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Role of Cytokines in Experimental Neurodegenerative and Neuroinflammatory Disorders

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ABSTRACT

Altered expression of cytokines in response to body injury has diverse actions that can exacerbate, mediate, reduce or inhibit neuronal and myelin damage as well as influence the disease development in a variety of nervous system disorders, such as Alzheimer's disease (AD), multiple sclerosis and Guillain-Barré Syndrome (GBS). In these studies, we attempted to explore the possible roles of tumor necrosis factor (TNF)-α and interleukin (IL)-18 in experimental neurodegenerative and neuroinflammatory disorders.

The role of TNF-α in kainic acid (KA)-induced excitotoxic neurodegeneration has been studied by comparing TNF receptor 1 (TNFR1) knockout (TNFR1-/-) mice with wild-type (WT) mice. After nasal application of KA, TNFR1-/- mice showed significantly severer seizures than WT mice. In addition, obvious neuronal damage, enhanced microglial activation and astrogliosis in hippocampus as well as increased locomotor activity were found in TNFR1-/- mice compared with WT controls. Moreover, CC chemokine receptor 3 expression on activated microglia was increased in TNFR1-/- mice after KA treatment as measured by flow cytometry. These data suggest that TNF-α may play a protective role via TNFR1 signalling in KA-induced neurodegeneration.

Epidemiological studies concerning gender differences in AD support the higher prevalence and incidence of AD in women. The influence of age and gender on excitotoxic neurodegeneration has been investigated by treating C57BL/6 mice (aged females and males as well as adult females and males) with KA. The results showed that aged female mice were more sensitive to KA-induced excitotoxicity associated with severer seizure activity, increased locomotion and rearing in open-field test, prominent hippocampal neuronal damage, enhanced astrocyte proliferation compared with aged males, adult females and adult male mice, respectively. In addition, higher level of brain-derived neurotrophic factor in hippocampi of aged female mice was observed. These results denote that aged female mice are more sensitive to KA-induced excitotoxicity.

IL-18 participates in the fundamental inflammatory processes, especially during aging. Based on the above results, we were interested in studying the role of IL-18 in KA-induced neurodegeneration in aged female C57BL/6 mice. We found that aged female IL-18-/- and WT mice showed similar responses to KA insult as demonstrated by comparable seizure activities, behavioral changes and neuronal cell death. However, aged female IL-18-/- mice failed to exhibit as strong microglial activation as WT mice. Interestingly, even though the number of activated microglia was less in KA-treated IL-18-/- mice than in KA-treated WT mice, the proportion of microglia that expressed the cytokines, TNF-α, IL-6 and IL-10 was higher in KA-treated IL-18-/- mice. Deficiency of IL-18 attenuates microglial activation after KA-induced excitotoxicity in aged brain, while the net effects of IL-18 deficiency are balanced by the enhancement of TNF-α, IL-6 and IL-10 production.

To further explore the role of IL-18 in the neurodegeneration and neuroinflammation, another animal model - experimental autoimmune neuritis (EAN) was induced by immunization of mice (IL-18-/-) with P0 protein peptide 180-199. The clinical course was not significantly different between IL-18-/- and WT mice. The splenic mononuclear cell (MNC) proliferation was also similar in both animal groups. However, the percentages of interferon-γ, IL-10 and IL-12 positive cells were decreased among infiltrating MNC of cauda equina in IL-18-/- mice. This indicates that IL-18 deficiency inhibits the production of both Th1 and Th2 cytokines in the target organ of EAN.

In summary, TNF-α may play a protective role via TNFR1 signalling in KA-induced neurodegeneration, while IL-18 may not be a key inflammatory cytokine in experimental neurodegenerative and neuroinflammatory disorders.

LIST OF PUBLICATIONS

I. Ming-Ou Lu, **Xing-Mei Zhang**, Eilhard Mix, Hernan Concha Quezada, Tao Jin, Jie Zhu, and Abdu Adem. TNF-α receptor 1 deficiency enhances kainic acid-induced hippocampal injury in mice.
Journal of Neuroscience Research. 2008, 86(7):1608-14.

II. **Xing-Mei Zhang**, Shun-Wei Zhu, Rui-Sheng Duan, Abdul H. Mohammed, Bengt Winblad, and Jie Zhu. Gender differences in susceptibility to kainic acid-induced neurodegeneration in aged C57BL/6 mice.
NeuroToxicology. 2008, 29(3):406-12.

III. **Xing-Mei Zhang**, Tao Jin, Hernan Concha Quezada, Eilhard Mix, Bengt Winblad, and Jie Zhu. Kainic acid-induced microglial activation is attenuated in aged interleukin-18 deficiency mice.
Journal of Neuroinflammation 2010, 7:26.

IV. Rui-Sheng Duan, **Xing-Mei Zhang**, Eilhard Mix, Hernan Concha Quezada, Abdu Adem, and Jie Zhu. IL-18 deficiency inhibits both Th1 and Th2 cytokine production but not the clinical symptoms in experimental autoimmune neuritis.
Journal of Neuroimmunology 2007, 183:162-7.

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer’s disease
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CA	cornu ammonis
CCR3	CC chemokine receptor 3
CE	cauda equina
CNS	central nervous system
COX	cyclooxygenase
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
ELISA	enzyme-linked immunosorbent assay
FCM	flow cytometry
GBS	Guillain-Barré syndrome
GFAP	glial fibrillary acidic protein
IFN-γ	interferon-γ
IL	interleukin
IL-18-/-	IL-18 knockout
KA	kainic acid
KO	knockout
MHC	major histocompatibility complex
MNC	mononuclear cells
MS	multiple sclerosis
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
PBS	phosphate-buffered saline
PD	Parkinson’s disease
p.i.	post immunization
PNS	peripheral nervous system
ROS	reactive oxygen species
RNS	reactive nitrogen species
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFR1-/-	TNFR1 knockout
WT	wild-type

1 INTRODUCTION

1.1 PART I: KAINIC ACID-INDUCED NEURODEGENERATIVE ANIMAL MODEL

1.1.1 Overview of Excitotoxicity Induced by L-Glutamate

L-glutamate, the major excitatory transmitter in the brain and spinal cord, is associated with learning, cognition, memory and neuro-endocrine functions [1, 2]. The glutamate receptors can be divided into two broad categories: the ionotropic receptors that mediate fast postsynaptic potentials by activating ion channels directly, and the metabotropic receptors that results in the expression of slow postsynaptic potentials through second messengers [3, 4]. The action of glutamate on the ionotropic receptors is always excitatory [5, 6]. There are three major subtypes of ionotropic glutamate receptors: α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate, and N-methyl-D-aspartate (NMDA), named according to the types of synthetic agonists that activate them, respectively [7, 8]. The NMDA glutamate receptor is blocked by specific antagonists such as D(-)-2-amino-5-phosphonovalerate (APV) [9, 10]. Both AMPA and kainate receptors are blocked by 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) [11, 12]. Thus the AMPA and kainate receptors are sometimes referred to together as non-NMDA receptors. The ion channel of NMDA receptor is a tetrameric structure that results from up to seven genes coding for seven subunits termed GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B [13, 14]. The AMPA receptor family is composed of four subunits, GluA1–4 [15, 16]. The kainate receptor family comprises five genes, divided into two subfamilies, including GluK4-5 and GluK1–3. GluK4 and GluK5 exhibit higher affinity for kainate than GluK1–3 [17, 18]. Herein, we used the new nomenclature for glutamate receptors recommended by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) [19]. NC-IUPHAR recommended and previous nomenclatures of ionotropic gluatamate receptor subunits are listed in **Table 1**.

Excessive amounts of glutamate are highly toxic to neurons, an action termed glutamate excitotoxicity [20, 21]. Glutamate excitotoxicity is triggered primarily by excessive Ca^{2+} influx arising from overstimulation of the NMDA subtype of glutamate receptors, followed by the generation of reactive oxygen species (ROS) as well as mitochondrial dysfunction, leading to neuronal apoptosis and necrosis [22, 23]. There is increasing realization that the mitochondrial dysfunction occupies the center stage in these processes [23, 24]. In many cell types, glutamate neurotoxicity is induced by NMDA as well as non-NMDA receptors [20, 21, 25].

Table 1. NC-IUPHAR recommended and previous nomenclatures of ionotropic gluatamate receptor subunits.

Ionotropic glutamate family	NC-IUPHAR subunit nomenclature	Previous nomenclatures	Human gene name	Human chromosomal location
NMDA	GluN1	GLU_{N1}, NMDA-R1, NR1, GluRξ1	GRIN1	9q34.3
	GluN2A	GLU_{N2A}, NMDA-R2A, NR2A, GluRε1	GRIN2A	16p13.2
	GluN2B	GLU_{N2B}, NMDA-R2B, NR2B, hNR3, GluRε2	GRIN2B	12p12
	GluN2C	GLU_{N2C}, NMDA-R2C, NR2C, GluRε3	GRIN2C	17q25
	GluN2D	GLU_{N2D}, NMDA-R2D, NR2D, GluRε4	GRIN2D	19q13.1
	GluN3A	GLU_{N3A}, NMDA-R3A, NMDAR-L, chi-1	GRIN3A	9q31.1
	GluN3B	GLU_{N3B}, NMDA-R3B,	GRIN3B	19p13.3
AMPA	GluA1	GLU_{A1}, GluR1, GluRA, GluR-A, GluR-K1, HBGR1	GRIA1	5q31.1
	GluA2	GLU_{A2}, GluR2, GluRB, GluR-B, GluR-K2, HBGR2	GRIA2	4q32-q33
	GluA3	GLU_{A3}, GluR3, GluRC, GluR-C, GluR-K3	GRIA3	Xq25-q26
	GluA4	GLU_{A4}, GluR4, GluRD, GluR-D	GRIA4	11q22
Kainate	GluK1	GLU_{K5}, GluR5, GluR-5, EAA3	GRIK1	21q22.11
	GluK2	GLU_{K6}, GluR6, GluR-6, EAA4	GRIK2	6q16.3-q21
	GluK3	GLU_{K7}, GluR7, GluR-7, EAA5	GRIK3	1p34-p33
	GluK4	GLU_{K1}, KA1, KA-1, EAA1	GRIK4	11q22.3
	GluK5	GLU_{K2}, KA2, KA-2, EAA2	GRIK5	19q13.2

1.1.2 KA Administration Induces Recurrent Seizures in Rodents

Kainic acid (KA) is a non-degradable analog of glutamate and 30-fold more potent in neurotoxicity than glutamate [26, 27]. Administration of KA to rodents caused a well characterized seizure syndrome, as described by Ben-Ari and other research groups [28, 29]. The seizure activity caused by intravenous, intraperitoneal injections or microinjection into amygdala or hippocampus is divided in several distinct phases. During the first 20-30 min, the animals have “staring” spells, followed by head nodding and numerous wet-dog shakes for another 30 min. One hour after KA administration, the animal starts recurrent limbic motor seizures, including masticatory and facial movements, forepaws tremor, rearing and loss of postural control. The seizures then become progressively severer, with a reduction in the intermission. In the following l-2 h, the animal displays a full status epilepticus [28-30]. In the past few years, our research group has developed a model of KA-induced hippocampal injury by intranasal administration of KA into C57BL/6 mice [31, 32]. Within 15 min after intranasal administration, the C57BL/6 mice are catatonic and staring. Myoclonic twitching and frequent rearing and falling follow this behavior. Within 30-40 min after administration, the mice have continuous tonic-clonic seizures, which continued for 1-5 h. Some serious cases die in this period. After 5 h of administration, mice assume a hunched posture and are immobile for the next few hours.

1.1.3 KA Treatment Changes Behaviors of Rodents

CA1 pyramidal neurons receive two distinct excitatory inputs that are capable of influencing hippocampal output and involving in spatial memory and memory consolidation [33, 34]. Damage in CA1/CA3 region of hippocampus induced by KA mainly results in the spatial learning deficits [35, 36]. KA-treated Wistar rats are impaired in the water maze and object exploration tasks, and hyperactive in the open field test, which can be improved by physical exercise and the selective cyclooxygenase (COX)-2 inhibitor [37, 38]. Intraperitoneal injections of KA into the developing rat brains induce the impaired short-term spatial memory in the radial-arm maze, deficient long-term spatial learning and retrieval in the water maze, and a greater degree of anxiety in the elevated plus maze [39, 40]. Mice with a single unilateral injection of KA into the dorsal hippocampus exhibit a decrease in depression-like behavior in the forced swimming test and retarded acquisition as well as impaired retention of visual-spatial information in the Morris water maze test [41]. Our research group also reported that intranasal KA administration to C57BL/6 mice induced the elevated level of spontaneous activity in the open field test [31, 32].

1.1.4 KA Insult Causes Hippocampal Neurodegeneration

Systemic injection of KA to rodents caused the selective neuronal vulnerability in the hippocampus, as reviewed by the research groups of Ratte and Wang [42, 43]. The hilar neurons are sensitive to KA-induced neurotoxicity, but neuron loss in the other areas of the hippocampus differs between animal species and strains [44-46]. In rats, the systemic injections of KA produced widespread neuronal death, primarily in the hippocampus hilus, CA1 and CA3 areas [25, 47]. High doses of KA can also induce neurotoxicity in the medial amygdaloid nuclei [48]. Mouse strains vary significantly in their sensitivity to KA-induced neurodegeneration [45, 46, 49, 50]. In general, the C57BL/6, C57BL/10, and (C57BL/6 x CBA/J) F1 strains are resistant to KA-induced neurodegeneration, while the FVB/N, ICR and DBA/2 J strains are vulnerable [46]. Systemic administration of KA to mice leads to neuronal damage, mainly in the hippocampus [32, 43, 45]. The vulnerable mice, such as FVB/N and DBA/2J, show extensive degeneration in most of the brain regions, including the neocortex, striatum, hippocampus, and nuclei in thalamus, hypothalamus and amygdale [46]. C57BL/6, the “relatively” resistant mouse strain, reveals significant neuronal damage in CA1 and CA3, and to a lesser extent, in the polymorphic layer of the dentate gyrus by 12 h post-treatment of KA systemically detected by cupric-silver and Fluoro-Jade B staining [51, 52]. Utilizing the traditional Nissl staining, we found that neuronal damage was restricted to the hippocampus, especially CA3 area 1 day after intranasal KA treatment [31, 32]. CA3 region has the highest abundance of kainate receptors, the activation of which can elevate the concentration of ROS and impair the normal function of mitochondria [53-55]. CA3 neurons are directly excited by stimulation of their KA receptors and indirectly, by increased glutamate efflux secondary to KA stimulation of mossy fibers [56, 57]. CA3 synchronization produces spreading epileptiform activity that extends to CA1 and other limbic structures [58, 59].

1.1.5 KA Mediates the Generation of Oxidative Stress

KA receptors have both presynaptic modulatory and direct postsynaptic excitatory actions [60, 61]. The activation of KA receptors produces membrane depolarization and results in alteration in intracellular calcium concentrations, which is required to trigger the neuronal death cascade (**Figure 1**) [62]. KA can also induce the release of lactate dehydrogenase (LDH), and a decrease in 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), which result in damage of mitochondrial function [43]. KA administration increases the generation of ROS and reactive nitrogen species (RNS). There is growing evidence that free radical generation plays a key role in the neuronal damage [63]. KA has been shown to immediately induce COX-2 expression that might be involved in hippocampal neuronal death, while COX-1 might participate in KA-induced cortical neuronal death [64]. Early induced COX-2 facilitates the recurrence of hippocampal seizures, and late synthesized COX-2 stimulates hippocampal neuron loss after KA administration [65]. COX-2 knockout mice are resistant to neuronal death after KA treatment [66]. COX catalyzes the first step in the synthesis of prostanoids, including prostaglandins (PGs), prostacyclin, and thromboxanes. PGE(2) is pathologically increased in the brain after KA treatment, and has been proven to be closely associated with neuronal death [67]. In addition, lipid peroxides play critical roles in the initiation and modulation of inflammation and oxidative stress upon KA insult. Seizures can induce the products of lipid peroxidation, such as F(2)-isoprostanes and Isofurans, which have been thought to be the reliable indices of oxidative stress in vivo [68].

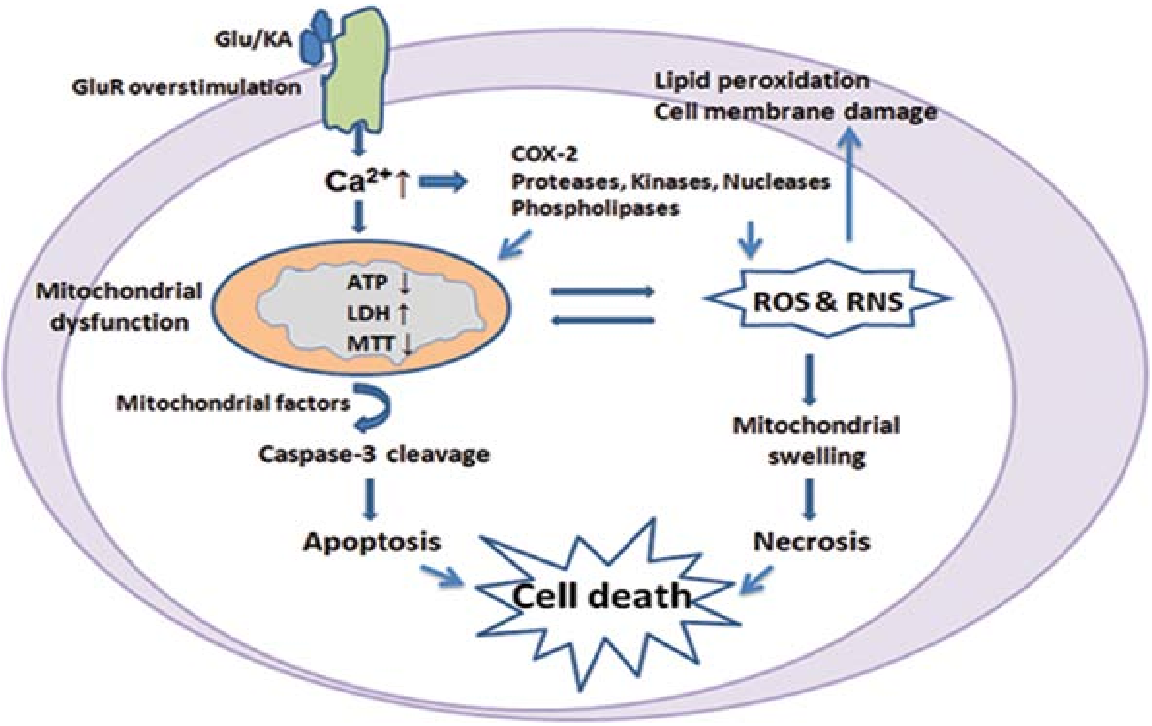

Figure 1. Schematic overview of KA-mediated neuronal death.

1.1.6 Glial Cells Are Activated upon KA Injury

KA-induced neuronal death is accompanied by increased activation of microglia and astrocytes [32, 69, 70]. Additionally, the activated glial cells cluster at the hippocampal lesions and the immunostaining reactivity is particularly strong around areas of debris (**Figure 2**).

1.1.6.1 Microglia

Microglia account for approximately 20% of the total glial population in the central nervous system (CNS). Microglia are the main effector cell type of the immune and inflammatory responses in the CNS, as earlier reviewed by Streit and his colleagues [71]. The normal role of microglia could be partly connected to neuroprotection, whereas in pathological conditions microglia may become disease-promoting cells. Upon neuronal injury, microglia rapidly acquire changes in morphology and secrete a variety of soluble mediators [72, 73]. Some studies suggested that the activated microglia might exert a neuroprotective function, especially in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE) by creating a microenvironment for reparative and regenerative processes [74]. Evidence is also accumulating that activated microglia induce and/or exacerbate neuropathological changes in several CNS diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) through secreting proinflammatory and neurotoxic factors [75, 76]. In KA-induced hippocampal neurodegeneration, microglial activation is generally believed to contribute to the neuronal death [70, 77, 78]. A recent study showed that IκB kinase/nuclear factor kappa B (NF-κB) dependent microglial activation participated in KA-mediated injury in vivo through induction of inflammatory mediators [77]. However, whether microglial activation initiates the disease progression or merely responds to neuronal death is still unclear.

1.1.6.2 Astrocytes

Astrocytes, the most numerous glial cells, have been regarded as passive supporters of neurons in CNS for decades. Studies of the last 20 years, however, challenged this assumption by demonstrating that astrocytes possess functional neurotransmitter receptors [79, 80]. These findings have led to a new concept of neuron-glia intercommunication where astrocytes play an undoubted dynamic role by integrating neuronal inputs and modulating synaptic activity, and so contribute to disease development [81]. Astrocytes have functional receptors for the excitatory neurotransmitter glutamate and respond to physiological concentrations of this substance with oscillations in intracellular Ca^{2+} concentrations and spatially propagating Ca^{2+} signals [82-84]. The expression of glial fibrillary acidic protein (GFAP) has been shown to steadily increase from one/three days up to one month after intra-hippocampal or intraperitoneal injection of KA [85, 86]. Astrogliosis induced by excitotoxicity has been considered as a marker for neurotoxicity [69, 87]. It is believed that astrocytes produce growth factors to prevent neurons from death and to promote proliferation and differentiation of precursor cells [88-90]. Activation of transcription factors, including nuclear factor erythroid-2-related factor 2 (Nrf2) and NF-κB, in astrocytes induces the neuroprotective molecule expression and confers protection to neighboring neurons [91-93]. Old astrocyte specifically induced substance (OASIS) is involved in the endoplasmic reticulum stress response [94]. A recent study showed that OASIS expressed in astrocytes plays important roles in protection against neuronal damage induced by KA [95].

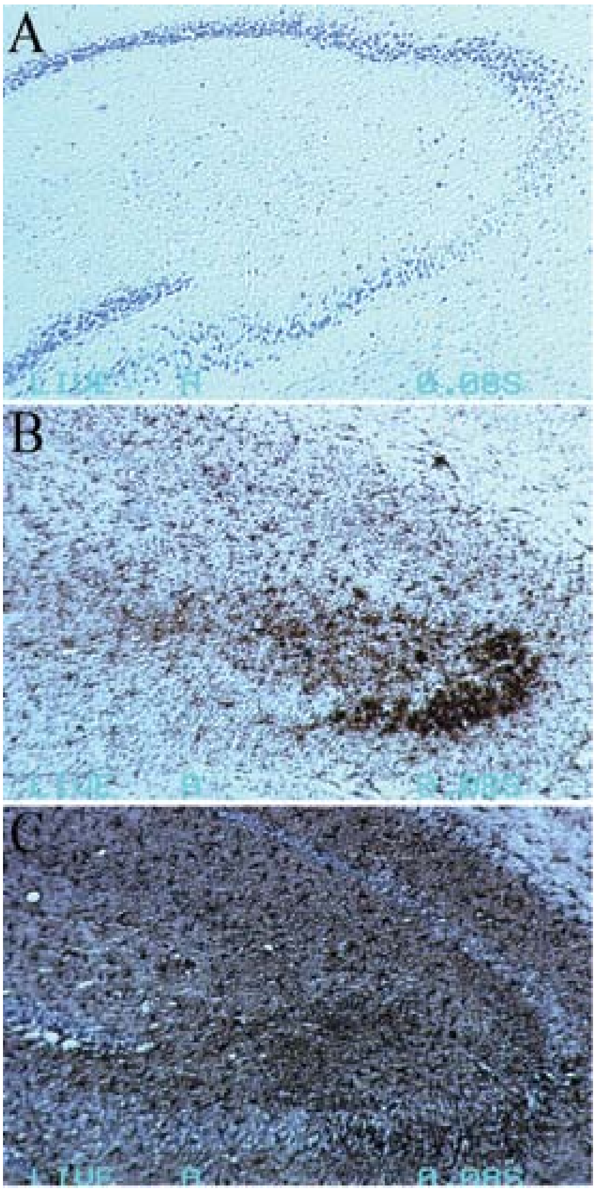

Figure 2. Glial cells activation accompanied neuronal death 7 days after KA (45 mg/kg body weight) treatment to C57BL/6 mice. (A) Obvious neuron loss was showed in CA3 area of hippocampus by Nissl´s staining. (B) CD11b positive cells (microglia) accumulated in the lesioned CA3 area. (C) GFAP positive cells (astrocytes) spread the whole hippocampus, especially in CA3 area.

1.1.7 Altered Cytokine Expression Affects KA-Induced Injury

Altered expression of cytokines in response to brain injury has diverse actions that can exacerbate, mediate, reduce or inhibit neuronal damage and influence the disease development in a variety of CNS disorders, such as AD, MS, viral or bacterial infections, ischemia, stroke, and various forms of encephalopathies [96-100]. Cytokines can be divided into pro-inflammatory and anti-inflammatory cytokines, which play the neurodestructive and neuroprotective roles, respectively. It is the balance between destructive and protective factors that ultimately determines the net result of the neuro-immune and neuro-inflammation interaction, as reviewed by Kerschensteiner and his co-workers [101]. Results from studies using KA model also indicated that manipulation of pro- and anti-inflammatory cytokines can modify the outcome with regard to the seizure activity, behavioral changes as well as the neuropathological consequences [32, 102-104].

1.1.7.1 TNF-α

Tumor necrosis factor-α (TNF-α) is mainly produced by microglia and astrocytes in the CNS. Its functions are mediated through two receptors, TNF receptor (TNFR) 1 (p55) and TNFR2 (p75), both of which are expressed on various cells types [105]. TNF-α over-expression participates in the pathogenesis of several CNS disorders, such as AD [106], bacterial meningitis [107], MS [108] and cerebral malaria [109]. TNF-α potentiates excitotoxic injury to human fetal brain cells [110]. In contrast to its well known deleterious roles, multiple lines of evidence suggested that TNF-α also exhibit neuroprotective properties. This implies an intricate biological function of TNF-α in modulating immune and inflammatory responses. TNF-α knockout worsens Listeria infection in the CNS [111] and TNF-α receptor knockout enhances the neuronal damage after excitotoxic [103, 112], ischemic [113] or traumatic injury [114]. Several neuroprotective molecules were identified as TNFR1 targets, including members of the Bcl-2 family, DNA repair machinery and cell cycle, developmental, and differentiation factors, neurotransmitters and growth factors, as well as their receptors [115]. The mechanisms by which TNF reduced neuron loss after brain injury may involve the up-regulation of proteins, such as neuronal apoptosis inhibitor protein (NAIP), which maintain calcium homeostasis and reduce free radical generation [116]. Study also proved that the protective roles of TNF-α in KA-induced neurodegeneration are via TNFR2 signalling [112].

1.1.7.2 IL-18

Interleukin (IL)-18 is most closely related to IL-1β. The similarities between both cytokines comprise structure, receptor complex, and pro-inflammatory properties [117]. IL-18 serves as a link between innate and adaptive immune responses, such as stimulating the expression of adhesion molecules, inducing the production of chemokines (IL-8) and cytokines (TNF-α and IL-6), stimulating the activity of NK cells, and promoting T helper 1 (Th1) cells responses in combination with IL-12, Th2 responses in combination with IL-4, and Th17 responses in combination with IL-23 [118]. IL-18 and IL-18 receptor (IL-18R) mRNA have been found in brain tissue and in cultured astrocytes and microglia [119]. IL-18 enhanced postsynaptic AMPA receptor responses in CA1 pyramidal neurons via the release of glutamate, thereby facilitating basal hippocampal synaptic transmission [120]. IL-18 deficient mice showed a diminished microglial activation and reduced dopaminergic neuron loss after acute 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine treatment [121]. The roles of IL-18 in KA-induced model are controversial. Levels of IL-18 and IL-18R in hippocampus increase progressively from day 1 and peaked at day 3 post-KA treatment [122]. Interestingly, intracerebellar coinjection of IL-18 counteracts the effect of IL-1β in KA-induced ataxia in mice [123]. We showed that exogenous IL-18 administration aggravated the KA-induced injury in normal C57BL/6 mice, while in the condition of IL-18 deficiency, IL-12 could overcompensate the function of IL-18 and worsen the seizure activity as well as hippocampal neurodegeneration [32].

1.1.8 Influence of Aging and Gender on KA-induced Injury

Accumulating results have demonstrated an increased sensitivity of aged individuals to excitotoxic neurodegeneration [52, 124, 125]. The currents induced by AMPA displayed a significant increase with age, which might mediate neurotoxicity associated with age-related neuropathies [126]. Aged CA1 failed to exhibit any tolerance to domoic acid (DOM) following preconditioning [127]. Several factors that change with age might influence brain sensitivity to excitotoxic damage, including the density of glutamate receptors, the uptake and release of neurotransmitters, and cellular metabolic processes [128]. Significant decreases have been detected for the NMDA and kainate receptor binding sites in most of the cortical areas in aging mice [129].

There is a gender-associated sensitivity in the aged brain to the neurodegeneration [130], which might involve alterations in estrogen levels or aged-related decline in the activity of NMDA receptors. Aged female Long-Evans (LE) rats show greatly enhanced susceptibility to KA-induced seizures even at doses four-fold lower than that of adult controls [125]. After injections of KA into the hippocampus of rats, only females exhibited the increase in daily food intake and body weight [131]. Estrogen can profoundly improve spatial reference memory in aged females and this improvement may be related to enhanced hippocampal synaptic plasticity [132]. The epidemiological studies concerning gender differences in AD also support the higher prevalence and incidence of AD in women [133, 134]. A recent study using positron emission tomography explored gender differences in the regional cerebral–metabolic rate of glucose in the patients with AD and found that, at the same level of severity of cognitive impairment, men showed a significantly greater hypometabolism than women. This suggests that men can compensate more pathological changes than women and men are less likely to express neurodegeneration as clinical dementia [135].

1.1.9 Therapeutic Strategies

Considering that oxidative stress is central to KA-induced excitotoxic damage, anti-oxidant and anti-inflammatory treatments may attenuate or prevent the KA-mediated neurodegeneration **(Figure 3)**. The potential role of COX-2 inhibitors as a new therapeutic drug for the neuron loss after KA treatment has been studied. The selective COX-2 inhibitors, celecoxib, NS398, rofecoxib and SC58125, can suppress an elevation of PGE(2) and block hippocampal cell death [37, 66]. Free radical scavengers are well known to prevent neuron loss induced by exposure to excitotoxins. Edaravone (Ed), a newly developed free radical scavenger, could inhibit lipid peroxidation and prevent neuron loss when administered after the onset of seizures in a KA-induced neurodegenerative animal model [136]. The pineal secretory product, melatonin, has free-radical-scavenger and antioxidant properties, which attenuates KA-induced neuronal death, lipid peroxidation, and microglial activation [137]. Several phospholipase A (2) inhibitors, quinacrine and chloroquine, arachidonyl trifluoromethyl ketone, bromoenol lactone, cytidine 5-diphosphoamines, and vitamin E, have been shown to prevent the neurodegeneration in KA-mediated neurotoxicity [138]. The other drugs tested experimentally include fluoxetine, ethyl pyruvate and statins, whose neuroprotective effects are associated with their anti-inflammatory effects. The transcription factor Nrf2 can guard the redox homeostasis and demonstrate its antioxidant properties against excitotoxicity, which has also been pharmacologically targeted to prevent KA-induced neuronal death [139]. Since glutamate excitotoxicity contributes to a variety of disorders in the CNS, the anti-oxidant and anti-inflammatory drugs merit further investigation. Additionally, targeting the pro-inflammatory cytokines, by blocking the unique signal transduction of the specific cytokine is another potential therapeutic strategy.

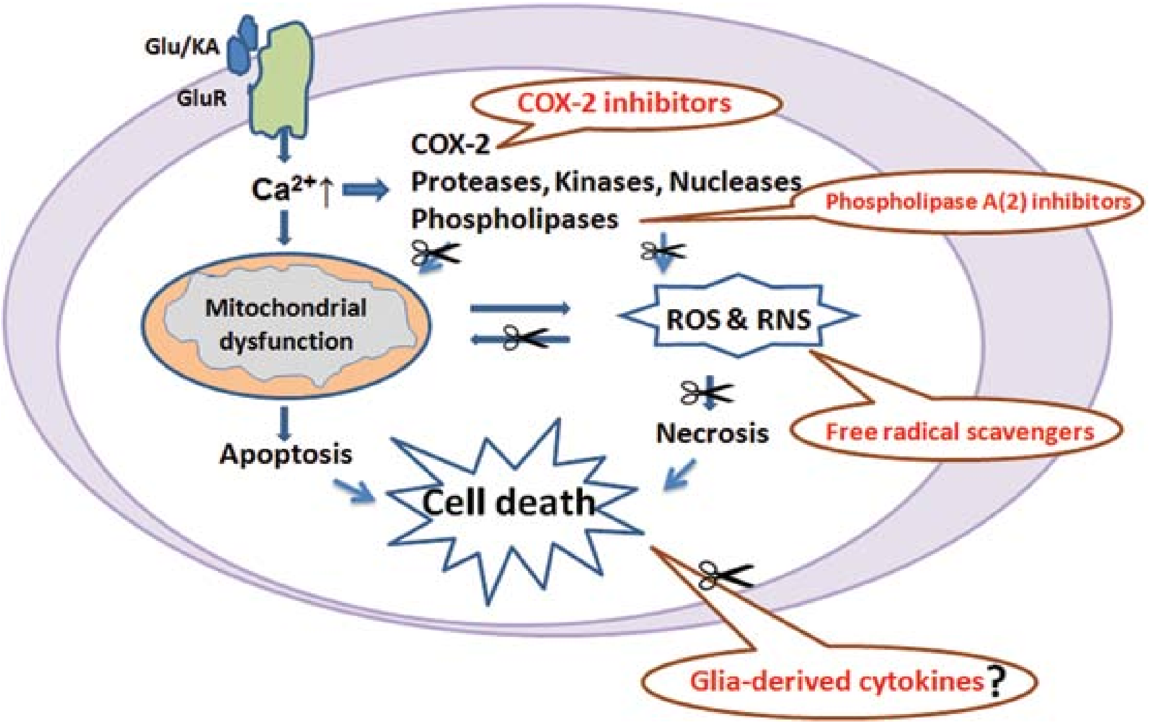

Figure 3. Schematic illustration of anti-oxidant and anti-inflammatory treatments to KA-mediated neuronal death.

1.2 PART II: GUILLAIN-BARRÉ SYNDROME AND ITS ANIMAL MODEL

Guillain-Barré syndrome (GBS) is an immune-mediated inflammatory disease of the peripheral nerves, involving both myelin sheath and axons. It is the most common cause of the acute flaccid paralysis worldwide [140]. GBS is characterized by acute progressive and symmetrical motor weakness of the extremities as well as of bulbar and facial musculature. The progressive phase of syndrome lasts from a few days to four weeks [141]. Approximately 85% of patients with GBS achieve a full functional recovery within 6 to 12 months. Some patients have persistent weakness, areflexia and paresthesia [142]. GBS is considered as an organ-specific immune-mediated inflammatory disorder emerging from a synergistic interaction of cellular and humoral immune response to the peripheral nerve antigens [143]. In GBS, activated T cells and macrophages in circulation cross the blood-nerve barrier and initiate an inflammation in the peripheral nervous system (PNS), which results in demyelination and axonal loss. Cytokines and chemokines, adhesion molecules, nitric oxide, and matrix metalloproteinases (MMP) contribute to this process.

Experimental autoimmune neuritis (EAN) is a $CD4^+$ T cell-mediated demyelinating inflammatory disease of the PNS, which serves as an animal model for the human GBS. EAN can be induced in rats, mice, rabbits and monkeys by active immunization with whole peripheral nerve homogenate, myelin proteins P0 or P2 and their neuritogenic peptides [144-146], and by adoptive transfer of P0 and P2 as well as their peptide-specific $CD4^+$ T cell lines [147, 148]. The pathological hallmark of EAN is the infiltration of the PNS by inflammatory cells including lymphocytes and macrophages, which results in multifocal demyelination of axons. The course of EAN appears to be determined by the temporally and spatially regulated expression of various cytokines produced by infiltrating immune cells and Schwann cells [149, 150].

Cytokines involved in the pathogenesis of EAN and GBS include TNF-α, interferon-γ (IFN-γ), IL-12, IL-18, IL-10, IL-4 and TGF-β. Th1 cytokines are dominant in the inflamed nerves during the acute phase of disease [150, 151]. Elevated serum concentration of TNF-α showed a positive correlation with the disease severity in GBS patients [152]. IFN-γ receptor knockout mice showed less EAN severity [153]. Both IL-12 deficient mice and mice treated with anti-IL-18 monoclonal antibody exhibited reduced clinical severity of EAN through impaired Th1 responses in inflamed nerves [154, 155]. Treatment by IL-10 ameliorated the inflammatory response in EAN by inhibiting Th1 and enhancing Th2 responses [156]. The balance of pro- and anti-inflammatory cytokines may determine the outcome of EAN.

2 AIMS OF THE STUDIES

To identify the role of TNF-α/IL-18 in the experimental neurodegenerative and neuroinflammatory disorders.

Specific aims:

Study I: To study the role of TNF-α in KA-induced neurodegeneration.

Study II: To investigate how age and gender affect the susceptibility to KA-induced excitotoxic neurodegeneration in C57BL/6 mice.

Study III: To investigate the role of IL-18 in KA-induced neurodegeneration in aging condition.

Study IV: To study the role of IL-18 in EAN.

3 METERIALS AND METHODS

3.1 ANIMALS (STUDIES I-IV)

TNFR1 KO mice, male, 5-6-week-old, and the corresponding matched wild-type (WT) C57BL/6 mice were used in Study I. Aged (19-20-month-old) and adult (7-8-month-old) C57BL/6 mice, both female and male were used in Study II. Aged female IL-18 KO mice (18-19-month-old) and young male IL-18 KO mice (4-week-old), were used in Studies III and IV, respectively. All mice were housed on a 12-h light-dark schedule with water and food available ad libitum.

3.2 KA ADMINISTRATION AND ASSESSMENT OF CLINICAL SIGNS (STUDIES I-III)

Mice were partially anesthetized with Isofluen. KA dissolved in distilled water/saline (10 mg/1.3 ml) was slowly and gently dropped by micropipette into the noses of the mice. Doses of KA used were 40 mg/kg body weight in Study I, 20 or 30 mg/kg in Study II, and 25 mg/kg in Study III. Age- and body weight-matched control mice received the same amount of distilled water/saline intranasally as controls. Mice were monitored continuously for 5 h to register the onset and extent of seizure activity. Seizures were rated as follows: 0, normal; 1, immobilization; 2, rearing and falling; 3, seizure for less than 1 h; 4, seizure for 1-3 h; 5, seizure for more than 3 h; and 6, death.

3.3 INDUCTION OF EAN AND ASSESSMENT OF CLINICAL SIGNS (STUDY IV)

The neuritogenic P0 protein peptide 180-199, corresponding to amino acids 180-199 of rat PNS myelin P0 protein was synthesized by solid-phase stepwise elongation using a Tecan/Syro peptide synthesizer (Multisyntech, Bochum, Germany). Mice were immunized twice on days 0 and 7 post-immunization (p.i.) by subcutaneous injection of 120 μg of P0 peptide 180-199 and 0.5 mg of Mycobacterium tuberculosis (Difco, Detroit, USA) in 25 μl saline and 25 μl Freund's incomplete adjuvant (ICN Biomedicals, Aurora, USA). All mice received 400, 200, and 200 ng pertussis toxin (Sigma, St. Louis, USA) by intravenous injection on days -1, 0 and +2 p.i., respectively. Clinical signs of EAN was assessed immediately before immunization (day 0) and thereafter every second day until day 27 p.i. and scored as follows: 0, normal; 1, flaccid tail, decreased tone in whole tail or mild limb weakness; 2, severe limb weakness or mild hind limb paralysis; 3, moderate hind limb paraparesis; 4, severe hind limb paralysis; 5, severe tetraparesis.

3.4 HISTOPATHOLOGICAL ANALYSIS (STUDIES I-IV)

The surviving KA-treated mice as well as water/saline-treated control mice were anesthetized with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% buffered formaldehyde at different time points after administration of KA. The brains were kept in 10% sucrose until being frozen. Coronal sections (12-µm slices) from -1.15, -1.94, and -2.80 mm, respectively, relative to the bregma were prepared according to the information in Franklin's brain atlas [157]. Sections were stained by Nissl's method (Studies I-III) and Fluoro-Jade B (FJB) staining (Study III) to evaluate degenerating neurons. FJB is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration. For assessment of severity and extent of neurodegeneration in the hippocampus according to Nissl's staining, sections were scored using a semiquantitative grading system: 0, normal; 1, slight shrinkage of neurons (1-4% pyknotic neurons in area CA3); 2, moderate shrinkage of neurons (5-15% pyknotic neurons in area CA3); 3, severe shrinkage of neurons (more than 15% pyknotic neurons in area CA3); 4, slight loss of neurons (5-10% neuron loss in area CA3); 5, moderate loss of neurons (11-40% neuron loss in area CA3); and 6, severe loss of neurons (more than 40% neuron loss in area CA3). In Study IV, sciatic nerves were dissected, fixed in 4% formaldehyde and embedded in paraffin. Multiple longitudinal sections (6-µm slices) were stained with haematoxylin-eosin for evaluation of the extent of mononuclear cell (MNC) infiltration. Tissue areas were measured by image analysis and the results were expressed as cells per mm^2 tissue section.

3.5 IMMUNOHISTOCHEMISTRY OF BRAIN SECTIONS (STUDIES I-III)

Frozen hippocampal sections were prepared as described for histopathological analysis. After washes with Tris buffer, the sections were blocked by "protein block" (DAKO A/S, Glostrup, Denmark) at room temperature (RT) for 30 min. Subsequently, they were exposed to appropriate primary and secondary antibodies. Sections were stained by using the avidin-biotin technique (Vectastain Elite Kit; Vector Labs, Burlingame, CA, USA). Peroxidase-substrate solution DAB (Sigma-Aldrich, Stockholm, Sweden) was added until the desired intensity of color developed.

Omission of primary antibodies and incubation with an isotype matched normal IgG served as negative controls.

3.6 ISOLATION AND CULTURE OF MNC AND PROLIFERATION ASSAY (STUDY IV)

The spleens of mice from each group were removed and single cell suspensions of MNC from individual mice were prepared by grinding through a wire mesh. Erythrocytes were osmotically lysed. MNC were washed three times before being suspended in RPMI-1640 medium with 2 mM glutamine and 25mM HEPES (Gibco, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 50 μ g/ml gentamycin (Gibco). MNC suspended in 200 μl portions were cultured in triplicates in round-bottomed 96-well polystyrene microtiter plates at a cell density of 2×10^6 cells/ml in a humidified atmosphere with 5% CO_2 at 37ºC in the presence of P0 180-199 peptide (10 μg/ml), ConA (5 μg/ml, Sigma) or the same volume (10 μl) of PBS. After 60 h incubation, cells were pulsed with ^{3}H-methylthymidine (1 μCi/well, Amersham, Little Chalfont, UK) and cultured for additional 18 h. Cells were harvested onto glass fiber filters (Titertek, Skatron, Lierbyen, Norway). ^{3}H-methylthymidine incorporation was measured in a liquid β-scintillation counter and results were expressed as counts per minute (cpm). Values used were the means from three separate wells.

3.7 ISOLATION AND FCM ANALYSIS OF MICROGLIA (STUDIES I AND III)

The surviving KA-treated mice, water/saline-treated mice and untreated mice were perfused with PBS and sacrificed. The hippocampi were dissected and dissociated by pipetting. Next, after trypsinization at 37ºC for 15 min, FBS (10%, final concentration) was added to inactivate trypsin activity. The tissues were then dissociated with repeated pipetting in Krebs-Ringer buffer (KRB) solution [120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14 mM D-glucose, 2.5 mM MgSO4, 0.3% bovine serum albumin (BSA) containing DNase I (Sigma-Aldrich)]. Cell suspensions were passed through a 70-µm pore-size strainer and spun down. The cell pellets were resuspended in 30% Percoll in PBS and centrifuged at 500 x g for 20 min. The resulting pellets were resuspended, passed through a 40-µm pore-size strainer, collected, and stained for flow cytometry (FCM).

Microglia enriched cell suspensions were washed with PBS containing 1% BSA (BSA/PBS), permeabilized, fixed and incubated with the appropriate combinations of antibodies. After washing, cells were analyzed by FACSCalibur flow cytometer and CellQuest software (both from Becton Dickinson). The percentage of positive cells of each molecule on microglia was determined. At each time point, microglia from all groups of mice (WT mice with or without KA treatment, and KO mice with or without KA treatment) were collected and analyzed on the same day with the same cytometer settings.

3.8 ISOLATION AND FCM ANALYSIS OF MNC FROM CE (STUDY IV)

Briefly, cauda equina (CE) fragments of spinal cords were carefully removed, transferred to RPMI-1640, ground and passed through a 70 μm cell nylon mesh. The resultant cells were suspended in 27% percoll in PBS and centrifuged at 1000×*g* for 30 min at 4°C. The pellet was washed with BSA/PBS, permeabilized, fixed and incubated with the appropriate combinations of antibodies. After washing, cells were analyzed by FACSCalibur flow cytometer and CellQuest software. The percentage of the positive cells of each molecule on infiltrating MNC was determined.

3.9 OPEN-FIELD TEST WITH ZONE MONITORING (STUDIES I-III)

The open field test is used to measure exploratory behavior and spontaneous motor activity of the animals. The apparatus consisted of four identical Plexiglas boxes (34 x 34 x 18 cm) with a lower and a higher row of photosensors connected to a computer that automatically recorded the activity of the animals inside of the arena. Light bulbs (25 W) provided the illumination for each arena. Four mice were tested simultaneously, one per arena. At the beginning each mouse was placed into the center of open-field arena and its locomotion as well as rearing were recorded every 5 min during 60 min. Locomotion was registered each time the mouse interrupted the lower row of photosensors and rearing was recorded each time it interrupted the higher row. All tests were carried out between the hours of 9:00-15:00.

3.10 ELISA (STUDY II)

Halves of the hippocampal and cortical samples were homogenized in ice-cold lysis buffer, and the total protein, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) levels were measured using the supernatants of the tissue homogenate solutions. The total protein level was measured by using the Pierce BCA Protein Assay Reagent kit. NGF and BDNF levels were assessed using a commercially available ELISA assay kit (Promega, Sweden). Briefly, standard 96-well flat-bottom ELISA plates were incubated with the corresponding captured antibody overnight at 4°C and 1× Block & Sample buffer for 1 h at RT. Wells containing the standard proteins and supernatants of brain tissue homogenates were incubated for 6 h at RT. They were then incubated with second antibody for 2 h at RT. A species-specific antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant for 1 h at RT. A TMB One Solution was used to develop color. This reaction was terminated with 1N hydrochloric acid and the absorbance was recorded at 450 nm in a plate reader.

3.11 STATISTICS (STUDIES I-IV)

Non-parametric tests (Kruskal-Wallis test for more than two groups and Mann-Whitney test for comparisons between two groups) were used to analyze the clinical, pathological and immunostaining data, and parametric tests was used to analyze flow cytometry, ELISA, and behavioral results. All tests were two-tailed. Data are presented as mean ± SD for parametric tests and median with percentiles (10% - 90%) for non-parametric analysis. The level of significance was set at $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 STUDY I: TNF-α RECEPTOR 1 DEFICIENCY ENHANCES KA-INDUCED HIPPOCAMPAL INJURY IN MICE

After nasal application of a single dose of 40 mg KA per kilogram body weight, TNFR1-/- mice showed significantly severer seizures than WT mice. In addition, obvious neuronal damage, enhanced microglial activation and astrogliosis in the hippocampus as well as increased locomotor activity were found in TNFR1-/- mice compared with WT controls on day 8 after KA delivery. Moreover, CC chemokine receptor 3 (CCR3) expression on activated microglia were increased on day 3 after KA treatment in TNFR1-/- mice measured by flow cytometry.

Neuronal injury is the main characteristics of KA-induced excitotoxic neurodegeneration. Inflammatory processes, such as the production of cytokines and related inflammatory molecules have been implicated in the pathogenesis of KA-induced neurodegeneration [31, 102]. TNF-α is a pleiotropic inflammatory cytokine and appears to play a pivotal role in the inflammatory conditions. Our results showed that TNFR1-/- mice exhibited significantly severer seizures and neuronal damage in hippocampus than WT mice after KA administration. Consistent with the severer neuronal damage, TNFR1-/- mice showed more difficulty in familiarizing the environment as demonstrated by more spontaneous motor activity compared with WT mice. These findings suggest that TNF-α has a protective role for neurons after KA insult via TNFR1. The ability of TNF-α to induce changes in ion currents is linked to ATP release and may be causally related to the neuroprotective characteristic of TNF-α during excitotoxicity [158]. Moreover, the induction of neuronal apoptosis inhibitor protein (NAIP) gene may also contribute to the neuroprotective properties of TNF [116]. In addition, TNF-α clearly possesses the ability to simultaneously activate both cell death and survival pathways, and this balance ultimately determines whether TNF-α promotes neurodegeneration or neuroprotection. Defining the dual role of TNF-α in the CNS may depend on determining which conditions amplify NF-κB survival pathway and which might attenuate NF-κB activity while simultaneously promoting other pro-apoptotic TNF-α signals [159].

Microglia are the major producers of TNF-α in the brain and may play a role in certain pathological conditions in the brain [160]. TNF-α is a strong activator of microglia in vivo [161], an event that results in substantial microglial production of nitric oxide [162] as well as further TNF-α secretion [163]. In our present study, TNFR1-/- mice showed an increased number of activated microglia on 8 days after KA treatment, although a decrease of activated microglia on day 3 after KA treatment was found, which seemed more likely to be the consequence of massive death of microglia following an earlier wave of activation induced by KA. Correspondingly, a slightly lower TNF-α production is found on day 3 after KA administration. This biphasic response of TNF-α and its receptor is in agreement with another study. Scherbel et al. reported that after cortical contusion for 24-48 h, TNF-α knockout mice recovered faster than the respective controls. However, between 1 and 4 weeks, TNF-α knockout mice demonstrated greater neurological dysfunction [164]. Together with our findings, it is concluded that TNF-α deficiency might exert a protective effect by limiting microglia recruitment at an early stage of neurodegeneration following KA administration, while later the microglia might increase as a reactive response to the injured neuronal cells in TNFR1-/- mice.

Activated microglia express CCR3 and other chemokine receptors, and migrate along the concentration gradient of chemokines into the inflammatory sites in the CNS [165]. On day 3 after KA administration, the increased CCR3 expression on CD11b positive microglia was shown in our TNFR1-/- mice compared with WT mice. Thereby more microglia may be attracted to the injured area with the consequence of microglial release of toxins to worsen the neuronal damage.

Astrocytes are the most numerous non-neuronal cells in the CNS. Astrogliosis is associated with neurotoxicity and strong neuronal activity [87, 166]. Excitotoxicity can lead to astrogliosis, a feature of KA-induced hippocampal injury secondary to neuronal injury. Astrogliosis is believed to be mainly neuroprotective [86]. In our study, KA treatment led to enhanced astrocytosis in TNFR1-/- mice when compared with WT mice. This suggests that a deficiency of TNFR1 may indirectly influence astrocyte activity.

Taken together, TNF-α might be detrimental at an early stage of KA-induced neurodegeneration by activating microglia. However, a beneficial effect of TNF-α via TNFR1 on neurons in KA-induced excitatory injury is suggested by comparison of clinical and histological changes between WT and TNFR1-/- mice. Our evidence highlights the complicated function of TNF-α, although the factors and pathways responsible for this beneficial effect remain to be resolved.

4.2 STUDY II: GENDER DIFFERENCES IN SUSCEPTIBILITY TO KA-INDUCED NEURODEGENERATION IN AGED C57BL/6 MICE

In the present study, we have observed that aged female mice were more sensitive to KA-induced excitotoxicity compared with aged males, adult females and adult male mice. When the mice were treated with 30 mg/kg bodyweight KA intranasally, only one out of five aged female mice survived. When the mice were treated with 20 mg/kg bodyweight KA intranasally, the aged female mice still displayed severe seizure activity and showed prominent hippocampal neuronal damage seven days after KA treatment. In addition, only KA-treated aged female mice exhibited differences from other three groups of mice in behavioral test, which also indicated that hippocampal functions in aged female mice were damaged more than the other mice [167, 168]. Additionally, the level of BDNF expression in the hippocampus of KA-treated aged female mice was significantly increased when compared with other groups of mice.

Gender differences in susceptibility to glutamate excitotoxicity have been discussed in the past few years. Our study evidenced that aged female C57BL/6 mice were highly sensitive to excitotoxic neurodegeneraion induced by KA. Some research groups reported aged-related sensitivity to excitotoxicity in male animals [127]; however, these studies have included only male subjects. It is still uncommon for aged males and females to be compared in the same study. Besides the pivotal reason of the different strains of animals used, this discrepancy might be also due to different routes and doses of KA administration. The dose of KA we used in the present study was much lower than we applied in our previous studies [31], thus, only the mice with high sensitivity to KA insult could be induced severe clinical, pathological and behavioral changes. The mechanism behind gender disparity to KA exposure needs further investigation and whether altered estrogen levels in aged female animals contribute to neurodegenerative diseases is still controversial. Recent reports have showed that although estradiol administration in KA-treated rats has beneficial effects on cell survival, it has diverse effects on exploratory behavior, object, and spatial memory [169]. In vitro studies suggested that 17-beta estradiol and raloxifene, a selective estrogen receptor modulator, could alter microglial and astrocyte produced molecules involved in neuroinflammation and neurodegeneration [170]. However, ovariectomy proved to be ineffective in altering seizure incidence, latency, or severity after KA treatment [125].

Although a few studies indicated that aged rats were less susceptible to the excitotoxic effect of kainic and quinolinic acids [171], many researchers have shown an increased sensitivity of aged individuals to excitotoxic neurodegeneration. It has been reported that KA-induced response via AMPA receptors was greater in the aged than in the young rats [172]. The currents induced by AMPA displayed a significant increase with age, which might mediate neurotoxicity associated with age-related neuropathies [126]. Aged CA1 failed to exhibit any tolerance to domoic acid (DOM) following preconditioning [127]. Hesp and his colleagues also reported that the aged rats lost tolerance to KA administration which was triggered by a selective reduction in constitutive KA-sensitive G-protein activity [173].

The age-related increase of astrogliosis may be secondary to modest synaptic degeneration. The roles of astrocytes in neuronal trauma are complicated and seemingly contradictory. There is ample evidence to support a beneficial component of astrocyte reactivity as well as an inhibitory one [86, 174]. Further, these dual roles may be more critical in the aged animals [175].

The trophic factors produced by activated astrocytes mainly include NGF, basic fibroblast growth factor (bFGF), BDNF and neuregulins [176]. The role of BDNF and NGF in maintaining survival and promoting plasticity of neurons in the CNS is well established. Our results showed that BDNF level in the hippocampus of aged female mice was remarkably higher than that of aged males after KA treatment, in agreement with the study by Matsuki et al which suggested that female animals were more sensitive to the induction of BDNF mRNA in the retrosplenial cortex by dizocilpine-induced neurotoxicity [177]. Our previous studies found that on five days after KA treatment, the expression of most molecules (F4/80, MHCII, and iNOS) dropped to untreated levels, indicating a short time window of aggravating phase in our neurodegenerative model [69]. Seven days after KA administration, elevated BDNF levels in severe injured aged female mice may represent a compensatory mechanism to the greater damage. BDNF can suppress oxyradical production and stabilize cellular calcium homeostasis in neurons [178], while the mechanism whereby BDNF protects neurons against excitotoxic injury in vivo is not known.

In summary, our present results denote the disparity of aging and gender in KA-induced hippocampal neurodegeneration. Aged female mice are more sensitive to KA-induced excitotoxicity.

4.3 STUDY III: KA-INDUCED MICROGLIAL ACTIVATION IS ATTENUATED IN AGED IL-18 DEFICIENT MICE

We investigated the role of IL-18 in the pathogenesis of KA-induced excitotoxic neurodegeneration in the aged situation by using IL-18-deficient aged (18 to 19 months old) mice and age-matched WT mice. The study was motivated by the fact that most common neurodegenerative disorders such as AD and PD are typically diseases of higher ages and by the hypothesis that findings of KA-induced neurodegeneration of young individuals may deviate from findings in older subjects. Since previously we also found that aged female C57BL/6 mice were more sensitive to KA treatment than aged male mice [179], we performed this study on female animals.

Our results show that IL-18 deficiency does not influence susceptibility to KA-induced injury in aged female mice, since KA-induced seizures, behavioral changes and histopathological changes including astrogliosis were similar in IL-18-/- mice and WT controls. However, we found also that aged IL-18-/- mice show lower microglial activation in response to KA than do WT mice as demonstrated by lower numbers of CD11b-positive and MHC-II-positive cells. On the other hand, in these activated microglia the percentages of TNF-α-, IL-6- and IL-10-positive cells were significantly higher in IL-18-/- mice than in WT mice. These results are in contrast to our previous findings in KA-induced excitotoxic neurodegeneration of IL-18-deficient young mice [32]. Young (6 to 8 weeks old) IL-18-/- mice were more sensitive to KA administration than age-matched animals with normal expression of IL-18. In that situation we concluded that excitotoxic injury in IL-18 deficient mice might be due to overcompensation by other microglia-derived, disease-promoting factors, of which IL-12 is one candidate. Our present finding that aged IL-18-/- mice display similar susceptibility to KA treatment as WT mice might be due to the balancing influence of other microglia-derived cytokines such as TNF-α, IL-6 and IL-10.

However, we also found in young animals that exogenous IL-18 administration could aggravate KA-induced neurodegeneration, when high dose of IL-18 was applied [32]. A recent study of Jeon and co-workers gave hints in the same direction by showing that levels of IL-18 and its receptor in hippocampal homogenates increased from day 1 post-KA onward [122]. Other researchers have found that lower IL-18 concentrations are associated with improved activities of daily living in 65- to 80-year-old men, suggesting that IL-18 might play an active role in age-related functional impairment [180]. Moreover, increased concentrations of the proinflammatory cytokines IL-1α, IL-18 and IFN-γ in hippocampus are accompanied by deficits in long-term potentiation in older rats [181]. The imbalance between pro-inflammatory and anti-inflammatory cytokines in the aged brain significantly contributes to age-related deficits in synaptic function [182].

Upon neuronal injury, microglia, as the main effector cells of the immune system in the CNS, acquire changes in morphology and expression of surface antigens and soluble molecules [73]. In the present study, lower KA-induced microglial activation in the hippocampi of aged IL-18-/- mice was found compared with WT as detected by immunohistochemistry and flow cytometry. This finding was in agreement with studies of Sugama and co-workers, which showed that stress-induced microglial activation was reduced in IL-18-/- mice [183]. IL-18 null mice also showed diminished microglial activation and reduced dopaminergic neuron loss following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment [121]. Interestingly, even though the number of activated microglia was smaller in KA-treated IL-18-/- mice than in KA-treated WT mice, the proportion of microglia cells that expressed the cytokines TNF-α, IL-6 and IL-10 was higher in the KA-treated IL-18-/- mice, which might account for the similar neuropathological and clinical outcome of the IL-18-deficient, aged mice.

In conclusion, deficiency of IL-18 attenuates microglial activation in KA-induced excitotoxicity in aged brain, while the net effects of IL-18 are balanced by other cytokines, such as TNF-α, IL-6 and IL-10. IL-18 may participate in KA-induced hippocampal neurodegeneration in young animals, but IL-18 does not seem to represent a key cytokine in this process in aged individuals.

4.4 STUDY IV: IL-18 DEFICIENCY INHIBITS BOTH TH1 AND TH2 CYTOKINE PRODUCTION BUT NOT THE CLINICAL SYMPTOMS IN EAN

IL-18 has been reported to serve as a link between innate and adaptive immune responses. But IL-18 does not seem to represent a key cytokine in our KA-induced neurodegenerative animal model. In the present study, we introduced another animal model-EAN, to further test the role of IL-18 in experimental neurodegenerative and neuroinflammatory disorders. EAN has been suggested to be associated with dysregulation of the cytokine network including a predominance of proinflammatory Th1 cytokines [150]. Previous studies pointed to a pathogenic role of IL-18 in EAN and its human analogue GBS [155, 184]. We found that IL-18-/- mice were similar to WT mice in clinical course and severity of disease. Also antigenically and mitogenically stimulated splenic MNC proliferation was not different between IL-18-/- mice and WT EAN mice. However, cells producing cytokines IFN-γ, IL-10 and IL-12 were decreased in the CE infiltrates of IL-18-/- EAN mice compared with WT EAN mice, suggesting that IL-18 may act as a co-inducer of both Th1 and Th2 cytokines in our EAN model.

Since a balance of cytokine production is likely to determine the outcome of EAN [150], the observed net effect of no influence of IL-18 deficiency on EAN severity may reflect the "new-level" balance of Th1 and Th2 cytokines in EAN. Our results are in accordance with the finding of Jiang et al. [185], who reported that experimental autoimmune uveitis (EAU) in IL-18 deficient mice was accompanied by decreased production of IFN-γ, TNF-α and IL-10 in supernatants of lymph node cell cultures.

Macrophages and T cells are the main components of infiltrates in the PNS of GBS and EAN [186]. Macrophages are the main source of TNF-α in the inflammatory process of both GBS and EAN [187]. Although IL-18 can promote TNF-α production [188] and a decreased TNF-α production has been found in supernatants of lymph node cell cultures in IL-18-/- mice with EAU [185] and collagen-induced arthritis [189], our data showed only an insignificant tendency of decreased TNF-α levels in the infiltrating cells of CE in IL-18-/- EAN mice. This indicates that TNF-α production in the inflamed nerves of EAN may not be dependent on IL-18 in contrast to IFN-γ, IL-12 and IL-10 production.

In brief, IL-18 deficiency does not prevent mice from EAN despite decreased levels of Th1 and Th2 cytokines in IL-18 deficient EAN mice. These findings indicate that IL-18 is a co-inducer of both Th1 and Th2 cytokines in EAN and possibly in GBS as well as in other T-cell-mediated autoimmune diseases.

5 CONCLUSIONS

1) TNF-α plays a protective role in KA-induced excitotoxic injury in brain through TNFR1 signalling.

2) There is a disparity of aging and gender in response to KA-induced hippocampal neurodegeneration. Aged female mice are more sensitive to KA-induced excitotoxicity.

3) Deficiency of IL-18 attenuates microglial activation in KA-induced excitotoxicity in aged brain, while the net effects of IL-18 are balanced by other cytokines, such as TNF-α, IL-6 and IL-10.

4) IL-18 deficiency does not prevent mice from EAN despite decreased levels of Th1 and Th2 cytokines.

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