 h after SE in the CA3-CA1 pyramidal cell regions, dentate granule cells, and molecular layers of the DG, and to some extent also in the CA3 *stratum radiatum* and *stratum oriens*. In P9 rats, no changes in COX-2 immunoreactivity were detected 24 h after the KA injection in any hippocampal region compared to their age-matched controls. In addition to the hippocampus, COX-2 immunoreactivity was also pronouncedly enhanced in the lateral and basolateral nuclei of the amygdala, and piriform cortex of P21 rats 24 h after SE, but not in the age-matched control rats, in which COX-2 immunoreactivity remained unchanged in these brain areas. In contrast, in P9 rats, the COX-2 immunoreactivity was not changed 24 h after SE in any brain region compared to the control rat.

III. In OHCs, KA treatment (5 μM, 24 h) resulted in pronouncedly enhanced COX-2 immunoreactivity, in comparison to control slices, especially in the CA3c

subregion, which contained numerous heavily COX-2 immunoreactive neurons. Also the CA3a/b and CA1 subregions show a few COX-2 immunoreactive cells. The concentration of PGE2 in the culture medium of KA-treated slices was significantly increased 4 h after the initiation of KA treatment compared to the culture medium of untreated, control slices.

5.3.2 Cytokine expression (III, IV)

Expression of various cytokine mRNAs were investigated after KA treatment in the hippocampus both *in vitro* in OHCs after 1 h, 6 h and 24 h KA (5 μM) treatment (IL-1β, TNF-α and IL-10) (III), and *in vivo* in P9 and P21 rat hippocampus 4, 8 and 24 h after KA-injection (IL-1β, TNF-α,

IL-10, MMP-9, GDNF, IFN-γ, and TGF-β1) (IV). In addition, in P21 rats, mRNA expression of the cytokines in the hippocampus was measured 3 d after SE and IL-1β protein expression with ELISA 8 h after KA-injection (IV).

III. The expression of neither the proinflammatory cytokines TNF-α, IL-1β, nor the anti-inflammatory cytokine IL-10 mRNA in OHCs was significantly increased from the control level at any studied time-point. It should be noted, that despite efforts to minimize variation in culture and treatment processes, the variation of cytokine mRNA expression was high between different samples of the same treatment group. Interestingly, IL-1β mRNA expression decreased over time, the difference compared to control level being significant 24 h after KA treatment.

IV. Expression of mRNA of the classic pro-inflammatory cytokines IL-1β and TNF-α increased rapidly and pronouncedly in the P21 rat hippocampus after SE. Both cytokine mRNAs were significantly increased already at the earliest studied time-point (4 h after KA-injection). TNF-α mRNA expression decreased thereafter, being still significantly enhanced at 8 h, but returning to the control level by 24 h. Meanwhile, IL-1β mRNA expression continued to rise, peaking at 8 h, when it was almost 25-fold higher compared to the controls, and at 24 h its expression still remained significantly higher than in the control group. To confirm the enhanced expression after SE at the protein level, the IL-1β protein expression in the hippocampus of P21 rats was studied with ELISA 8 h after KA injection. The concentration of IL-1β protein was 95.3 ± 7.8 pg/mg (mean ± SEM) in the control group, and it significantly ($p<0.05$, unpaired t-test) increased to 124.3 ± 10.1 pg/mg (mean ± SEM) in the KA-treated group (8 h after injection). In contrast, in P9 rats, IL-1β mRNA expression did not significantly change from the control group 4 h or 8 h after KA injection, but at 24 h its expression was slightly, but significantly enhanced. Likewise, TNF-α mRNA expression in the P9 rat hippocampus after KA-induced SE increased more gradually and moderately than in P21 rats, enhancement being significant first 8 h after KA injection, and remained elevated up to 24 h. In P21 rats, expression of the anti-inflammatory IL-10 mRNA was significantly increased at 4 h but thereafter it sharply decreased,

returning to the control level already at 8h. In P9 rats, however, the increase in IL-10 mRNA expression was again more gradual, being significant first 8 h after SE, and thereafter returned to the control level by 24 h.

The two age groups had more similarities in the MMP-9 mRNA expression profile. In both age groups, the expression of MMP-9 mRNA was significantly enhanced 8 h after KA. However, in P21 rats the expression decreased to control level at 24 h, whereas in P9 rats at the same time-point it remained significantly enhanced. In P21 rats, GDNF expression was significantly enhanced at 4 h after KA injection, and it remained elevated at 8 h and 24 h, whereas in the P9 rat hippocampus its expression remained unaltered after SE at all time-points studied. In both age groups, the expression of neither IFN-γ nor TGF-β1 was significantly altered compared to their respective age-matched controls at any time-point studied.

To study whether the SE-induced increase in expression of the studied cytokines was prolonged beyond the acute phase, their mRNA expression was measured also 3 d after SE in P21 rats and in their age-matched control group. However, the expression of all the cytokines did not significantly differ from those of the controls 3 d after SE.

5.3.3 Glial cell activation (III, IV)

The activation of glial cells, i.e. microglia and astrocytes, was studied in OHCs after KA treatment (III) and in the postnatal rat hippocampus after SE (IV). In order to visualize glial cells, immunocytochemistry was used to detect the microglial antigen Iba1, and the astrocytic antigen GFAP. In OHCs, microglial and astrocytic activation was observed 24 h after KA treatment (5 μM). Iba1 immunoreactivity revealed numerous heavily Iba1-stained cells scattered throughout the OHC, and these microglial cells had an activated appearance showing an enlarged cell body and thicker processes in increased numbers. Also enhancement of GFAP immunoreactivity was observed in groups of astrocytes, together with a pronounced change in morphology. These cells exhibited thickened, heavily GFAP-immunopositive processes after KA treatment. It is of interest to note that astrocytes exhibiting active appearance tended to be grouped together and that these groups were spread throughout the pyramidal cell layers. In the hippocampus of P21 rats 3 d after SE, the morphology of microglia and astrocytes changed from the resting appereance of the control hippocampi to the active appearance. The active appereance was similar to that seen in KA-treated OHCs. It is of importance to note that while activated astroctyes were spread throughout the hippocampus, Iba1 staining was heaviest in the microglia in the CA1 region. In contrast, in P9 rats, both microglia and astrocytes retained the resting appereance 3 d after SE. A summary of the results concerning the activation of inflammatory processes in the P9 and P21 rat hippocampus after KA-induced SE is presented in Table 4.

Table 4. Comparison of activation of inflammatory processes after KA-induced seizures in the hippocampus between P9 and P21 rats.

	P9	P21
COX-2 expression	Unaltered	Pronouncedly, but transiently increased
Glial cells (microglia and astrocytes)	Retain inactive morphological appereance	Morphology changes to active appearance
Cytokine mRNA expression	Mild, delayed increase	Fast, pronounced, but transient increase

Abbreviations: COX-2, cyclooxygenase-2; mRNA, messenger RNA.

5.3.4 Effect of a COX-2 selective inhibitor on excitotoxic nerve cell damage *in vitro* (III)

In order to investigate the hypothesized neuroprotective effects of COX-2 inhibition, slices were treated with the COX-2 selective inhibitor NS-398 before adding KA. NS-398 (90 μM) treatment was successful in inhibting the KA-induced increase in PGE2 production, attenuating PGE_2 release to the control level. However, neuronal death was not ameliorated as evidenced by FJB and thionin stainings. In fact, KA-induced neuronal loss in the CA3a/b region was exacerbated after NS-398 pre- and co-treatment. Slices were also treated with NS-398 alone to rule out neurotoxicity by NS-398 or its dissolvent DMSO. No discernible differences in FJB and thionin stainings were found between control and NS-398 (90 μM)-treated OHCs.

6 DISCUSSION

6.1 Methodological considerations

6.1.1 Animal models of human epilepsy

The objective in inducing SE in animals is to mimic human TLE as closely as possible. The difference is that in experimental animals SE has to be provoked with extrinsic and artificial methods, whereas SE in humans is an intrinsic and spontaneous pathological process. Still, it is possible to gain valuable information from experimental animal epilepsy models, and from this conclusions about human epilepsy can be drawn, albeit with some careful consideration.

The KA model of SE has been used for over three decades (Nadler, 1981; Ben-Ari, 1985). The shared features between human TLE and the animal KA model are that in both seizures originate in and remain confined to the limbic system, and that similar neuronal loss and sclerosis occur in the vulnerable areas in the limbic system, for example in hippocampal pyramidal cell regions (Nadler, 1981; Ben-Ari 1985; Vincent and Mulle, 2009). The hallmark of epilepsy is the spontaneous recurrence of seizures. In the adult rat, KA-induced SE is followed by a latent phase of 4-8 seizure-free weeks after which the first spontaneous seizures occur, but epileptogenesis continues beyond this, and frequency of seizures increases in a sigmoid fashion (Williams et al., 2009).

The plastic developing brain is a considerably different environment compared to the mature adult brain. When using immature animals instead of adults, it is necessary to be mindful of certain factors which might affect the results. For example, stress caused by maternal deprivation leads to more prominent seizure-induced neuronal degeneration and worsening of cognitive impairments in immature rats (Huang et al., 2002). Therefore, it is important that the control and the treated immature animals are separated from their mothers for an equal amount of time, as has been done in our experiments. When comparing pathology resulting from KA-induced SE between adult and immature rat brain it is evident that neuronal degeneration is more severe in the adult brain than in the immature brain (Haas et al., 2001). However, sensitivity to convulsants influencing amino acid receptors is argued to decrease with age (Mares et al., 2004). Lower doses of KA are used in developing rats than in adult rats to maintain low mortality. To manage the mortality issue in immature rats, one option is to use as low a KA dose as possible. However, the number of animals that cannot be used due to failure to develop SE may increase. Another option is to use a higher dose of chemoconvulsant and stop SE chemically, e.g. with paraldehyde treatment (Kubova et al., 2001). After suppression of SE with paraldehyde, some seizures still occur and continuation of ictal activity has been confirmed with EEG recordings (Kubova et al., 2004; Druga et al., 2005). However, paraldehyde treatment dose-dependently modulates the long-term outcomes of cognitive functions after SE (Kubova et al., 2005). Our studies concentrated in the acute phase

after SE (up to 7 days) and therefore avoided this. We explored both of these options and experienced the advantages and disadvantages of both approaches as mentioned above.

6.1.2 Organotypic hippocampal slice cultures as *in vitro* model of epileptic activity

Organotypic hippocampal slice cultures prepared from P6-7 rats correspond to the human perinatal hippocampal tissue. OHCs retain morphological organization and the majority of intrinsic neuronal connections intact (Caeser and Aertsen, 1991; Frotscher et al., 1995). Additionally, during the first weeks of culturing, the cultures continue to develop parallel to the *in vivo* hippocampal tissue, including proliferation of granule cells, outgrowth and organization of axons and dendrites, and the maturation of receptors and synapses (Caeser and Aertsen, 1991; Frotscher et al., 1995; Holopainen and Lauren, 2003; Holopainen, 2005). In contrast to cell cultures, all neuron types and different glia of the *in vivo* hippocampus are found in OHCs. However, external connections are lost and the severing of the input to the DG causes mossy fiber sprouting in the molecular layer and enhanced excitability of the dentate granule cells (Caeser and Aertsen, 1991; Frotscher et al.,1995; Bausch and McNamara, 2000; Lindroos et al., 2005). Also systemic factors such as the infiltrating components of the immune system become absent.

OHCs also present with similar features to their *in vivo* counterparts after KA treatment. KA treatment of cultures induces epileptiform activity verified by electrophysiological recordings, mossy fiber sprouting and cell death as comparable to that *in vivo* (Bruce et al., 1995; Routbort et al., 1999; Lindroos et al., 2005). However, part of the toxic effect is conveyed by direct local application of KA, which causes stronger KAR activation and activation of also AMPARs (Kristensen et al., 2001). While animal data might correspond better to human TLE, the advantages of OHCs are the better control over experimental conditions and the ease of carrying out pharmacological experiments.

6.2 Death mechanism in excitotoxic neurodegeneration in the developing hippocampus

KA (5 μM) treatment of OHCs caused irreversible neuronal damage and the CA3 was the most severely damaged hippocampal region (I, III). The higher density of KA receptors in the CA3 region compared to CA1 and DG regions in young rats might make the CA3 region more vulnerable to KA-induced damage, especially as part of the toxic effect is caused by direct exposure to KA (Bahn et al., 1994; Kristensen et al., 2001; Ritter et al., 2002). The initial KA exposure seems to induce a lasting epileptiform electrical acitivity which maintains excitotoxicity as neuronal degeneration is not reversed during recovery but even exacerbates, as is seen in our study and elsewhere (Bruce et al., 1995; Lahtinen et al., 2001). Degenerating neurons were detected 12 h, but not 6 h after application of KA treatment, which suggests a time window of a few hours after insult during which it is possible to apply treatment to save neurons from degeneration. *In vivo*, KA-induced SE caused neuronal damage in the CA1 region of P21 rats. It remains unknown why neuronal

degeneration was not detected in the CA3 region as has occurred in other studies in P21 rats, where degenerating neurons were found in both CA3 and CA1 regions after i.p. KA injection (Rizzi et al., 2003; Ravizza et al., 2005). Usually, an i.p. KA injection leads to neuronal damage in the CA1 region while an intracerebroventricular or intramygdaloid injection of KA damages more prominently the CA3 region in the adult hippocampus (Nadler et al., 1978; Sperk, 1994). CA3 neurons are susceptible to locally applied KA, possibly because the density of KA1 subunit containing KARs is highest in CA3 in the postnatal rat hippocampus (Bahn et al., 1994; Ritter et al., 2002). On the other hand, CA1 neurons might be prone to systemic excitotoxic injury because of regional differences in astrocyte function impairment during excitotoxicity in the hippocampus. It has been suggested that astrocytes in the CA1 region are more sensitive than in the DG. This manifested after ischemia, a different model of excitotoxicity, as a more rapid decrease in GLT-1 expression and glutamate transport activity in CA1 compared to DG, exposing CA1 neurons to enhanced excitotoxicity (Ouyang et al., 2007).

The mechanism of excitotoxic nerve cell death in the adult brain has been a subject of much debate and it seems the outcome varies greatly depending on the type, strength, and length of the insult. In the developing brain, this phenomena has remained incompletely known, but it would seem that the acute neuronal loss is mostly necrotic in the first days after the excitotoxic insult, while apoptotic mechanisms take over later, possibly as a response to the loss of neuronal connections (Sankar et al., 1998; Humphrey et al., 2002; Wasterlain et al., 2002, Young et al., 2004). This is confirmed by our results both *in vitro* and *in vivo*, as expression of all studied proteins involved in apoptosis, except that of bcl-2, remained unaltered acutely after KA excitotoxicity (up to 7 days post-treatment) and electron microscopy revealed morphological features of necrosis in the degenerating neurons in OHCs 24 h after starting KA treatment. The transient decrease in bcl-2 expression at 24 h after KA-induced SE in P21 rats suggests a minor reaction in the apoptotic machinery at the mitochondrial level, which, however, did not affect the end result. It is evident that the effects of the initial insult are long-lasting as remodelling of the developing injured hippocampus in the form of delayed apoptosis is detectable after over 2 months from the initial insult (Humphrey et al., 2002).

Constitutive expression of proteins that indicate ongoing apoptosis (i.e. active form of caspase-3 and cleaved PARP) was detected in control hippocampi both *in vitro* and *in vivo*, which confirms that apoptosis is a physiological process in the developing hippocampus, and suggests that it has a role in regulating the size of the brain cell population during development at these ages (for references, see Kuan et al., 2000).

6.3 Inflammatory processes in excitotoxic nerve cell damage and epileptogenesis

A wide array of inflammatory mediators was activated by KA-induced excitoxicity both *in vitro* and *in vivo*. COX-2 expression and PGE_2 production increased, glia underwent morphological changes from resting to active appearance, and the expression of many cytokines augmented. These reactions have been detected also in other studies in the

developing hippocampus after KA treatment (Tocco et al., 1997; Rizzi et al., 2003; Yoshikawa et al., 2006). The individual contribution of the activation of these different processes to neuronal degeneration is somewhat an open question. Overall, inflammation provoked by lipopolysaccharide seems to exacerbate seizure-induced injury in the developing hippocampus (Auvin et al., 2007). COX-2 induction and the following increase in PGE_2 production are generally viewed as harmful events. However, inhibition of COX-2 has in some cases exacerbated neuronal injury (Baik et al., 1999; Kunz and Oliw, 2001a) and PGE_2 can act on the prostaglandin E receptor 1 (EP1) or 2 (EP2), of which EP1 ligation promotes neuronal death, whereas EP2 affords neuroprotection in glutamate receptor-mediated excitotoxicity (McCullough et al., 2004).

The activation of glia can also lead to both harmful and protective effects. The possibility of this dual nature of their response is due to the great variety in their receptors, useful for surveying the surrounding micro-environment, and the diversity of signal molecules they themselves emit, e.g. both pro-and anti-inflammatory cytokines (Giaume et al., 2007; Hanisch and Kettenmann, 2007). It might be that the degree of neuronal damage determines the outcome, i.e. if the damage is moderate, glia support the recovery of neurons, but if the damage is excessive, glia work to destroy the neurons that are beyond rescue to prevent them from causing harm to other neurons, e.g. electrical dysfunction (Hanisch and Kettenmann, 2007). Preliminary data suggests that microglial inhibition by minocycline protects neurons in the hippocampus from damage caused by KA-induced seizures (Heo et al., 2006). Both pro-inflammatory and anti-inflammatory cytokines are induced by seizures as are growth factors (Jankowsky and Patterson, 2001). The fact that even the classical pro-inflammatory cytokines (e.g. IL-1β and TNF-α) can have trophic effects makes the verdict on the benignity of cytokine induction complicated (Allan et al., 2005; Vezzani et al., 2008). The volume of cytokine expression or the balance in expression of pro-inflammatory cytokines vs. anti-inflammatory cytokines and growth factors could be important for the end result, i.e. excessive cytokine expression or favoring pro-inflammatory cytokine expression could lead to neurotoxicity. For example in our study (IV) in P21 rats that did present with neuronal degeneration after SE, pro-inflammatory cytokine expression (IL-1β and TNF-α) outweighed the expression of the anti-inflammatory Il-10 and the growth factor GDNF. Conversly, in the P9 rats, in which neurodegeneration was not detected, IL-10 expression was more pronounced and pro-inflammatory cytokine expression was mild. Our data show that COX-2 and cytokine induction is transient after SE in the P21 hippocampus, quieting down by the third day post-SE, which is corroborated by other studies (De Simoni et al., 2000; Hewett et al., 2006; Jung et al., 2006). In contrast, hippocampal glial reaction after SE persists (Hüttmann et al., 2003; Shapiro et al., 2008). According to our study (IV), astroglial reaction remains universal in the hippocampus, whereas microglial response is targeted to the damaged CA1 region 3 d after SE. This suggests that the initial fulminant and general inflammatory response develops into a more moderate microglial response targeted to the damaged area. In another study, long-term glial reactivity after KA-induced seizures has been associated with neuronal injury and neurobehavioral impairment (Somera-Molina et al., 2007).

Taken together, our results together with the findings of others suggest that the initial excessive COX-2 and cytokine reaction is harmful, the targeted microglial response at injury-site denotes reaction to injury in the subacute phase, but at least long-term glial reactivity is deleterious.

Another crucial aspect to consider about inflammatory processes in epilepsy is their effect on seizure threshold and, more generally speaking, their effect on epileptogenesis and epilepsy itself. Sustained glial activation in the developing brain has been suggested to increase susceptibility to seizures in adulthood (Somera-Molina et al., 2007). Furthermore, aberrant neurogenesis caused by seizure-induced inflammation has been reversed by microglial inhibition with minocycline (Yang et al., 2010). Regarding cytokines and epileptogenesis, chronic IL-1β expression has been associated with the development of spontaneous limbic seizures after prolonged febrile seizures in P11 rats (Dube et al., 2010). Conversly, the naturally occurring IL-1Ra has mediated anticonvulsant effects in a variety of limbic seizure models, including intrahippocampal KA and bicuculline injection, and electrical kindling (Vezzani et al., 2002). Additionally, transgenic mice overexpressing IL-6 in astrocytes showed increased sensitivity to seizures induced by glutamatergic agonists (Samland et al., 2003). Compared to wild-type animals, transgenic rats overexpressing MMP-9 had higher sensitivity to pentylenetetrazole kindling-induced seizures, whereas in MMP-9 knockout mice the sensitivity was lower (Wilczynski et al., 2008). These findings suggest that long-term glial activity through sustained pro-inflammatory cytokine induction may lead to aberrant neurogenesis in the developing brain forming excitatory circuits and thus contributes to increased susceptibility to seizures in maturity.

6.4 Developmental differences in nerve cell damage and activation of inflammatory processes induced by kainic acid

Previous studies have suggested that the immature brain is more resistant to seizure-induced damage than the more mature juvenile brain. It seems that the age of around two weeks (P14) is the turning point, since many studies in postnatal rodents have reported neuronal degeneration after seizures in the limbic structures after, but minor or no damage before this age (Nitecka et al., 1984; Cavalheiro et al., 1987; Sperber et al., 1991; Sankar et al., 1998 ; Haas et al., 2001; Cilio et al., 2003; Druga et al., 2005). However, more recently, it has been argued that the extent of damage caused by seizures in the developing brain is primarily model-dependent, depending on e.g. convulsant used, and type and severity of seizures elicited (Wasterlain, 2006). When SE is induced by i.p. KA injection, the outcome does seem to be quite age-specific. The immature brain resists neuronal damage from KA-induced SE well, but the more mature juvenile brain is more prone to neuronal degeneration. In our study (II), KA-induced SE damaged the hippocampus of the juvenile rat (P21) but not so in the immature rat (P9), a result which is in line with other studies (Rizzi et al., 2003; Ravizza et al., 2005). The maturational stage of our OHCs, which were taken from P6-7 rats and cultured for 7 days can be considered to lie somewhere between the immature and juvenile hippocampus, and were

damaged by KA. However, topical KA treatment differs from systemic administration, and KA-induced damaged has been detected when immature P7 rats were given an intracerebral injection (Humphrey et al., 2002; Dong et al., 2003). This is possibly due to direct toxicity of KA and the fact that topical KA activates AMPA-receptors as well (Kristensen et al., 2001).

In the developing hippocampus, activation of inflammatory processes by KA-induced seizures is highly age-dependent. COX-2 mRNA upregulation in the hippocampus after KA-induced seizures has been detected in P14 and P21 but not in P7 rats (Tocco et al., 1997). This age-specificity was confirmed at the protein level in our study (II), as COX-2 protein was induced in P21 rats but not in P9 rats. COX-2 was also induced in the OHCs by KA treatment together with increased PGE_2 release into culture medium, which suggests sufficient maturity of P6-7 + 7 DIV OHCs to elicit this response. Activation of microglia and astrocytes has also been detected in P15 and P21 rats, but excluding P9 rats, at 4 and 24 h after KA-induced SE (Rizzi et al., 2003). We detected similar age-dependent glial activation (in P21 but not in P9) also 3 d after KA-induced SE, with the difference that microglial activation was most prominent in the damaged CA1 region. This suggests a sustained glial reaction and a targeted microglial response in the juvenile brain, and lack of delayed glial activation in the immature brain after KA-induced SE. Both types of glia were also activated in the OHCs 24 h after KA, and furthermore, heavily GFAP-immunopositive astrocytes with active morphology tended to be grouped together (III). This is supported by the finding that astroglia form networks in pathological situations and these networks can supply energy for high neuronal demand, or, on the other hand, amplify neuronal injury by allowing intercellular diffusion of death signals (Giaume et al., 2010). This has been shown at a molecular level in the developing hippocampus (OHCs, 14 DIV), when bicuculline treatment enhanced parallel gap junction expression and function, and epileptiform discharges (Samoilova et al., 2003). Expression of IL-1β, TNF-α and IL-6 has been studied in P9, P15, and P21 rats and all of these cytokines were induced 4 h after SE in P21 rats, but only IL-1β in P15 rats, and none in P9 rats (Rizzi et al., 2003). Similar tendency has been discovered in hypoxia, a different setting of excitotoxicity, where MCP-1 and IL-18 were more pronouncedly increased in hippocampal endothelial cells and perivascular areas of P21 than of P9 mice (Qiu et al., 2007). In our *in vitro* work (III), IL-1β, TNF-α or IL-10 mRNA expression was not induced, which may be due to technical aspects (high variance between OHCs) or the immaturity of OHCs in this respect. In our *in vivo* work (IV), pro-inflammatory (IL-1β and TNF-α) cytokine mRNA expression was robustly induced as well as that of IL-10, GDNF and MMP-9 in the P21 rat hippocampus. Compared to the juvenile rats, in P9 animals, the pro-inflammatory cytokine mRNA expression was increased, but only mildly and more gradually, while IL-10 and MMP-9 mRNA expression were induced similarly as in P21 rats. In summary, it would seem that KA-induced seizures activate inflammatory processes to a greater degree in the more mature juvenile brain than in the immature brain. Furthermore, the pro-inflammatory reaction is favored over anti-inflammatory elements after KA-induced SE in the juvenile brain and vice versa in the immature brain.

The reason for the faster and stronger inflammatory response after SE in the more mature developing brain compared to the immature brain can only be speculated. It seems that the machinery of the innate immunity system in the brain matures during development and a certain developmental stage is required to mount an effective inflammatory reaction in response to environmental disturbances. This can be beneficial in some cases, but harmful in others, as seems to be the case in acute seizures. The immature brain seems to respond to SE with a plastic reaction instead of an inflammatory one, which, however, may lead to formation of functionally aberrant neuronal circuits. The long-term outcome of both of these age-dependent responses seems to be similar: disturbed brain maturation and neuronal connectivity, and an enhanced susceptibility to seizures. An illustration of the suggested age-dependent consequences of SE is shown in Figure 3.

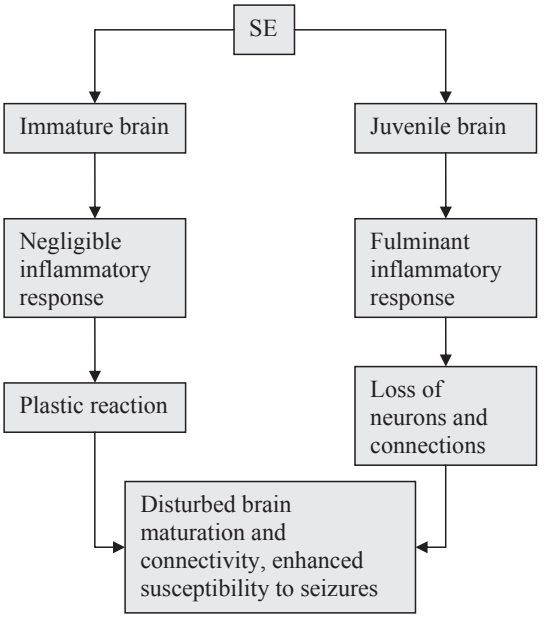

Figure 3. Suggested age-dependent consequences of SE.

6.5 Potential of anti-inflammatory drugs as neuroprotectants in epilepsy

Non-steroidal anti-inflammatory drugs, i.e. COX-inhibitors, present a straightforward treatment option as they are already in wide use. As it is, their potential to procure neuroprotection in epilepsy has been tested, but the outcomes have been variable. In *in vitro* models, for example in primary cortical cultures, COX-2 selective inhibitors flubriprofen and NS-398 afforded protection from NMDA- but not from KA-induced neurotoxicity (Hewett et al., 2000). In another cell culture study, NS-398 prevented KA-induced death in hippocampal neurons but not in cortical neurons (Kim et al., 2001). *In vivo* studies with celecoxib and rofecoxib have reported neuroprotection from seizure-

induced damage (Kunz and Oliw, 2001b; Kawaguchi et al., 2005; Hewett et al., 2006; Jung et al., 2006). However, in other studies, COX-2 selective inhibitors have, in contrast, exacerbated KA-induced neurotoxicity (Baik et al., 1999; Kunz and Oliw 2001a). The question of the importance of the timing of the COX-2 inhibition treatment in respect to the excitotoxic insult has been raised, as pre-treatment has been shown to be insufficient to block the second phase of PGE_2 increase and only post-treatment is suggested to afford neuroprotection (Gobbo and O'Mara, 2004; Takemiya et al., 2006). Furthermore, seizure severity and neuronal damage induced by KA have been enhanced in COX-2 deficient and wild-type mice chronically pre-treated with celecoxib (Toscano et al., 2008). Our *in vitro* work (III), where COX-2 pre- and co-treatment in respect to KA did not ameliorate neurodegeneration and even slightly enhanced neuronal loss, is in line with these observations. Taken together this suggests that the acute PGE_2 spike after KA is a beneficial and required response for neuronal survival, while sustained increase in the late-phase PGE_2 production is harmful and should be countered with COX inhibitor treatment in order to achieve neuroprotection from seizures.

Other candidates for anti-inflammatory neuroprotective treatment include IL-1 receptor antagonists, immunosuppressants, and microglia inhibitors. The microglia-inhibiting tetracycline antibiotic minocycline, has inhibited caspase-dependent and -independent cell death pathways and protected neurons from KA-induced hippocampal damage in mice (Heo et al., 2006). *In vitro,* post-treatment of OHCs with the IL-1 receptor antagonist inhibited microglial activation and afforded neuroprotection in NMDA (50 μM for 4 h)-mediated neurotoxicity (Vogt et al., 2008). The 5-lipoxygenase (5LOX) enzyme, which catalyzes the production of both leukotriens and anti-inflammatory lipoxins, has been shown to be induced within 3 h after KA injection (Manev et al., 2000). Treatment of relatively mature OHCs (21-25 DIV) with a low dose of immunosuppressant tacrolimus (0.1 μM) after KA exposure (5 μM, 18 h) has attenuated the expression of 5LOX and caspase-3 proteins leading to neuroprotection (Lee et al., 2010). These tentative findings further highlight the advantages of post-treatment over pre-treatment and present multiple options to be explored further in order to achieve protection from neurodegeneration in epilepsy via anti-inflammatory treatment. Future studies with COX-inhibitors are needed and should aim at screening more compounds, establishing the optimal timing of treatment, and performing *in vivo* studies in developing animals.

7 CONCLUSIONS

Inducing neuronal degeneration in the developing rat hippocampus with KA, as was done in our work, is a viable model to gather comparable information about molecular mechanisms of and contributing factors to neuronal death in the developing human brain caused by SE and other excitotoxic brain pathologies.

Our results show that KA induced region-specific and age-dependent neuronal degeneration in the developing hippocampus. In OHCs, irreversible neuronal damage was detectable by FJB first at 12 h after commencement of KA treatment. The most vulnerable areas of the hippocampus, according to our *in vitro* and *in vivo* results, were the pyramidal cell layers CA3 (in OHCs) and CA1 (in P21 rats). There was a clear age-dependency in neuronal damage, as P9 rats suffered no detectable neurodegeneration.

The predominant death mechanism in KA-induced excitotoxic neurodegeneration was necrosis. Selected proteins involved in caspase-dependent apoptosis were not triggered to a significant extent and ultrastructual analysis of dying neurons revealed necrotic morphology in the intracellular environment. However, caspase-independent apoptosis or other recently discovered cell death mechanisms with overlapping features of necrosis and apoptosis cannot be completely excluded.

Inflammatory processes were activated pronouncedly and rapidly, but largely transiently, in the hippocampus in OHCs and juvenile rats. COX-2 was induced and PGE_2 production increased, glial cells underwent a morphological change from resting to active appearance and cytokine mRNA expression increased *in vivo*. However, activation of inflammatory processes after KA-induced SE was age-dependent, since COX-2 was not induced and glial cells retained the resting appearance in the immature rat hippocampus. Also, cytokine mRNA expression was more pronouncedly and rapidly induced in the juvenile P21 hippocampus, compared to the more moderate and delayed increase in the immature P9 hippocampus. Furthermore, glial cell morphological change occurred only in the P21 hippocampus 3 d after SE. Remarkably, microglial activation was most prominent in the damaged CA1 area 3 d after SE, at which time COX-2 and cytokine expression was abated, suggesting a regulated shift from fulminant initial reaction to a more moderate and targeted response. In addition to possibly contributing to neuronal degeneration, acute activation of inflammatory processes might have epileptogenic effects.

COX-inhibitors have yielded somewhat mixed results when tested against excitotoxicity in the adult brain, while their effects on excitotoxicity in the developing brain are much less known. Pre- and co-treatment (in respect to KA treatment) with the COX-2 selective inhibitor NS-398 did not alleviate neuronal degeneration in OHCs, but even slightly exacerbated it. This can be due to ineffectivity of this compund against the specific effects of KA or the timing of the treatment. Reviewing results of other studies, it seems that a more optimal time-point for intervention with COX-inhibitors is a few hours after the insult. The hypothesis in this is that the initial boost in PG production

following KA might be required as a defensive mechanism, while a sustained production increase is harmful and should be prevented in order to achieve neuroprotection.

Other anti-inflammatory drugs have shown promise in affording neuroprotection from excitotoxic insults, which highlights the role of inflammation in excitotoxic neuronal damage. However, the vast majority of these studies have been conducted only in the adult brain. Overall, studies with anti-inflammatory drugs in the developing brain are required to reveal an effective treatment to avert the harmful acute and chronic consequences of epileptic seizures in pediatric patients.

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