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Functional analysis of astroglial IKK/NF-κB signaling in brain development and homeostasis

DISSERTATION

zur Erlangung des Doktorgrades Dr. rer. nat.
der Fakultät für Naturwissenschaften
der Universität Ulm

vorgelegt von

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aus Ulm

2012

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Tag der Promotion: 21.12.2012

Die Arbeiten im Rahmen der vorliegenden Dissertation wurden im Institut für physiologische Chemie der Universität Ulm durchgeführt und von Herrn Prof. Dr. Thomas Wirth betreut.

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Zusammenfassung

Neuroinflammation ist ein essentieller Prozess, der dem Schutz des zentralen Nervensystems vor Pathogenen und der Bewältigung von Verletzungen dient. Neuroinflammation tritt häufig auch als Begleiterscheinung bei neurologischen Erkrankungen auf, wobei der Beitrag neuroinflammatorischer Prozesse zur Pathogenese dieser Erkrankungen im Detail aber nur unvollkommen verstanden ist. Astrozyten sind Gliazellen, die in mehrere zentrale Schritte neuroinflammatorischer Reaktionen involviert sind, die aber auch wichtig für die Entwicklung und Homöostase des Gehirns sind. Die NF-κB-Transkriptionsfaktoren sind bekannt für ihre Schlüsselposition bei der Regulation von Entzündungsantworten, können aber auch die Proliferation, Differenzierung und das Überleben von Zellen entscheidend beeinflussen. Eine Reihe früherer Studien legt nahe, dass NF-κB in Astrozyten eine wichtige Rolle in neuroinflammatorischen Prozessen spielt. So konnte in Tiermodellen gezeigt werden, dass die genetische Inhibition des NF-κB-Signalwegs in Astrocyten inflammatorische Prozesse und deren Folgen in pathologischen Zuständen abschwächen kann.

In der vorliegenden Studie wurde ein neuartiges Mausmodell zur Aufklärung der Rolle von NF-κB in Astrozyten bei neuroinflammatorischen Prozessen entwickelt. Dieses konditionale Modell basiert auf der Astrozyten-spezifischen Expression einer konstitutiv aktiven Mutante der IκB Kinase 2 (IKK2), die essentiell für die Aktivierung des kanonischen NF-κB-Signalwegs ist. Dadurch wird eine dauerhafte NF-κB-Aktivierung in Astrozyten erzielt, welche eine starke inflammatorische Reaktion hervorruft. Diese ist charakterisiert durch die Produktion von Inflammationsmediatoren, die Aktivierung von Mikroglia und die Infiltration peripherer Immunzellen. Als pathologische Konsequenzen dieses neuroinflammatorischen Phänotyps wurden Defekte der Hirnentwicklung und eine Beeinträchtigung der neuralen Homöostase gefunden. Die Aktivierung von NF-κB in Astrocyten während der Hirnentwicklung führte zur Ausbildung eines Hydrozephalus aufgrund einer beeinträchtigten ependymalen Ziliogenese. Die Aktivierung von NF-κB in Astrozyten im voll entwickelten Gehirn führte dagegen nicht zur Hydrozephalusentstehung, resultierte stattdessen aber in einer Atrophie des Cerebellums und in einer Ataxie. Als Ursache wurde eine Störung der Unterstützungsfunktionen der Astrozyten-

verwandten Bergmann-Glia identifiziert, welche zur exzitotoxischen Degeneration der zerebellären Purkinje-Neuronen führt.

Die vorliegende Studie untermauert enscheidend die Sichtweise, dass Astrozyten neben Mikroglia wichtige Zellen des angeborenen Immunsystems des zentralen Nervensystems sind, welche über die Aktivierung von NF-κB Neuroinflammation induzieren können. Die Studie zeigt darüber hinaus zum ersten Mal einen kausalen Zusammenhang zwischen dem NF-κB-Signalweg und Hydrozephalusentwicklung, die häufig als Komplikation bei Erkrankungen mit einer starken neuroinflammatorischen Komponente auftritt. Schließlich bietet die NF-κB-vermittelte Aktivierung von Bergmann Glia eine bemerkenswerte mechanistische Erklärung für die spezifische Degeneration von Purkinje-Zellen, wie sie in einer Gruppe seltener neurodegenerativer Erkrankungen auftritt, den Autoimmunitäts-/Inflammations-induzierten zerebellären Ataxien.

Damit erweist sich das GFAP/IKK2-CA-Mausmodell als nützliches Modellsystem für die weitere Erforschung von Hydrocephalusentstehung und Autoimmunitäts-/Inflammations-induzierten zerebellären Ataxien. Darüber hinaus bietet es die idealen Voraussetzungen, um in Kombination mit Modellen neurologischer Erkrankungen, z.B. der Alzheimer-Krankheit, die Rolle inflammatorischer Prozesse in diesen Krankheiten zu untersuchen.

Summary

Neuroinflammation is an essential defense response to pathogens or injury in the central nervous system. It is also found in various types of neurological disorders, including neurodevelopmental and neurodegenerative diseases, where it is supposed to be involved in disease pathogenesis, although its precise contribution is only partially understood. Astrocytes are glial cells that are implicated in several central steps of the neuroinflammatory response, but they are also important in brain development and homeostasis. The NF-κB transcription factors are the key regulators of the inflammatory response that are also involved in cell proliferation, differentiation and survival. NF-κB activation in astrocytes is thought to be critical for neuroinflammatory responses, as previous studies showed that genetic inhibition of NF-κB signaling in astrocytes reduces neuroinflammation and its consequences in models of neuropathological insults.

In the present study a novel mouse model was developed to further elucidate the role of astroglial NF-κB signaling in neuroinflammation. This model conditionally expresses a constitutively active mutant of the IκB kinase 2 (IKK2), the activating kinase of canonical NF-κB signaling, specifically in astrocytes. This results in constitutive activation of NF-κB in astrocytes, which is sufficient to induce a prominent inflammatory response characterized by the production of inflammatory mediators, activation of microglia and infiltration of peripheral immune cells. Analysis of the pathological consequences of this neuroinflammatory phenotype revealed defects in brain development and an impaired neural homeostasis. Activation of astroglial NF-κB signaling during brain development resulted in hydrocephalus formation due to an impaired ependymal ciliogenesis. By contrast, activation of astroglial NF-κB in the fully developed brain did not cause hydrocephalus, but resulted in an atrophy of the cerebellum and ataxia. This was caused by a disruption of the support function of the astrocyte related Bergmann glia, which results in the excitotoxic degeneration of the cerebellar Purkinje neurons.

In conclusion, the present study strengthens the view that astrocytes along with microglia are key players of the innate immunity of the central nervous system, which are able to induce neuroinflammation by activating NF-κB signaling. With the demonstration of impaired ependymal ciliogenesis, the study also provides the first causal link between NF-κB signaling and hydrocephalus formation, which is a

frequent complication of disorders with a strong neuroinflammatory component. Finally, the study shows that NF-κB mediated activation of Bergmann glia might provide a mechanistic explanation for the specific degeneration of Purkinje cells in a group of rare neurodegenerative diseases, the autoimmune/inflammatory cerebellar ataxias.

As future perspective, the described mouse model might serve as a very useful tool to study hydrocephalus formation and autoimmune/inflammatory ataxias. In addition, it can be used in combination with other models of neurological disorders like Alzheimer's disease, to investigate the role of inflammatory processes in these diseases.

1 Introduction

Inflammation is an essential physiological response of the organism to various kinds of pathological insults, like invading pathogens and tissue injury. It induces diverse processes that are required for the resolution of such insults, in particular the infiltration and activation of cells of the innate and adaptive immune system at the site of inflammation. These cells can then eliminate invading pathogens and defective cells, thereby limiting damage and enabling tissue regeneration. On the other hand, the requirement for a fast and strong reaction on threats makes the inflammatory response prone to aberrant and overshooting activation, which can also increase tissue damage or can even cause autoinflammatory or autoimmune disease. Thus, the inflammatory response needs to be tightly controlled, which is especially important in the CNS, where damage is often largely irreversible due to its very limited regenerative capacity. Not only in acute brain injury and chronic neurodegenerative diseases, but also neurodevelopmental and psychiatric diseases inflammatory responses are found in the CNS. Therefore investigation of the regulation and consequences of neuroinflammation is of great interest for a better understanding and treatment of diseases that are of outstanding medical and social relevance, such as stroke, multiple sclerosis, Alzheimer's or Parkinson's disease.

1.1 Inflammation and immune responses in the CNS

Like other organs, the CNS needs inflammatory and immune reactions to cope with environmental threats like injuries and pathogens. The high structural complexity and limited regenerative capabilities of the CNS require an especially tight control of the local environment and potentially damaging inflammatory and immune reactions. Therefore some specific features have evolved that separate the CNS from the rest of the organism and make the CNS an "immune privileged" organ with reduced inflammatory and immune responses. Nevertheless, under certain conditions this barrier has to be opened to fight invading pathogens or for tissue repair after injury. But also in many other CNS diseases, inflammation and a leakiness of this barrier is found, and is believed to contribute to these diseases, e.g. in neurodegenerative disorders like Alzheimers disease (Wilson et al., 2010).

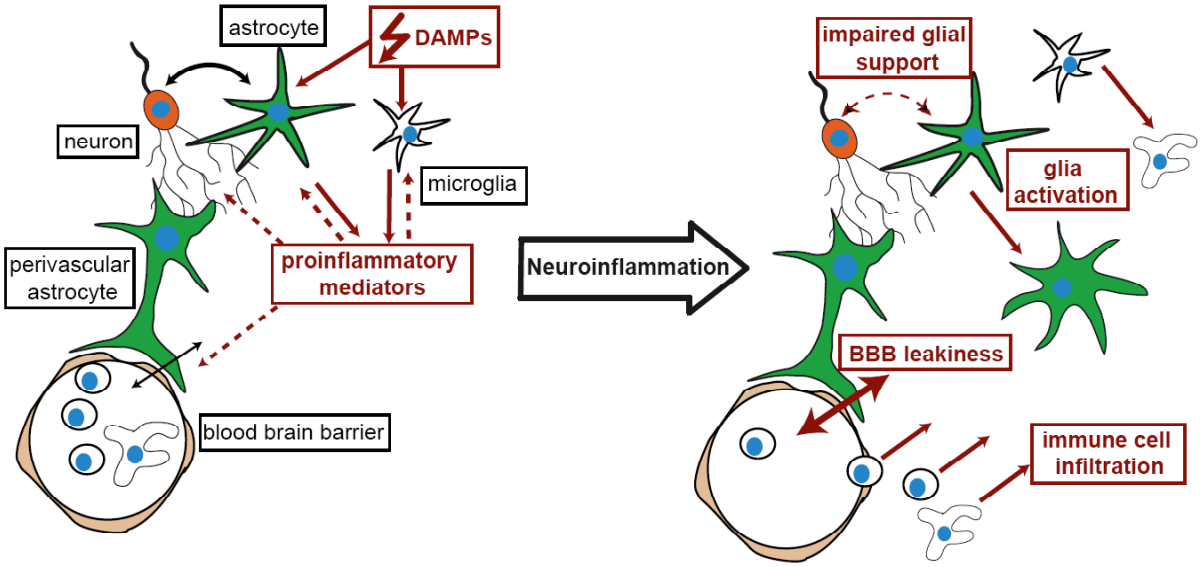

Figure 1: The inflammatory response in the CNS

Danger associated molecular patterns (DAMPs) activate proinflammatory signaling in microglia and astrocytes (left). This induces proinflammatory mediators like chemokines and cytokines (left), which act on various cell types (left, dashed lines). These factors then initiate immune cell infiltration and glial activation, which results in blood brain barrier (BBB) permeabilization and impairs the glial support for neuronal metabolism and synaptic transmission (right).

Key players in the induction of this specific neuroinflammatory response, which is described in detail in the next sections, are glial cells, especially microglia and astrocytes (Figure 1).

1.1.1 The immune privilege of the CNS

Already in the 19th century it was recognized that the CNS is an anatomical compartment with unique properties, first of all with a highly restricted access of soluble substances and peripheral cells, which is controlled by the blood-brain-barrier (BBB), a specialized endothelium of the brain blood vessels. The brain is also characterized by the absence of a lymphatic system, low levels of MHC protein expression and low numbers of professional antigen presenting cells, which altogether results in a reduced immune responsiveness of the CNS (Wilson et al., 2010).

The CNS compartment contains a special extracellular fluid, the cerebrospinal fluid (CSF), which fills the ventricular system of the brain. It provides a route for metabolite transport and protects the brain's fragile tissue structure by supporting its weight and

dampening concussions. The CSF is mainly secreted by a highly vascularized structure in the lateral ventricles, the so-called choroid plexus. The CSF flows through the ventricular system and is supposed to be finally resorbed in the subarachnoid villi. The CSF is slightly hyperosmotic due to an active transport of Cl^- from the blood into the CSF, but in contrast to blood plasma and other extracellular fluids it contains only low amounts of proteins (Speake et al., 2001; Brown et al., 2004).

1.1.1.1 The blood-brain barrier

The blood-brain barrier (BBB) is a specialized endothelium of brain blood vessels, which tightly controls the transport of metabolites, signaling molecules and other substances from the blood into the brain, as well as the invasion of peripheral immune cells (Wilson et al., 2010). The BBB (see Figure 2) actually consists of three main cellular components, the vascular endothelial cells, pericytes and astrocytes (Correale and Villa, 2009).

Endothelial cells connected by tight junctions assemble the actual diffusion barrier that completely prevents passive diffusion of water-soluble molecules. Therefore, under normal physiological conditions, nearly all substance exchange between blood and brain occurs transcellularly via transporters/channels for small molecules like water, ions, glucose and amino acids or via caveolae mediated vesicular transcytosis for proteins and other larger molecules (Ohtsuki and Terasaki, 2007; Correale and Villa, 2009; Wolburg et al., 2009).

The role of pericytes, which are located between the endothelial cells and the basement membrane, is not well studied. They seem to play a role in the development of blood vessels and the formation of the blood brain barrier (Correale and Villa, 2009).

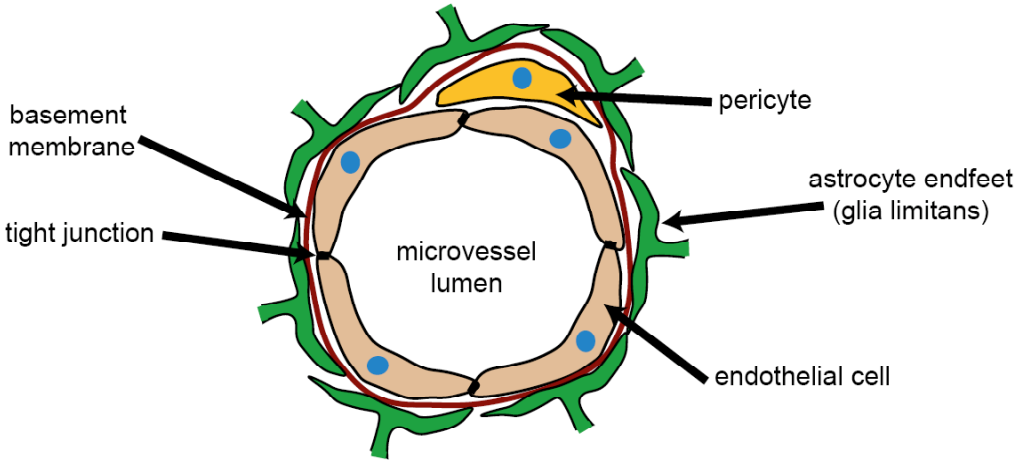

Figure 2: Structure of the blood-brain barrier (BBB)

The BBB consists of a diffusion barrier of endothelial cells linked by tight junctions, which is surrounded by a basement membrane and a layer of astrocyte endfeet (glia limitans). Pericytes are located between the endothelial cells and the basement membrane.

The outer border of the BBB is the glia limitans, consisting of astrocyte foot processes that ensheath about 90% of the cerebral microvasculature. Via this close association, astrocytes are thought to contribute to the special properties of the endothelium of the blood brain barrier, like the remarkably completely sealed tight junctions. This close association is also important for the regulation of the CNS water and ion homeostasis by astrocytes. Furthermore, astrocytes adjust the local blood flow according to neuronal requirements by sensing neuronal activity and secreting mediators that control vessel dilatation. In case of pathological events affecting the brain parenchyma, they also mediate the local breakdown of the BBB and the recruitment of immune cells (Correale and Villa, 2009; Sofroniew and Vinters, 2010).
The features mentioned above describe the BBB at the microvessels of the brain parenchyma, which is slightly different at certain specialized areas, namely in the meningeal zone and the circumventricular organs like the choroid plexus and the subcommissural organ. These regions, where the major exchange between the vascular system and the CSF is taking place (production and resorption of the CSF), show an altered structure and increased leakiness. Importantly, these sites are major entrance gates for immune cells in the course of diverse inflammatory reactions (Correale and Villa, 2009; Wilson et al., 2010).

1.1.1.2 The innate immune system of the CNS

Because of the strict separation from the bloodstream, under normal physiological conditions the CNS is not under restricted surveillance of the peripheral immune system. Instead, the CNS has its own specialized innate immunity.

In the classical perspective the myeloid microglial cells, which account for about 10 % of all glia (Barres, 2008), are generally regarded as specialized tissue macrophages that are the most important cells of this defense system. Beyond microglia, which reside in the brain parenchyma, there are smaller populations of macrophages at blood vessels (perivascular macrophages), at the meninges and at the circumventricular organs (Ransohoff and Perry, 2009).

While these other populations are phenotypically more closely related to classical macrophages, microglia possess a very distinct appearance and have specific functions in the normal physiological state. These features might be partially due to the lack of exposure of serum proteins, leading to a rather inactivated ("ramnified") phenotype. These ramnified microglia have a typical "glial" appearance, i.e. thin, branched processes. With these processes, microglia monitor the local environment. Upon certain signals, e.g. exposure to serum components due to a breakdown of the BBB or danger signals like extracellular ATP or TLR ligands, microglia get activated, start to produce proinflammatory mediators and change into an amoeboid, actively phagocytic phenotype (Ransohoff and Perry, 2009). Additionally, especially in infectious and autoimmune disease, activated microglia upregulate MHC class II proteins and costimulatory factors, turning them into dendritic cell-like antigen presenting cells (Tambuyzer et al., 2009). These activated microglia can exert typical immune cell functions like phagocytosis of pathogens and antigen presentation to induce an adaptive immune response. They can also remove cellular debris and apoptotic cells in acute injury and neurodegeneration. Indeed, in many neurodegenerative disorders a prominent activation of microglia is observed, which can have both protective and detrimental effects (Ransohoff and Perry, 2009; Tambuyzer et al., 2009).

Beyond these classical macrophage/immune cell functions, microglia are supposed to be involved in several developmental and homeostatic functions in the brain, namely developmental neuronal apoptosis, synapse elimination, angiogenesis, axon growth, neurogenesis and astrocyte differentiation (Ransohoff and Perry, 2009; Pont-Lezica et al., 2011).

In addition to microglia and other macrophage related cells, also astrocytes are important players in the innate immune response of the CNS, as will be discussed in more detail below. The production of proinflammatory chemokines, cytokines, complement proteins and cell adhesion molecules by astrocytes is critical for the recruitment and activation of microglia and peripheral immune cells (Farina et al., 2007). Furthermore, some evidence indicates that also astrocytes can express MHC class II proteins and might therefore act as antigen presenting cells (Dong and Benveniste, 2001; Williams et al., 2007).

1.1.2 Initiation of inflammation in the CNS

Despite the potentially dangerous side effects in the CNS, like the irreversible loss of neurons, certain situations require an inflammatory response to avoid uncontrolled pathogen spreading or to repair injured tissue.

As in other organs, inflammation in the CNS is usually initiated by the detection of highly conserved pathogen derived or tissue intrinsic alarm signals, so called pathogen/danger associated molecular patterns (PAMPs/DAMPs). PAMPs are conserved pathogen molecules that under normal conditions do not occur in the tissue, like LPS, peptidoglycans, or double stranded RNA. DAMPs are endogenous molecules that are released from ruptured cells when the tissue is damaged, e.g. intracellular components like chromatin, heat shock proteins (HSPs), adenosine or ATP (Marsh et al., 2009; Amor et al., 2010).

All these signals activate pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), intracellular nucleotide-binding oligomerization domain-like receptors (NLRs), adenosine receptors or the receptor of advanced glycation end-products (RAGE). Thereby proinflammatory signaling cascades like the IKK/NF-κB pathway are activated. In the CNS, especially astrocytes and microglia are important for the induction of an inflammatory response via PRR activation (Farina et al., 2007; Amor et al., 2010).

1.1.3 Mediators and effectors of the inflammatory response

Activation of these proinflammatory signaling pathways results in the induction of a large number of inflammatory mediators, like chemokines, cytokines, cell adhesion molecules and enzymes for the production of prostaglandins, NO and other small molecule mediators of inflammation. These factors initiate a sequential activation of the innate and adaptive immune system (Figure 3). Furthermore, this primary response also results in induction of acute phase proteins, effector proteins that provide a rapid initial defense to protect the tissue from damage and to control pathogen spreading and proliferation.

Among these acute phase proteins are antimicrobial proteins like components of the complement cascade like C3, C4, C9, mannose binding lectin, serum amyloid A and LPS binding protein. Other acute phase proteins limit cell damage when the tissue is injured, like antiproteases, components of the coagulation cascade and proteins that counteract oxidative damage like haptoglobin, ceruloplasmin and MnSOD (Gabay and Kushner, 1999; Lakota et al., 2011).

Chemokines like MCP-1/CCL2, RANTES/CCL5, IP-10/CXCL10 and SDF-1/CXCL12 and cell adhesion molecules like ICAM-1, VCAM-1 and several integrins are crucial for the recruitment of immune cells, i.e. for transendothelial migration and intraparenchymal trafficking of these cells in the CNS (Wilson et al., 2010).

The breakdown/leakiness of the BBB, which is observed during neuroinflammation, also seems to be partially mediated by the chemokine MCP-1, and by inflammation induced matrix metalloproteases (Candelario-Jalil et al., 2009; Semple et al., 2010).

While chemokines and cell adhesion molecules are directing immune cells to the site of inflammation, additional inflammatory mediators are required to activate these cells to induce a full immune response. Major mediators of this activation step are cytokines like TNFα, IFNγ and several interleukins, which exert various effects on both infiltrating leukocytes and microglia, like induction of proliferation, overall cell differentiation and upregulation of specific immune response genes.

As an early event in the immune response, phagocytosis by macrophages and microglia is induced by cytokines like TNFα, M-CSF, GM-CSF, TGF-β, and IFN-β/-γ.

As a prerequisite for the induction of an adaptive immune response, antigen presentation pathways are upregulated by cytokines like TNFα, IFN-γ, IL-1β, IL-6 and IL-12 (Town et al., 2005; Tayal and Kalra, 2008; Tambuyzer et al., 2009).

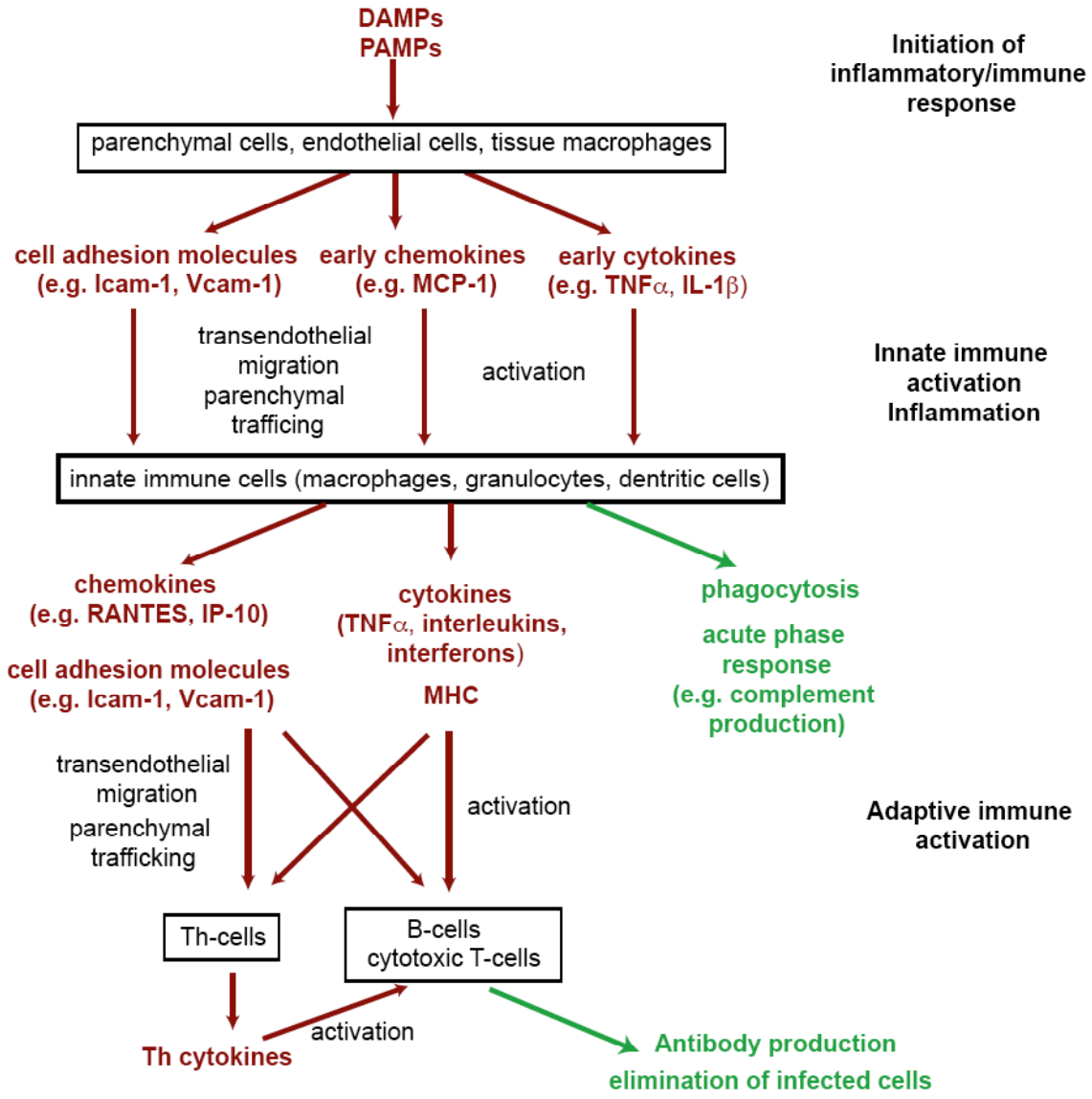

Figure 3: Molecular mechanisms of the activation of the immune system

Danger/pathogen associated molecular patterns (DAMPs/PAMPs) initiate inflammation by inducing the production of cell adhesion molecules, chemokines and cytokines in the tissue cells, resulting in the recruitment and activation of innate immune cells. These cells execute effector functions like phagocytosis and the acute phase response, and produce factors required for the initiation of an adaptive immune response, including chemokines, cytokines and MHC class II proteins.

Many of these cytokines are produced by macrophages and act in an autocrine manner, but also in a paracrine manner to activate other recruited immune cells. Various interleukins induce proliferation of B- and T-cells, and also maturation and differentiation of B- and T-cells is dependent on interleukins that are produced by antigen presenting cells or helper T-cells. For example plasma cell differentiation and

immunoglobulin production is induced by factors like IL-4 and IL-6. On the other hand, IFN-γ, IL-12 and IL-18 are involved in T_H1 polarization of helper T-cells, whereas IL-4 promotes T_H2 differentiation (Gutcher and Becher, 2007; Tayal and Kalra, 2008).

While the early cytokines TNFα and IL-1 act in a positive autoregulatory feedback loop to increase the inflammatory activation (M1 polarization) and cytokine production by macrophages and microglia, other cytokines have anti-inflammatory properties, especially TGF-β and IL-10, but also IL-4. They are involved in the resolution of inflammation and reduce inflammatory macrophage activation or induce an alternative, non-inflammatory macrophage activation, known as M2 polarization (Tayal and Kalra, 2008; Ransohoff and Perry, 2009).

1.1.4 Consequences of inflammation for CNS homeostasis

Beside their functions on immune cells, many proinflammatory factors, including chemokines, cytokines and cell adhesion molecules, exert additional effects on neural cells in both pathological and normal physiological states of the CNS. There exists a complex interaction network between the CNS and the immune system, by which both neuroinflammation and peripheral inflammation can alter brain function, which in turn can influence physiological processes in the periphery by neuronal signaling.

A well-known neuro-immune connection is the prostaglandin E mediated induction of fever by pyrogenic inflammatory cytokines like IL-1, IL-6 and TNFα in the hypothalamus (Steinman, 2008). These cytokines are also responsible for the behavioral alterations that accompany infections and other inflammation associated diseases. This “sickness behavior” is characterized by decreased motor activity, social withdrawal, reduced food and water intake, as well as alterations of the sleep rhythm, cognitive functions and pain sensitivity (Dantzer et al., 2008).

Several chemokines including MCP-1/CCL2, RANTES/CCL5, IP-10/CXCL10 and SDF-1/CXCL12 are also implicated in body temperature regulation and other neuroendocrine functions of the hypothalamus-pituitary system, like stress responses, water balance and food intake regulation (Callewaere et al., 2007).

The mechanistic details of these behavioral alterations are not or only partially understood, but several studies demonstrated the impact of inflammatory factors on differentiation and electrophysiology of neurons and glial cells.
IL-1, IL-6, IL-18, TNFα, IFN-γ, SDF-1/CXCL12 and nitric oxide were described to regulate proliferation and cell death of neural progenitor cells (NPC) under certain conditions. IL-6, IL-18, TNFα, IFN-γ and nitric oxide also influence differentiation of NPCs, usually shifting the balance from neuronal to astrocytic commitment. Additionally, in specific experimental settings these mediators could inhibit neurite outgrowth (Das and Basu, 2008; Whitney et al., 2009). Several chemokines are implicated in NPC migration, most prominently SDF-1/CXCL12, but also MCP-1/CCL2 (Liu et al., 2007; Tran et al., 2007; Li and Ransohoff, 2008).
Remarkably, several inflammation related molecules are able to modulate synaptic plasticity. The complement factors C1q and C3, and MHC I proteins are involved in the developmental elimination of synapses, while cytokines and chemokines like TNFα, IL-1, IL-18 and MCP-1/CCL2 can modify excitability of certain types of neurons (Boulanger, 2009; Kerschensteiner et al., 2009).

Finally, and especially important in the context of neurodegenerative diseases, inflammatory mediators can trigger neuronal cell death by several mechanisms.
The most direct way by which inflammatory mediators can kill cells is the activation of the extrinsic apoptosis pathway via death receptors of the TNFR1 family and subsequent caspase 8 activation, which then activates effector caspases directly or via the mitochondrial apoptosis pathway. This type of cell death can be induced by the proinflammatory cytokine TNFα and related death receptor ligands like FasL, TRAIL or TWEAK. Although these death receptor ligands are found in the CNS and they are implicated in pathogenesis of CNS disorders, there is sparse evidence for a major involvement of such a direct apoptotic death receptor signaling in neuronal cell death (Yakovlev and Faden, 2001; Haase et al., 2008; Mc Guire et al., 2011).
Instead, inflammation seems to promote neuronal cell death mainly indirectly by causing adverse environmental conditions that trigger the intrinsic apoptosis pathway, necrosis or atypical cell death types. Two major factors that impair neuronal survival in inflammation are oxidative and excitotoxic stress.
Inflammation can impair the function of mitochondria and antioxidative protection, e.g. by the glutathione system. Thereby levels of reactive oxygen species (ROS) in

the cell increase, resulting in oxidative damage of DNA, proteins and lipids, which can eventually lead to apoptotic or necrotic cell death. As neurons possess a high metabolic activity, they are very sensitive to mitochondrial dysfunction and produce large amounts of ROS, rendering them highly vulnerable to oxidative stress.

Beside this passive increase of oxidative stress, PRR signaling and inflammatory cytokines like TNFα and IL-1β are also known to induce iNOS and NADPH oxidase to actively generate NO and related reactive nitrogen species (RNS) or ROS respectively. These serve both as signaling molecules and antimicrobial agents, but both are also toxic to cells, if the protective antioxidant systems are overwhelmed (Halliwell, 2006; Roberts et al., 2009; Roberts et al., 2010).

Excitotoxicity is a neuron-specific type of cell death, which is caused by prolonged hyperexcitation, usually because of a deregulation of glutamatergic synaptic signaling. Inflammation contributes to excitotoxicity mainly by impairing reuptake of glutamate released from glutamatergic presynapses. Critical for this reuptake are glial cells, especially astrocytes (Tilleux and Hermans, 2007). The impaired reuptake in inflammatory conditions results in extracellular accumulation of glutamate, leading to an excessive activation of ionotropic glutamate receptors of the NMDA-, AMPA- and kainate receptor types. This results in excessive Ca^{2+} influx into the cytoplasm, which hyperactivates calcium-dependent signaling pathways e.g. via calmodulin. After prolonged stimulation it triggers activation of stress signaling pathways like ERK/JNK/p38 MAPK-cascades and NF-κB, thereby increasing ROS and NO production. Calcium influx also activates a group of cell death promoting proteases called calpains. Furthermore, depending on the cellular context, also caspases seem to be involved in some forms of excitotoxic cell death. Calpains and caspases finally cleave vital cellular structural proteins, as well as additional death promoting signaling proteins. One of these signaling proteins that was implicated in excitotoxic cell death is AIF, which induces DNA fragmentation similar as it is observed in classical apoptosis, but in a caspase independent manner (Lau and Tymianski, 2010; Wang and Qin, 2010; Kostandy, 2011).

1.1.5 The role of neuroinflammation in CNS disorders

Given the dual functions of many inflammatory mediators in the CNS and the immune system, the sensitivity of neurons for inflammatory damage and their limited regenerative capabilities, it is not surprising that neuroinflammation has a prominent role in various types of CNS disorders. These range from developmental defects over acute and chronic neurodegenerative pathologies to psychiatric diseases.

1.1.5.1 Neuroinflammation during CNS development

As many inflammatory mediators regulate migration, proliferation, differentiation and cell death not only of immune cells, but also of NPCs and immature neurons, the developing brain is especially vulnerable to inflammatory insults like prenatal and childhood infections and injury like perinatal subarachnoid bleedings, which are frequently occurring in preterm children.

Although the mechanistic connections are poorly understood, such inflammatory events are clearly associated with white matter defects (periventricular leukomalacia), cerebral palsy, autism and schizophrenia (Huleihel et al., 2004; Deverman and Patterson, 2009; Stolp and Dziegielewska, 2009). White matter damage and defects in neuronal maturation caused by inflammatory insults are also assumed to contribute to less defined motor, cognitive and behavioral impairments frequently found in preterm infants (Huleihel et al., 2004).

Another CNS defect, which is frequently associated with brain infections or injury, in particular during brain development, is hydrocephalus. Beside genetic developmental defects that cause hydrocephalus in about 1 of 1500 live births (congenital hydrocephalus), a large fraction of patients with inflammation-associated diseases develops an acquired hydrocephalus. For example, in 2 of 3 patients with tuberculous meningitis hydrocephalus occurs, as well as in 20-30 % of patients with subarachnoid bleedings (Mack et al., 1999; Zhang et al., 2006; Garg, 2010; Germanwala et al., 2010). This dilatation of the cerebral ventricles is a consequence of an increased pressure of the cerebrospinal fluid and results in an overall increased intracranial pressure, severe neurological symptoms and brain damage, which is finally lethal in 20-50% of non-treated patients (Poca and Sahuquillo, 2005). Proposed mechanisms that cause this increase of CSF pressure are an elevated production of CSF in the choroid plexus, an impaired resorption of CSF in the

subarachnoid villi because of fibrotic alterations of the subarachnoid space, or an impaired flow of the CSF through the ventricular system. The latter can be a consequence of obstructions of narrow parts of the ventricular system, or of defects of the ventricular epithelial cells, the ependymal cells, whose cilia are critical for the transport of the CSF through the ventricular system (Zhang et al., 2006).

1.1.5.2 Neuroinflammation in acute brain injury

Acute CNS damage is an important problem in public health, as it is a frequent and devastating consequence of cardiovascular disease (ischemic stroke) and all kinds of accidents with an injury of the head or spine (traumatic brain injury/TBI, spinal cord injury/SCI). This kind of injury is often lethal or else causes lasting disabilities due to the limited regenerative potential of the CNS. The primary tissue damage caused by these insults results in a massive release of DAMPs and therefore initiates a strong inflammatory response. Although it is important for the clearance of damaged cells and wound closure, this inflammatory response has a major contribution to a wave of secondary neuronal loss in the adjacent brain tissue (penumbra) found in these pathologies. This second wave of damage is an active cell death response of the tissue caused by mechanisms described above, mainly excitotoxic and oxidative damage (Guo et al., 2011; Helmy et al., 2011). A large number of studies demonstrated by different genetic and pharmacological approaches that dampening the inflammatory response by blocking e.g. IL-1 or TNFα signaling could reduce tissue damage in experimental TBI models. Nevertheless, other inflammatory mediators like IL-6 were shown to be protective in this paradigm, stressing the ambivalent role of inflammation in CNS injury (Helmy et al., 2011).

1.1.5.3 Neuroinflammation in chronic neurodegenerative disorders

The “classical” neurodegenerative diseases like Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD) and amyotrophic lateral sclerosis (ALS), but also many less common neurodegenerative diseases like the spinocerebellar ataxias (SCA) and prion diseases are traditionally thought to be caused by abnormal proteins that directly affect neuronal homeostasis and survival (“proteinopathies”). These proteins form putatively toxic aggregates like the intracellular tangles of hyperphosphorylated tau protein and the extracellular amyloid

plaques in AD, which were the first characteristic pathological alterations found in the brains of patients suffering from this disease. Nevertheless, the exact mechanisms causing such toxicities are generally poorly understood. During the last years it became increasingly evident that non-cell autonomous mechanisms are involved in many of these diseases. In particular subacute neuroinflammation was found as a common hallmark in the course of these chronic neurodegenerative diseases (Frank-Cannon et al., 2009; Ilieva et al., 2009; Glass et al., 2010; Garden and La Spada, 2012). However, under which circumstances such degeneration-associated neuroinflammation promotes disease progression as seen in acute injury, or when it has beneficial functions, is far from clarified.

For example, some studies suggest that the chronic use of non-steroidal antiinflammatory drugs (NSAIDs) reduces the risk of AD development. On the other hand, experimental approaches addressing the role of microglia in AD indicate that the observed activated microglia around amyloid plaques might rather be involved in the clearance of these potentially toxic aggregates (Frank-Cannon et al., 2009). For PD, experimental models suggest that neuroinflammation contributes to disease progression, as LPS administration into the substantia nigra can induce a loss of dopaminergic neurons as found in PD, and inhibition of TNFα signaling could reduce the loss of these cells in some PD models. But these models do not very closely resemble the human disease pathogenesis, and there is no clear evidence for a similar role in human PD yet, so these findings have to be regarded with caution (Frank-Cannon et al., 2009).

For HD and ALS, where good genetic mouse models are available, the role of inflammation is not well studied. While so far there is no evidence for an involvement of inflammation in HD progression, there are some hints that microglia activation contribute to disease progression in ALS, whereas different infiltrating T-cell populations might influence ALS pathogenesis in different directions (Frank-Cannon et al., 2009; Glass et al., 2010).

1.1.5.4 Autoimmune/autoinflammatory diseases of the CNS

Whereas for most of the neurodegenerative “proteinopathies” the contribution of inflammatory and immune responses are still unclear, there are several autoimmune disorders of the CNS known that are driven to a large extent by neuroinflammation.

The by far most frequent of these diseases is multiple sclerosis (MS), in which T- and B-cells destroy the myelin sheaths of CNS axons in an autoimmune reaction, finally resulting in progressive axonal degeneration and disabilities. The early (relapse-remitting) stage of the disease is characterized by phases of prominent inflammatory and autoimmune activity, demyelination and neurological impairments, and by quiescent phases with recovery. At later time points, after a decade or more, many patients develop a secondary progressive stage with a rather continuous axonal loss and progressive irreversible neurological deficits. The first stage is relatively well treatable with anti-inflammatory and immunosuppressive therapies, e.g. with IFN-β or Natalizumab, an antibody against $\alpha 4\beta 1$ integrin, that blocks infiltration of immune cells into the brain (Bhat and Steinman, 2009). Although the classical mouse model for MS, experimental autoimmune encephalitis (EAE), suggests that T-cells are the most important autoreactive cells in MS, recently Rituximab, an anti-CD20 antibody that depletes B-cells was proven to be very effective in the treatment of relapse-remitting MS (Bartok and Silverman, 2011). Whereas this first, clearly autoimmune stage of the disease is well treatable, there is no effective treatment for the secondary progressive stage of the disease or for primary progressive MS, that rather resemble classical neurodegenerative diseases (Bhat and Steinman, 2009; Bartok and Silverman, 2011).

There is a number of other autoimmune and autoinflammatory dieseases of the CNS described, although these are relatively rare and far less understood than MS.

Another autoimmune demyelinating disease of the CNS is neuromyelitis optica, which is associated with the appearance of autoantibodies against aquaporin 4, which seem to have a pathogenic role in this disease. It shares symptomatic similarities with MS and affects mainly the optic nerve and the spinal chord (Bhat and Steinman, 2009).

Paraneoplastic syndromes are autoimmune diseases that are occurring in certain types of cancer, e.g. small cell lung cancer (SCLC), Hodgkin's lymphoma or some types of breast or ovarian cancer. Interestingly, autoantibodies found in these diseases, are often directed against certain neural antigens, e.g. anti-Yo or anti-Hu antibodies. These syndromes frequently cause neurological disorders with neuroinflammatory and neurodegenerative pathologies like paraneoplastic cerebellar degeneration, opsoclonus-myoclonus or limbic encephalitis (Dalmau and Rosenfeld, 2008; Grant and Graus, 2009; Manto and Marmolino, 2009). Of note, cerebellar

ataxias, often associated with Purkinje cell degeneration, are also found in several other autoimmune diseases like celiac disease, autoimmune thyroiditis or systemic lupus erythematosus (Manto and Marmolino, 2009).

1.1.5.5 Neuroinflammation in psychiatric disorders

Acute infectious diseases and similar states of systemic inflammation are associated with changes in mood and cognitive function that partially overlap with symptoms of psychiatric diseases. This so-called “sickness behavior”, which is known for a long time, was linked to the action of inflammatory cytokines on the CNS during the last 20 years. This raised the question, whether also in classical psychiatric diseases inflammatory processes might play a role. Therefore in the recent years a number of clinical studies investigated whether there are associations between inflammatory markers, infectious or autoimmune diseases and psychiatric diseases. Indeed for several psychiatric disorders including major depression, bipolar disorder, schizophrenia and autism associations with inflammatory states were found, although in most cases it is not clear whether there is a causal link. Current knowledge is far away from understanding mechanistic relationships, as these diseases are very complex and the use of animal models is very limited for most of these diseases (Dantzer et al., 2008; Deverman and Patterson, 2009; Dean, 2011).

The only psychiatric diseases, in which the role of inflammation is relatively well understood, are depression and related mood disorders. Symptoms of depression and sickness behavior are to a large extent similar, and in fact clinical long-term cytokine therapies with IL-2 or IFN-α cause major depression as severe side effect in about one third of the patients, clearly showing that certain inflammatory mediators can themselves induce depression. Similar effects were found in rodent models, where e.g. LPS or IL-1β can induce depression-like behavior. Already early it was recognized that depression is associated with reduced levels of serotonin due to increased tryptophan degradation via the kynurenine pathway. It was shown that several cytokines, in particular IFN-γ and TNFα, induce the indoleamine-2,3-dioxygenase (IDO), the key enzyme of the kynurenine pathway, thereby linking inflammation to depression-related alterations in the tryptophan metabolism. Indeed it was demonstrated that inhibition of the IDO could block LPS induced depression-like behavior (Dantzer et al., 2008).

1.2 Astrocytes in CNS development, homeostasis and disease

For a long time, glial cells received little attention, as they were regarded as rather primitive bystander cells that exert some supportive functions for the neurons with their unique structural and functional characteristics.

However, the importance of glia is highlighted by the fact that glial cells seem to exceed neurons in numbers, although the estimations vary largely from a 1:1 ratio to up to 10-fold in the human brain, and there are prominent differences between brain regions and species (Lent et al., 2012). Moreover, it becomes increasingly clear that glial cells are a group of highly specialized, diverse cell types fulfilling many crucial functions in brain development and homeostasis. Remarkably, astrocytes are even directly involved in “neuronal” synaptic transmission (Parpura et al., 2012). Additionally, as described above, astrocytes and microglia are the main players in the specialized innate immune system of the brain that have important functions in pathological conditions (Farina et al., 2007). Except for microglia, the brain macrophages that are of hematopoietic origin, glia and neurons derive from a common developmental origin, the embryonal neuroectoderm (Kriegstein and Alvarez-Buylla, 2009).

1.2.1 Astrocyte-like glial cell types in the developing and adult brain

The most numerous population of cells in the CNS parenchyma are astrocytes, which were originally defined morphologically as large, highly branched stellate shaped cells. There were two major subgroups distinguished, protoplasmic and fibrous astrocytes (Sofroniew and Vinters, 2010). In the last decades, when specific marker antigens were discovered to distinguish different cell types, the intermediate filament “glial fibrillary acidic protein” (GFAP) was found to be expressed in these cells, but not in neurons, microglia or oligodendrocytes, the myelinating glial cells of the CNS. Therefore GFAP emerged as the now most accepted and used marker for astrocytes. However, not all astrocytes seem to express GFAP under every condition and there are morphologically and functionally different cells that also express GFAP, like different types of radial glia and the epithelial-like ependymal cells that line the ventricles. Despite approaches to define astrocytes by other markers, like the recently described aldehyde dehydrogenase 1-like 1 (Aldh1l1), there is no fully stringent discrimination between these cell types, and there is indeed evidence that

these cells rather belong to a continuous spectrum of related cellular entities (Cahoy et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Sofroniew and Vinters, 2010).
Already embryonic radial glia, which are both scaffolding cells and neural stem cells, express GFAP, and while the progenitors of neurons and oligodendrocytes lose this expression during their differentiation, other cells originating from these radial glia keep GFAP expression. Many of these cells differentiate in typical stellate astrocytes, but descendents of radial glia are also supposed to differentiate into ependymal cells, and to the adult stem cells of the subventricular zone ("B-type astrocytes") and the radial glia-like adult stem cells of the subgranular zone of the dentate gyrus (Kriegstein and Alvarez-Buylla, 2009; Sofroniew and Vinters, 2010).
Another population of GFAP expressing cells with radial glial morphology, which persist into adulthood, are the Bergmann glia of the cerebellum. Some studies suggest that these cells might also possess stem cell properties (Yamada and Watanabe, 2002; Koirala and Corfas, 2010).
Under normal physiological conditions in the adult animal only the GFAP expressing stem cells in the subventricular zone and subgranular zone are clearly giving rise to neurons. Nevertheless some studies found that under certain conditions also normal activated astrocytes can become neurogenic, further arguing for a very close relationship between all these GFAP expressing astrocyte-like cell types (Sofroniew and Vinters, 2010; Robel et al., 2011).

1.2.2 Functions of astrocytes in CNS homeostasis

Beside the important functions of astrocyte related stem cells in brain development, the classical parenchymal astrocytes have several important functions in normal physiological brain homeostasis (Figure 4).
As already described above, astrocyte endfeet that enwrap the brain's blood vessels form the glia limitans, an essential part of the blood brain barrier. This border position of astrocytes makes them crucial for the communication between the blood- and the brain-compartment. They are essential for adjusting the extracellular milieu of the brain to the requirements of the neurons by regulating water balance, pH and ion content of the interstitial fluid. They also take up glucose from the blood, store it as glycogen and metabolize it into lactate, which they supply neurons as a major energy source.

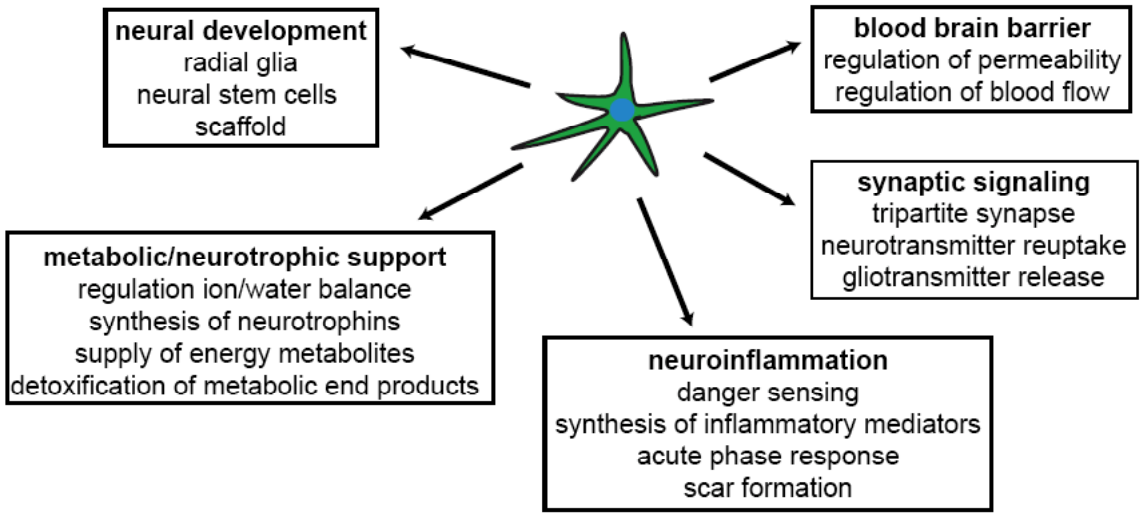

Figure 4: Physiological functions of astrocytes

They detect local environmental alterations, including direct sensing of neuronal activity, to adjust these parameters to the activity-dependent neuronal requirements. They release mediators to the blood vessels that regulate the local blood flow in order to adapt the supply of oxygen and nutrients to local brain activity (Sofroniew and Vinters, 2010; Parpura et al., 2012).

Astrocytes supply neurons not only with energy metabolites, but they also produce growth factors like EGF, IGF-1 and various neurotrophins like NGF, BDNF, GDNF and CNTF, that regulate proliferation, differentiation and survival of neural cells (Markiewicz and Lukomska, 2006; De Keyser et al., 2008).

Another key support function of astrocytes for neurons is the removal of potentially toxic metabolites and especially excess neurotransmitters from the extracellular space, to avoid hyperexcitation and resulting excitotoxic stress for neurons (Sofroniew and Vinters, 2010; Parpura et al., 2012).

During the last years it has become increasingly clear that astrocytes, beyond these support functions, are also directly involved in synaptic signaling.

For a long time, the signaling events at the synapse were thought to involve only two neurons, one pre- and one postsynaptic. However, recently the concept of the “tripartite synapse” emerged, which adds an astrocyte as a third player, as most synapses were found to be closely associated to astrocyte processes. Indeed it was shown that astrocytes can detect and respond to synaptic neurotransmitter release at a single synapse level (Perea et al., 2009; Panatier et al., 2011).

The perisynaptic astrocyte processes have similar neurotransmitter receptors as neurons, and synaptic neurotransmitter release induces a local cytoplasmatic Ca^{2+} influx, which can either result in a local response, or can spread over the whole astrocyte. These Ca^{2+}-waves can even propagate through neighboring astrocytes, as most astrocytes form gap junction connected syncytia with their neighbors. This global signal propagation is thought to be important for the adaptation of the already mentioned homeostatic functions to the neuronal activity, as well as for the communication between spatially and functionally separated neuronal networks. On the other hand, locally restricted responses of perisynaptic astrocyte processes modulate synaptic transmission at single synapse level by the release of so-called "gliotransmitters" like glutamate and ATP/adenosine (Perea et al., 2009; Panatier et al., 2011; Parpura et al., 2012).

These findings propose a more complex model of synaptic transmission, with complex bidirectional communication between neurons and astrocytes, in comparison to the simplistic unidirectional two-cell textbook-model.

1.2.3 Alterations of astrocyte functions in neuropathological conditions

As regulators of CNS homeostasis, astrocytes detect and respond with high sensitivity to alterations of the local environment. Therefore it is not surprising that astrocytes also react on all kinds of pathological conditions and that this response has an important influence on the consequences of these insults for the whole CNS.

Indeed astrocytes, in addition to microglia, can be regarded as part of the innate immune system of the brain that can detect many different types of endogenous and pathogen derived danger signals, as they express various types of TLRs, scavenger receptors and other PRRs. Activation of these receptors induces the expression of various inflammatory mediators in astrocytes, including the major inflammatory cytokines TNFα, IL-1β, IL-6 and chemokines like MCP-1/CCL2, RANTES/CCL5 and IP-10/CXCL10 (Farina et al., 2007). These factors on the one hand contribute to the recruitment and activation of immune cells, on the other hand they act in a paracrine manner on neural cells including astrocytes themselves. This also results in an increased permeability of the BBB, which is regulated by astrocytes (Correale and Villa, 2009; Sofroniew and Vinters, 2010).

Under pathological conditions, astrocytes undergo an activation process termed astrogliosis, which is induced by TLR-ligands, inflammatory mediators like IL-6, IL-1β, TNFα, IFN-γ and Lcn2, but also by growth factors like FGF2, TGF-β and EGFR-ligands and general stress factors like ROS, hypoxia, glucose deprivation and toxic proteins like Aβ (Correa-Cerro and Mandell, 2007; Lee et al., 2009; Sofroniew and Vinters, 2010).

These stimuli result in major morphological and functional alterations, characterized by an upregulation of GFAP and general hypertrophy of astrocytes. This process is fully reversible under moderate astrogliosis inducing conditions, whereas persistent and strong stimulation of astrocytes results in more prominent and irreversible structural alterations. In such a context the astrocytes start to proliferate and lose their normal domain architecture, in which a single astrocyte covers a certain volume unit without overlapping with the domain of the neighboring astrocyte. Strong astrogliosis distorts this normal brain microarchitecture and can finally result in the formation of a dense astroglial scar (Sofroniew and Vinters, 2010).

The functions of this cellular response of astrocytes to pathological insults are only partially understood and different studies often show conflicting findings. Astrogliosis might help to restrict the spreading of tissue damage and infectious agents by forming a physical barrier that protects the healthy tissue. Under some circumstances activation and proliferation of astrocytes seems to reduce oxidative and excitotoxic stress by the generally neuroprotective and homeostasis preserving functions described above. Furthermore, activated astrocytes cannot only enhance inflammatory responses but also seem to exert anti-inflammatory functions under certain conditions. Nevertheless, a large amount of evidence indicates that activation of astrocytes interferes with their normal protective physiological functions, thereby even increasing tissue damage and impairing tissue regeneration. By their proinflammatory function, astrocytes augment inflammatory tissue stress, e.g. oxidative stress. It is also well documented that most inflammatory conditions induce a downregulation of the astrocytic glutamate transporters EAAT1/GLAST and EAAT2/GLT-1, resulting in an impaired glutamate uptake by astrocytes and therefore increased excitotoxic stress. As astrocytes are also able to actively release glutamate as gliotransmitter, their dysfunction can even revert glutamate flow, resulting in an active enhancement of excitiotoxicity by astrocytes. Finally, disruption of the BBB and

altered distribution of astroglial water channels like Aqp4 can contribute to edema formation (Tilleux and Hermans, 2007; Sofroniew and Vinters, 2010).

1.2.4 Role of astrocytes in specific CNS disorders

Although astrocyte activation is found in a large variety of neurological diseases, the precise role of astrocytes in most of these diseases is not or only partially understood. So far, only few disorders could be identified, which are caused by primary astrocyte dysfunction.

Alexander's disease, which is caused by dominant gain-of-function mutations in the GFAP gene, is a severe neurological disorder with seizures and motor defects resulting in premature death. It is characterized by the intracellular accumulation of GFAP containing protein aggregates in astrocytes, the so-called Rosenthal fibers, and can be experimentally reproduced in mice by overexpression of GFAP (Sofroniew and Vinters, 2010; Allaman et al., 2011). Another brain pathology, which is likely caused primarily by astrocyte dysfunction, is hepatic encephalopathy. It develops in the course of severe liver dysfunction, which causes high blood ammonia levels. In the brain, ammonia is detoxified by astrocytes by metabolization to glutamine, which accumulates in the astrocytes and results in astrocyte swelling, disrupting their normal function and causing an increase of intracranial pressure (Allaman et al., 2011). Finally, astrocytes or related progenitor cells are most likely the origin of the most abundant and dangerous brain tumors, the glioma. These include the highly aggressive and basically non-curable glioblastoma multiforme, which is one of the cancer entities with the worst prognosis. In mouse models, glioblastoma formation was described to be induced e.g. by overexpression of c-Myc in GFAP positive cells, indicating that astrocytes or related cells are the origin of these tumors (Jensen et al., 2003).

Although astrocyte activation is seen in many and diverse other neurological disorders, the functional relevance of astrocytes in these diseases is only partially studied. Because of the complex functions of astrocytes and the involvement of other cell types, *in vitro* studies or pharmacological approaches are only of limited use to understand astrocyte functions in these diseases. The best way to address the

specific role of astrocytes in neurological diseases is the combination of *in vivo* disease models with genetic approaches that specifically target astrocytes. Most of these models use different variants of the GFAP promotor to express transgenes specifically in astrocytes and related GFAP expressing glial cells. This approach can be used for the direct overexpression of potentially functionally relevant genes, for the cell type specific conditional gene deletion with Cre/loxP-systems or for the cell ablation by “suicide genes” like the herpes simplex virus thymidine kinase (Pfrieger and Slezak, 2012).

In neurodevelopmental disorders, recent findings using such models suggest that astrocytes seem to be involved in the pathogenesis of fragile X syndrome, the most frequent inherited cause of mental retardation, and in Rett’s syndrome, an inherited autism spectrum disorder (Jacobs and Doering, 2010; Lioy et al., 2011). Expression of an artificial opioid receptor related G-protein coupled receptor in GFAP expressing cells was shown to induce early postnatal hydrocephalus, suggesting that astrocytes might under certain conditions be involved in hydrocephalus formation (Sweger et al., 2007).

In different mouse models of acute brain damage by mechanical injury or ischemia, conflicting roles of astrocytes were reported. For example, ablation of reactive astrocytes seems to exacerbate inflammatory CNS damage in traumatic brain injury (TBI), and focal ischemia results in larger lesions in animals lacking GFAP, which both suggests a protective function of astrocytes (Laird et al., 2008; Barreto et al., 2011). On the other hand, blocking proinflammatory signaling in astrocytes by inhibiting NF-κB activation reduces tissue damage in spinal chord injury (SCI) and retinal ischemic injury, demonstrating a damage promoting role of astrocytes by enhancing inflammation (Brambilla et al., 2005; Dvoriantchikova et al., 2009).

In *in vivo* models of several chronic neurodegenerative proteinopathies, including PD, ALS, and the polyglutamine diseases HD and SCA7, the astrocyte specific expression of the disease causing mutant proteins is sufficient to at least partially drive the corresponding diseases (Allaman et al., 2011). If these proteins induce disease specific neurotoxic properties in astrocytes, or if a general astrocyte dysfunction is underlying this non cell-autonomous neurodegeneration, is not clear yet. For the cerebellar Purkinje neurons, which are the main cell type affected by SCA7 and which are also relatively sensitive to various other types of

neurodegenerative insults, it was shown that manipulation of the closely associated Bergmann glia in various models was sufficient to induce neurodegeneration. This suggests that at least for Purkinje cells an unspecific astrocyte dysfunction is sufficient to drive neurodegeneration (Cui et al., 2001; Custer et al., 2006; Tao et al., 2011; Wang et al., 2011).
In autoimmune/autoinflammatory CNS disorders, the inflammatory functions of astrocytes might be of special importance. Indeed, blocking of astrocytic NF-κB activation in EAE improves disease outcome, by inhibiting the inflammatory functions of astrocytes (Brambilla et al., 2009b).

Although the role of astrocytes in CNS disorders is far from being fully understood, these studies indicate that astrocytes are of great importance for the development of many CNS disorders. They partially lose their physiological neuroprotective functions, and they can also gain adverse functions. Beside their ability to form scars that impair regeneration, mainly overshooting inflammatory responses seems to be a major mechanism of astrocyte mediated pathogenesis of CNS diseases.

1.3 The NF-κB transcription factor system

The key regulator of inflammatory responses is the nuclear factor κB (NF-κB) signaling pathway. The NF-κB transcription factors were first described in 1986 as a group of proteins binding to enhancer elements of the immunoglobulin κ light chain gene (Sen and Baltimore, 1986). They are activated by various cytokines and general stress factors and induce a broad spectrum of target genes that are involved most prominently in inflammatory and immune signaling, but also cell death, proliferation and differentiation.

1.3.1 Basic principles of the NF-κB signaling pathway

The activation pathways and the transcriptional responses induced by NF-κB signaling are highly complex and diverse, which is described in more detail below. In general, in the resting state, the dimeric NF-κB transcription factors are kept in the cytoplasm by binding to the inhibitor of NF-κB proteins (IκB), which mask the nuclear translocation domain of the NF-κB proteins. Activating stimuli cause the release of

the NF-κB dimers, allowing their nuclear translocation and binding to the DNA at specific binding sites with the degenerated κB consensus sequence 5'-GGGRN-A/T-YYCC-3' (N – any base, R – purine, Y – pyrimidine). At these sites NF-κB can regulate transcriptional activity in a positive or negative manner, depending on dimer composition, posttranslational modifications and the interaction with other proteins (Hayden and Ghosh, 2008).

The key step of NF-κB activation, the release of NF-κB dimers from the IκB proteins, is in most cases initiated by the phosphorylation of IκBs by the IκB kinase complex (IKK). This phosphorylation labels the IκB proteins for ubiquitination and subsequent proteasomal degradation. IKK proteins mediate the two major NF-κB activation pathways, the canonical and the non-canonical pathway (Hayden and Ghosh, 2008), which are described below.

1.3.2 Components of the NF-κB signaling pathway

The components of the NF-κB system are highly conserved among vertebrates, and also in invertebrates homologues of these proteins were found, best characterized in *Drosophila* (Minakhina and Steward, 2006).

1.3.2.1 The NF-κB family of transcription factors

In mammals, the NF-κB family consists of five members, namely p65 (RelA), RelB, c-Rel, p50 (NF-κB1, with its precursor p105) and p52 (NF-κB2, with its precursor p100), which can form a large variety of homo- and heterodimers. Of those RelA/p50, p50/p50 and RelB/p52 dimers are best characterized, but nearly all subunit combinations except some RelB containing heterodimers seem to occur (Hoffmann et al., 2006; Wietek and O'Neill, 2007). The large number of subunit combinations, their different DNA binding specificities and possibilities of posttranslational regulation enable a complex transcriptional response (Hoffmann et al., 2006). In the CNS, RelA and p50 are the dominant subunits (Mattson and Meffert, 2006; Huh et al., 2009), although all other subunits are reported to occur under specific conditions (Denis-Donini et al., 2005; Cao et al., 2008; Pizzi et al., 2009).

All NF-κB proteins share the conserved N-terminal Rel-homology domain (RHD), allowing DNA binding, dimerization and IκB interaction. RelA, RelB and c-Rel contain

a C-terminal transactivation domain, which is lacking in p50 and p52. Therefore, only dimers containing RelA, RelB or c-Rel can directly induce gene expression, whereas p50 and p52 homodimers and p50/p52 heterodimers usually act as transcriptional repressors. Instead of a transactivation domain, the precursors of p50 and p52, p105 and p100, have a C-terminal IκB like ankyrin-repeat domain, which relates them also to the IκB protein family. Processing of the precursor p105 to p50 occurs mainly co-translationally in a constitutive manner, while p100 is mainly processed stimulus dependent via the alternative NF-κB activation pathway (Hayden and Ghosh, 2008).

1.3.2.2 The IκB family

The IκB family consists of six proteins (IκBα, -β, -ε, -ζ, Bcl-3 and IκBNS) or eight, if the NF-κB precursors p100 and p105 are included. Their common structural feature is a domain containing multiple ankyrin repeats, which enables their interaction with NF-κB dimers (Ghosh and Hayden, 2008). Of the IκB family, mainly IκBα, the most abundant member, IκBβ and IκBε are classical inhibitor IκB proteins, which keep the NF-κB dimers inactive in the cytoplasm. IκBα and IκBε are themselves induced by NF-κB with different kinetics, thereby providing a two-step negative feedback loop important for shutting off NF-κB signaling (Hayden and Ghosh, 2008).

p105 can block the nuclear translocation of newly formed dimers containing this subunit till processing occurs, but it can also bind to existing NF-κB-dimers. In the latter case it is completely degraded similar to a classical IκB protein. Its C-terminus can also be transcribed from an alternative promotor, and can act as a classical IκB protein termed IκBγ. p100 is of special importance for the stabilization of its main binding partner RelB, but can also act on other NF-κB subunits (Ghosh and Hayden, 2008; Hayden and Ghosh, 2008).

The other members, IκBζ, Bcl-3 and IκBNS, modulate NF-κB transcriptional activity by interaction with NF-κB dimers in the nucleus. Bcl-3 possesses a transactivation domain that enables activation of transcription from promotors with bound p50 or p52 homodimers, that else act as transcriptional repressors. IκBζ and IκBNS do not possess a transactivation domain, they seem to specifically regulate transcription by binding to p50 homodimers at certain promotors, e.g. the IL-6 promotor (Ghosh and Hayden, 2008).

1.3.2.3 The IKK complex

The IKK complex is the central regulator of NF-κB signaling for most activating stimuli. It consists of the two enzymatically active subunits IKK1 (IKKα), IKK2 (IKKβ) and a regulatory subunit, the NF-κB essential modifier (NEMO or IKKγ). IKK1 and IKK2 are two closely related serine/threonine kinases containing several conserved domains, including the N-terminal kinase domain and domains for protein-protein interactions and regulation of kinase activity, in particular a leucine zipper, a helix-loop-helix-domain and the C-terminal NEMO binding domain (NBD). The kinase domain of both proteins contains two conserved serine residues at positions 176 and 180 in IKK1 or positions 177 and 181 in IKK2 respectively (in the human proteins). These residues can be phosphorylated by upstream kinases or by autophosphorylation, resulting in IKK complex activation. Mutation of these residues to alanine abolishes IKK activation, while the phosphomimetic mutation to glutamate results in constitutive activity of the IKK complex. Beyond these two kinases, there are two other related kinases belonging to this protein family, namely IKKi (IKKε) and TBK1, which are not part of the classical IKK complex (Scheidereit, 2006; Hayden and Ghosh, 2008).

The third IKK component, NEMO, is not structurally related to the other IKK proteins and does not possess a catalytic activity, but is an essential structural and regulatory component of the IKK complex. NEMO contains the N-terminal kinase binding domain, the minimal oligomerization/ubiquitin binding domain and a C-terminal zinc-finger domain (Scheidereit, 2006).

Various complexes of the different subunits, including IKK1/2 homo- and heterodimers can be found in specific conditions *in vitro* and *in vivo*, but the typical IKK complex *in vivo* seems to consist mainly of complexes of a $(NEMO)_2$(IKK1)(IKK2) stoichiometry, which can oligomerize into larger complexes. Nonetheless, whereas NEMO and IKK2 are indispensable for canonical NF-κB signaling, they seem not to be absolutely required for non-canonical NF-κB activation (Hayden and Ghosh, 2008). IKK1 vice versa seems to play only a minor role in canonical signaling, but is the central player in non-canonical signaling. This indicates the existence of different functional complexes with different properties, probably within the same cell. Furthermore, beyond this core complex, at least two other proteins, HSP90 and ELKS, were found closely associated to this complex, although their functional relevance is unclear (Hayden and Ghosh, 2008).

Whereas the classical IKK complex is located in the cytoplasm, at least NEMO and IKK1 can also be found in the nucleus. In the case of NEMO, nuclear/cytoplasmic shuttling is required for canonical NF-κB activation by DNA damage, while for IKK1 nuclear functions unrelated to IκB phosphorylation were described, e.g. phosphorylation of the histone H3 or the transcriptional coactivators CBP and SMRT (Scheidereit, 2006; Chariot, 2009).

Although the IκB proteins are the most important and best characterized IKK substrates, IKK1 and IKK2 also phosphorylate a spectrum of other proteins, like the mentioned nuclear IKK1 substrates, signaling molecules like IRS-1 and β-catenin, transcription factors like RelA, IRF7 and FOXO3a and others (Chariot, 2009).

1.3.3 Regulation of NF-κB signaling

The modulation of target gene expression by NF-κB is a complex process involving several levels of regulation.

NF-κB signaling is induced by a large variety of stimuli, e.g. cytokines like TNFα, IL-1β, BAFF, LT-β, CD40L, microbial components recognized by Toll-like receptors (e.g. LPS), oxidative stress and DNA damage (Oeckinghaus and Ghosh, 2009; Miyamoto, 2011; Morgan and Liu, 2011). Many of these stimuli also activate NF-κB in the CNS, but additionally there are CNS specific signals that induce NF-κB, like neuronal activity, neurotransmitters (e.g. glutamate), neurotrophic factors, but also neurotoxic proteins or peptides involved in CNS diseases like Aβ (Kaltschmidt et al., 2005).

Most of these signals converge at the level of the IKK complex on mainly two pathways, the canonical and the non-canonical or alternative pathway (Figure 5). Beside these pathways there are some conditions reported (“atypical pathways”), in which IKK independent mechanisms initiate NF-κB nuclear translocation (Figure 5). Downstream of NF-κB nuclear translocation a large variety of regulatory mechanisms modify the outcome of the NF-κB response, depending on the stimulus, the cell type, the physiological state of the cell and the specific target gene.

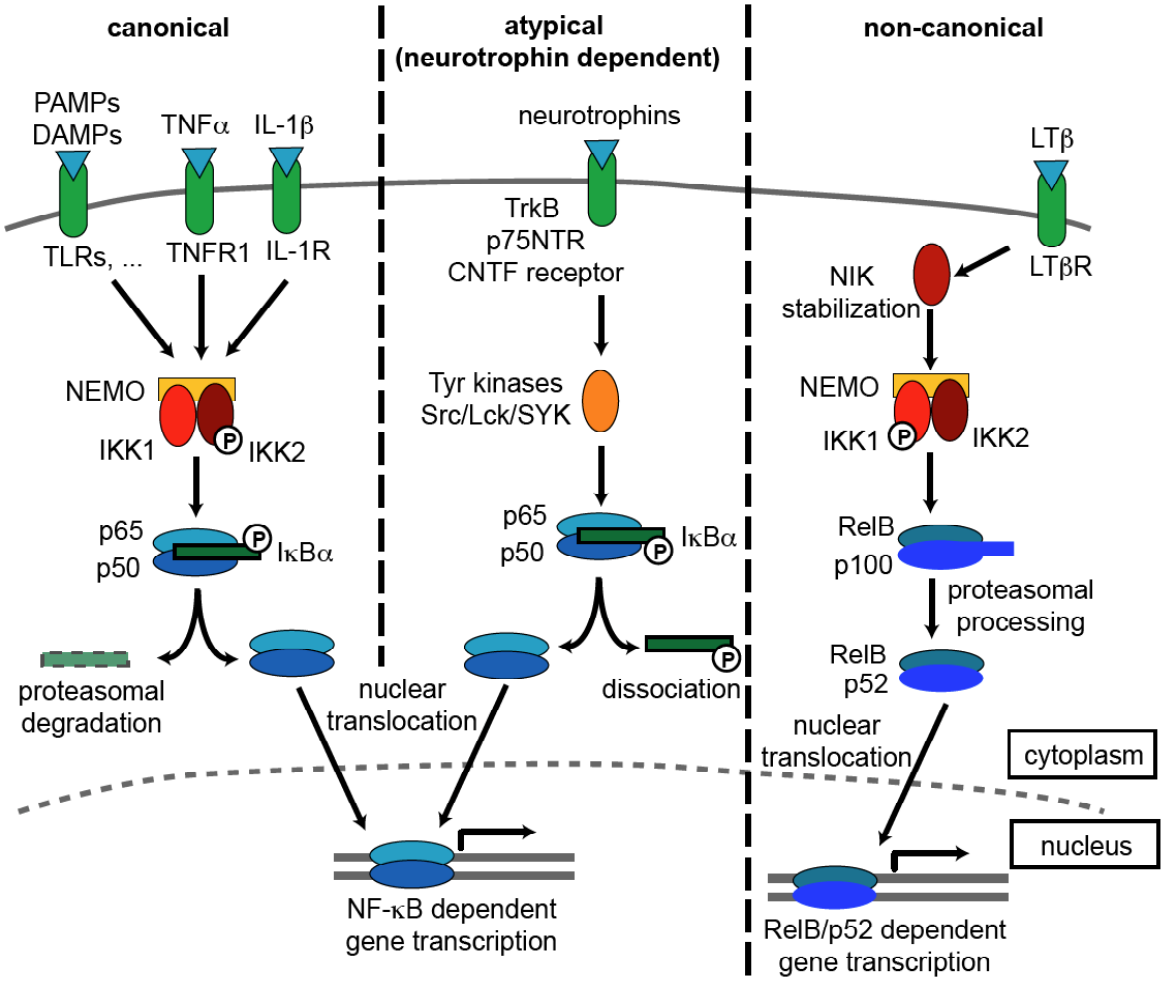

Figure 5: NF-κB activation by cytokines and neurotrophins in the CNS

Classical proinflammatory agents induce NF-κB activation via the canonical pathway, resulting in the IKK2-dependent phosphorylation (Ser-32/36) and degradation of IκBα (left). Certain growth factors like neurotrophins activate NF-κB in an atypical pathway by tyrosine kinases, resulting in Tyr-42 phosphorylation and dissociation of IκBα (middle). Non-canonical NF-κB signaling activates RelB/p100 dimers by NIK/IKK1 mediated processing of p100 to p52 (right).

1.3.3.1 The canonical NF-κB activation pathway

Many signal transduction pathways induce canonical NF-κB activation via the IKK complex, requiring NEMO and IKK2. Among these pathways are the well studied signaling cascades from the TNFα receptor super-family with the prototypical TNFR1, or the IL-1β receptor (IL-1R)/toll-like receptor (TLR) family. Upon ligand binding, these receptors recruit ubiquitin ligase complexes via adapter proteins (TRADD for TNFR1 and MyD88 and IRAK for IL-1R and TLRs). These ubiquitin ligase complexes like TRAF2/5/cIAP1/UbcH5 for the TNFR1 and TRAF6/Ubc13 for

the IL-1R/TLR then polyubiquitinate further adaptors like RIP1 (in TNFR1 signaling) or IRAK1 (in IL-1R/TLR signaling). The polyubiquitin chains built at these adaptors act as a scaffold to recruit the IKK complex, which binds via the NEMO UBD, and the TAB2/3/TAK1 kinase complex. This allows phosphorylation and activation of IKKs by TAK1 or by trans-autophosphorylation. Another player that was described to be involved in IKK activation by these receptors is the HOIP/HOIL-1L ubiqutin ligase complex, which can initiate NEMO oligomerization via linear polyubiquitin chains, resulting also in trans-autophosphorylation (Shih et al., 2011).

The IKK complex activated by these mechanisms can then phosphorylate IκB proteins with IKK2 as active kinase. These phosphorylations (Ser-32 and Ser-36 in human IκBα) are the signal for their ubiquitination by the SCF/βTRCP E3 ubiquitin ligase, which results in their proteasomal degradation (Shih et al., 2011).

Similar mechanisms activate NF-κB after activation of B-cell or T-cell receptors (Gupta et al., 2010).

A special case of NF-κB activation via NEMO/IKK2 is the activation by DNA damaging agents. After DNA damage, nuclear NEMO is sumoylated and phosphorylated by the DNA damage activated kinase ATM. This causes a monoubiquitination of NEMO that is a signal for the nuclear export together with ATM. In the cytoplasm, NEMO is polyubiquitinated and joins with IKK1 and IKK2 to reassemble an IKK complex in an preactivated state similar to the ubiquitinated IKK containing complexes described above, which then finally gets activated by TAK1 or autophosphorylation (Janssens and Tschopp, 2006; Miyamoto, 2011).

1.3.3.2 Non-canonical NF-κB activation

The non-canonical pathway is responsible for the activation of NF-κB-dimers that are inhibited by p100, mainly RelB/p100-dimers, but also RelA/p52 and RelB/p52 dimers that are inhibited by p100, which in this setting acts as a classical IκB protein and is also named IκBδ (Basak et al., 2007; Shih et al., 2011).

The non-canonical pathway is activated by certain cytokines like LT-β, BAFF or CD40L and is mediated by IKK1 and the kinase NIK, whereas it does not require IKK2 and NEMO. IKK1 activation is induced by phosphorylation by the upstream kinase NIK that is negatively regulated by a complex containing TRAF2/3 and cIAP1/2, which induces proteasomal degradation of NIK. NIK and activated IKK1

both phosphorylate p100, which results in proteasomal processing of p100, i.e. degradation of its IκB domain, allowing the nuclear translocation of the previously inactive NF-κB dimers (Hayden and Ghosh, 2008; Shih et al., 2011).

1.3.3.3 Atypical NF-κB activation pathways

Beyond these two major pathways, some stimuli activate NF-κB signaling by completely IKK independent mechanisms. Among these is UV radiation, which via p38 signaling induces CKII dependent phosphorylation, ubiquitination and degradation of IκBs (Neumann and Naumann, 2007). Oxidative stress induced by hydrogen peroxide activates both canonical and atypical NF-κB signaling, the latter mediated by several tyrosine phosphorylations of IκBs that result in the dissociation of the IκB from the NF-κB dimer (Morgan and Liu, 2011). One of these phosphorylations, at Tyr-42 in IκBα, also mediates NF-κB activation downstream of certain growth factors. These include the neurotrophins BDNF, NGF and CNTF that activate the tyrosine kinases Syk or Src, which then phosphorylate IκBα (Gutierrez and Davies, 2011).

1.3.3.4 Regulation of NF-κB transcriptional activity

The activation pathways described above are necessary for the nuclear translocation of NF-κB dimers, but are not always sufficient to induce NF-κB dependent transcription, which indicates the requirement of additional activation steps. Furthermore, the NF-κB dependent transcriptional response is not uniform, but depends on the cell type, physiological state of the cell, and the inducing stimulus.
The first layer of regulation specifies those potential NF-κB target gene promoters, to which the NF-κB dimers can actually bind. These are on the one hand defined by the physical accessibility of the site, e.g. the local chromatin structure, on the other hand by the exact binding site sequence and the NF-κB dimer composition, as the different NF-κB subunits seem to have different binding affinities depending on the exact sequence of the NF-κB binding site (Hoffmann et al., 2006). Furthermore, certain posttranslational modifications (e.g. Ser-276 of RelA) and the interaction with other transcription factors like STAT1 and IRF3 can alter the DNA binding affinity or specificity of NF-κB transcription factors (Wietek and O'Neill, 2007).

The second layer of transcriptional regulation by NF-κB in the nucleus is the modulation of the transcriptional activity by posttranslational modifications and the recruitment of cofactors. This kind of regulation can enhance, reduce or even reverse transactivation or transrepression abilities of a certain NF-κB dimer. A large number of posttranslational modifications for all NF-κB subunits have been described, most extensively studied for RelA, where several phosphorylations and acetylations were found. Furthermore ubiquitinations, methylations, a cysteine oxidation and a specific proline isomerization were described. These modifications alter the transcriptional activity, DNA-binding, IκB-binding or recruitment of cofactors, thus enabling a fine tuning of the NF-κB transcriptional response by other signaling pathways (Perkins, 2006; Huang et al., 2010). A well-studied example is the phosphorylation of RelA at Ser-276 by PKA or MSK1, which allows the recruitment of the histone acetylase and transcriptional coactivator p300/CBP. Another important phosphorylation site in RelA is Ser-536, by which IKKs, TBK1 or RSK1 can induce the recruitment of p300/CBP, similarly increasing RelA transcriptional activity. Phosphorylation of Ser-468 by GSK-3β, IKK2 or IKKi (IKKε) by contrast reduces transcriptional activity by stimulating its ubiquitination and degradation (Huang et al., 2010).
In some cases, the recruitment of cofactors can even reverse the direction of the transcriptional regulation. For example the atypical IκB protein Bcl-3, which contains a transactivation domain, can induce transcription by binding to the transcriptional repressor p50/p50 (Ghosh and Hayden, 2008). Further complexity is added by interaction of NF-κB with other transcription factor systems, e.g. p53, nuclear receptors, IRF- or STAT-proteins, which can result either in synergistic or competitive/repressive effects (Neumann and Naumann, 2007; Wietek and O'Neill, 2007; Oeckinghaus et al., 2011).

1.3.3.5 Inactivation of NF-κB signaling

Whereas much research focused on the complexity of its initiation, the inactivation of NF-κB signaling is studied in less detail. So far, two major principles to inactivate the NF-κB response are described, namely the nuclear export of NF-κB dimers by newly synthesized IκB proteins, and the proteasomal degradation of the activated NF-κB proteins.

IκBα, IκBβ and IκBε are induced by NF-κB, which provides a negative feedback loop, as they bind to the active NF-κB dimers and promote their nuclear export, thereby inactivating the NF-κB transcriptional response. Alternatively, nuclear NF-κB complexes can be ubiquitinated by the ECS ubiquitin ligase complex, containing the proteins Elongin B/C, Cullin-2, SOCS1 and COMMD1, or the ubiqutin ligase PDLIM2. This targets the active NF-κB for proteasomal degradation, also resulting in the inactivation of the NF-κB transcriptional response (Ghosh and Hayden, 2008; Wan and Lenardo, 2010).

To shut off NF-κB signaling, not only the active NF-κB proteins have to be degraded or exported from the nucleus, but also the upstream signaling cascade has to be reset to a basal inactive state. Apart of the negative feedback mechanisms affecting different signaling pathways upstream of IKK, there are also feedback mechanisms acting on the level of the IKK complex. First, IKK2 can autophosphosphorylate a serine cluster in its C-terminal HLH domain, which reduces IKK activity. Second, the phosphatases PP2A and PP2Cβ can remove the activating phosphorylations of IKK2. Furthermore, several chaperones and heat shock proteins like Hsp70 and Hsp27 can inhibit IKK signaling, probably by disrupting IKK complex assembly. A fourth mechanism is the removal of ubiquitinations that are crucial for IKK activation by deubiquitinases like CYLD or A20 (Scheidereit, 2006).

1.3.4 Functions of NF-κB signaling

As complex and diverse as its upstream regulatory mechanisms are the transcriptional targets and physiological functions of NF-κB signaling. Over the years hundreds of NF-κB target genes have been described, with functions in all kinds of cellular processes. One of the first discovered and most prominent functions of NF-κB is the regulation of inflammation and immune responses. In addition, functions in cell proliferation, differentiation and cell death/survival were described and seem to play a role in various neoplastic and degenerative diseases. Interestingly, beyond these functions, in the CNS very specific and distinct functions in neuronal signaling, learning and memory are emerging (Figure 6).

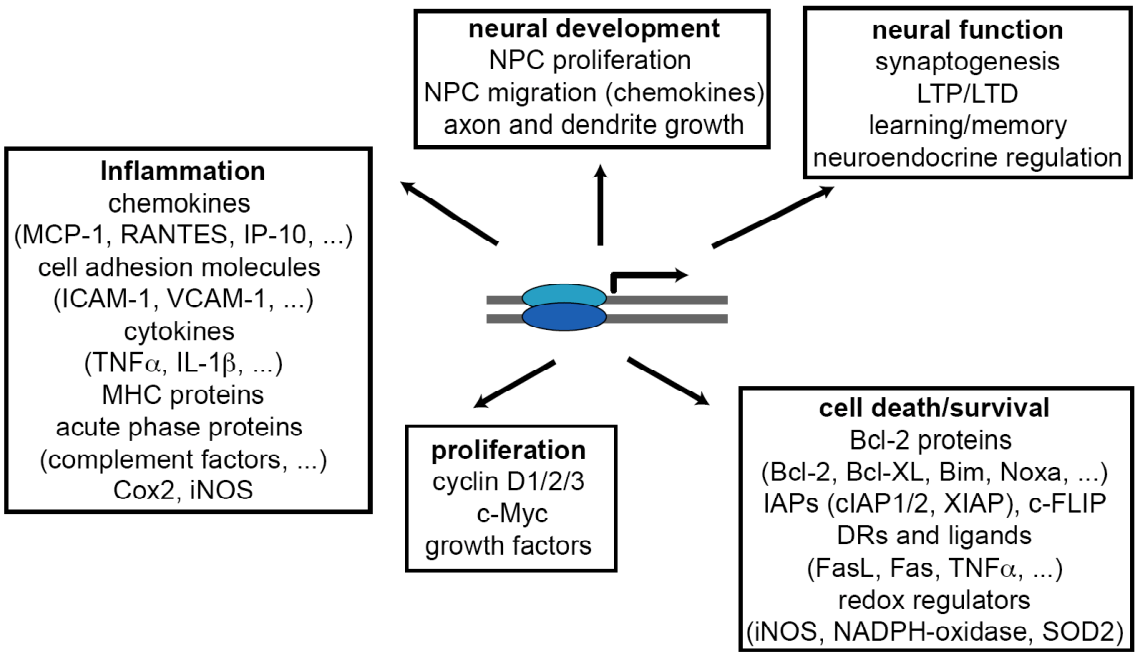

Figure 6: NF-κB functions in the CNS and related NF-κB target genes

Global inactivation of RelA or the components of the IKK complex is embryonically/perinatally lethal, and inactivation of other components of the NF-κB pathway results in a variety of often severe defects in development and immune responses. Because of these dramatic global defects, much knowledge about the specific functions of NF-κB is derived from *in vitro* models and conditional knock-out and transgenic mouse models that modulate NF-κB signaling in specific cell types (Gerondakis et al., 2006).

1.3.4.1 NF-κB as a master regulator of inflammation

A large proportion of NF-κB target genes is implicated in proinflammatory signaling and immune functions. Among those are genes for many chemokines like MCP-1 (CCL2), Rantes (CCL5) or IP-10 (CXCL-10), which mediate cell migration, especially the migration of immune cells to a site of inflammation. Several cell adhesion molecules like ICAM-1 or VCAM-1, which are important for the trafficking of immune cells, are also induced by NF-κB. Beside the recruitment, the activation of immune cells is mediated by NF-κB target genes like interleukins, TNFα and related factors and enzymes that are involved in the generation of small molecule inflammatory

mediators, like COX-2 and iNOS. Some of these NF-κB target genes are themselves activators of NF-κB signaling (e.g. TNFα, IL-1β), and provide a positive feedback loop, which allows the rapid and strong response to potential threats. NF-κB also induces many other genes required for the direct or immune cell mediated defense against invading pathogens, like acute phase proteins, complement factors and MHC proteins (Gupta et al., 2010).
Therefore, NF-κB can be regarded as an alarm switch, which leads to the orchestrated activation of multiple defense mechanisms required to cope with diverse threats like pathogens and tissue injury.

The key proinflammatory function of NF-κB is obvious in *in vitro* models, and is also demonstrated in many human diseases and animal models.
Increased NF-κB activation was found in a large number of disorders with inflammatory components, like autoimmune diseases, sepsis, cancer, but also acute and chronic neurodegenerative pathologies (Senftleben, 2008; Wong and Tergaonkar, 2009). Many anti-inflammatory drugs, including the classical NSAIDs like aspirin, exert their anti-inflammatory action at least partially by inhibition of NF-κB signaling (Senftleben, 2008; Gupta et al., 2010).
Genetic inhibition of NF-κB signaling by tissue specific conditional deletion of IKK2 or NEMO, or transgenic expression of non-degradable IκB proteins or dominant negative acting IKK2 mutants, prominently reduces inflammation and improves outcome in several inflammatory disease models, e.g. in models for sepsis, arthritis, lung inflammation and pancreatitis (Senftleben, 2008). This was also shown in CNS diseases or injuries, were genetic inhibition of NF-κB activation in neurons and/or glial cells can reduce inflammation and tissue damage e.g. in models of spinal chord injury, cerebral ischemia, and experimental autoimmune encephalitis (Brambilla et al., 2005; Herrmann et al., 2005; van Loo et al., 2006). Vice versa, genetic activation of NF-κB signaling by expression of a constitutively active IKK2 mutant can itself induce inflammation in several tissues, e.g. in the airway epithelium, the pancreas, the liver, the heart or the intestinal epithelium (Sadikot et al., 2003; Baumann et al., 2007; Vlantis et al., 2011; Maier et al., 2012; Sunami et al., 2012).

These findings have raised hopes that specific pharmacological inhibitors of NF-κB signaling might be potent tools for the treatment of diseases with hyperinflammatory

components. Indeed, preclinical studies with specific IKK/NF-κB-inhibitors show beneficial effects in certain conditions, especially in inflammatory/autoimmune diseases. For example the disruption of the IKK complex by a cell permeable peptide corresponding to the NEMO binding domain of IKK2 inhibits inflammation in mouse models for multiple sclerosis (experimental autoimmune encephalitis), inflammatory bone disease and arthritis (Senftleben, 2008). In the CNS, this peptide could also reduce inflammation in a mouse model for Parkinson's disease (Ghosh et al., 2007), while BMS-345541, a small molecule inhibitor of IKK2 improved outcome in cerebral ischemia (Herrmann et al., 2005).

Nevertheless, because of its broad spectrum of functions, strong and long-lasting inhibition of NF-κB signaling can also cause severe side effects that so far have prevented the clinical use of specific IKK/NF-κB inhibitors (Gupta et al., 2010).
Under certain conditions the loss of other NF-κB functions can mask the anti-inflammatory outcome of NF-κB inhibition and even can itself induce tissue damage and subsequent inflammatory reactions. Several animal models highlight these two faces of NF-κB signaling and its inhibition. In the liver, loss of NF-κB signaling highly sensitizes hepatocytes for TNFα induced apoptosis, but protects the liver tissue from damage by ischemia/reperfusion and chronic alcohol administration by reducing inflammation (Wullaert et al., 2007). Inhibition of NF-κB signaling in the intestinal epithelium increases tissue destruction in acute damage models, probably by increased apoptosis, resulting in an impaired epithelial barrier function. However, in chronic inflammation models inhibition of NF-κB signaling improved disease outcome by reducing the inflammatory response (Spehlmann and Eckmann, 2009). A similar picture is seen in the skin, where an inhibition of NF-κB signaling in keratinocytes can induce inflammation and keratinocyte apoptosis, which both can be blocked by inactivation of TNFR1. But also the classical proinflammatory role of NF-κB was observed in the skin, as IκBα deficient mice also develop an inflammatory skin disease, in this paradigm due to enhanced activation of NF-κB (Sur et al., 2008).

1.3.4.2 Specific functions of NF-κB in the central nervous system

NF-κB signaling was shown to be critically involved in processes like inflammation and cell death, which are implicated in the pathogenesis of neurodegenerative diseases. Furthermore, high basal NF-κB activity levels in neurons and neurological deficits in mouse models with impaired NF-κB signaling raised attention to the role of NF-κB in the central nervous system (Kaltschmidt and Kaltschmidt, 2009). This lead to the discovery of new specific functions of the NF-κB system in the CNS, in particular a prominent role in the unique differentiation and signaling processes in neurons.

At the earliest stage of neural differentiation, on the level of neural stem and progenitor cells, NF-κB seems to regulate both migration and proliferation. The findings on the role of NF-κB in proliferation of neural progenitor cells (NPCs) are controversial: while TNFα can induce NPC proliferation via NF-κB dependent signaling *ex vivo*, NF-κB dependent impairment of adult neurogenesis in the hippocampus is found in mice after stress exposure (Widera et al., 2008; Koo et al., 2010). NF-κB can also alter migration of NPC by the induction of chemokines like CXCL12 (SDF-1α) and CCL2 (MCP-1), which are NPC guidance molecules (Widera et al., 2008).

At later developmental stages, NF-κB signaling is involved in the regulation of axon and dendrite growth and branching in a complex manner, which is only partially understood yet.

For perinatal nodose ganglion sensory neurons it was shown that BDNF and CTNF induced axon growth and branching is mediated by NF-κB signaling, mainly by an atypical tyrosine kinase dependent NF-κB activation mechanism. In contrast to these findings, canonical NF-κB signaling via IKK2 could inhibit neurotrophin mediated axon outgrowth in several model systems. These studies suggest that the additional Ser-536 phosphorylation of RelA by IKK2 is responsible for this switch in function. Other studies in turn suggest a role of canonical NF-κB signaling in axon initiation and guidance, although this function is less well understood (Gutierrez and Davies, 2011). NF-κB signaling is not only involved in axonal development, but also in dendrite growth. Several studies show that NF-κB activation enhances outgrowth and

maintenance of dendrites in cortical pyramidal neurons, embryonic hippocampal neurons and cerebellar Purkinje cells (Gutierrez and Davies, 2011).

Beyond these functions in neuronal differentiation, NF-κB is also implicated in synaptic plasticity. Already early findings showed that the neurotransmitter glutamate could activate NF-κB in glutamatergic neurons, suggesting a role in the transcriptional response to synaptic signaling. Indeed, IKK, IκBs and NF-κB complexes are found at synaptic sites and p65 is actively transported to the nucleus after synapse stimulation in glutamatergic neurons, where it might modulate synaptic transmission by increasing PKA/CREB signaling (Kaltschmidt et al., 2005; Kaltschmidt and Kaltschmidt, 2009). Additionally recent work demonstrated that formation of dendritic spines and subsequent synapse formation is impaired when NF-κB activation is inhibited, pointing out that indeed NF-κB is a key factor for synaptic plasticity (Kaltschmidt and Kaltschmidt, 2009; Boersma et al., 2011; Gutierrez and Davies, 2011; Schmeisser et al., 2012).

These effects on synaptic signaling and plasticity translate into altered electrophysiological properties of the neurons and influence behavior and cognition. Overall or glutamatergic neuron specific inhibition of NF-κB signaling decreases LTP and LTD and results in impaired learning and memory in several mouse models, whereas inhibition of NF-κB preferentially in inhibitory GABAergic interneurons seems to result in opposite effects. Consequences of this altered neurophysiologic properties are not only found in hippocampal learning, but also in neuroendocrine functions of the hypothalamus, namely in the regulation of sleep and food intake, and also in models of stress behavior and addiction (Kaltschmidt and Kaltschmidt, 2009; Gutierrez and Davies, 2011).

Beside these highly specialized functions in neuronal development and synaptic signaling under physiological conditions, NF-κB is also important under pathological conditions as a regulator of neuroinflammation and cell death. In glial cells this classical role of NF-κB is often dominant, but there are also nervous system specific processes modulated by NF-κB signaling in glial cells. For example, myelination by Schwann cells in the peripheral nervous system, but not by oligodendrocytes in the CNS, is NF-κB dependent (Raasch et al., 2011). While the role of NF-κB in oligodendrocytes is not well studied, in microglia and astrocytes NF-κB is clearly

important under pathological conditions for their activation and for the production of inflammatory mediators.

As macrophage related cells of myeloid origin, microglia are important players in the inflammatory response of the brain. Their activation by pathological insults, pathogen associated molecular patterns (e.g. via TLR signaling) and/or proinflammatory cytokines induces signaling events that result in a prominent production of chemokines, cytokines and other proinflammatory mediators (e.g. prostaglandins and nitric oxide). These factors activate stress responses in neural cells and allow recruitment and activation of peripheral immune cells in the brain (Tambuyzer et al., 2009). Many of these inflammatory mediators are typical NF-κB target genes, therefore the importance of microglial NF-κB signaling in the CNS under pathological conditions is obvious (Ransohoff and Perry, 2009; Glass et al., 2010), although the distinction between NF-κB signaling in resident microglia and peripheral macrophages is difficult to assess *in vivo*. Nevertheless, genetic modulation of NF-κB signaling *in vivo* in myeloid cells shows prominent effects in different models of CNS disease. Inhibition of microglial NF-κB signaling by targeted deletion of IKK2(fl/fl) in myeloid cells driven by a LysM-Cre transgene massively reduced microglia activation and neuronal cell death induced by kainate excitoxicity and MCAO (Cho et al., 2008). Correspondingly, activation of NF-κB signaling by targeted deletion of IκBα in microglia exacerbated inflammation and their pathological consequences in experimental autoimmune encephalitis (Ellrichmann et al., 2012).

In astrocytes an important proinflammatory role and some more specific neural functions of NF-κB are described, which are discussed in detail below (see 1.4).

1.3.4.3 Functions of NF-κB signaling in proliferation and cell death

Beyond the classical function of NF-κB in inflammation and its specialized functions in the CNS, it is implicated in a number of other physiological and pathophysiological processes.

One of these processes with an outstanding role of NF-κB signaling is the regulation of cell survival and cell death. This is responsible for several major defects in mouse models deficient for components of the NF-κB pathway, most prominently the massive TNFα induced hepatocyte apoptosis that results in embryonic lethality of animals deficient of RelA, IKK2 or NEMO (Gerondakis et al., 2006). Accordingly,

several other cell types were shown to have increased apoptosis sensitivity when NF-κB signaling is impaired, including hematopoietic cells like macrophages and epithelial cells, e.g. in the intestine (Gerondakis et al., 2006; Spehlmann and Eckmann, 2009). Vice versa, many tumors show high levels of NF-κB activity, increasing apoptosis resistance of these cells (Dutta et al., 2006; Ben-Neriah and Karin, 2011). This classical antiapoptotic function can be explained by several well-established antiapoptotically acting NF-κB target genes like Bcl-2, Bcl-XL, c-FLIP, cIAP1/2 and XIAP (Dutta et al., 2006).

On the other hand, under certain conditions NF-κB activation can also increase apoptosis sensitivity, which was demonstrated e.g. after certain DNA damage inducing treatments of RelA deficient fibroblasts or the activation of NF-κB signaling in Burkitt's lymphoma (Campbell et al., 2004; Klapproth et al., 2009). The increase of cell death by NF-κB activation can have different reasons. First, NF-κB also regulates some proapoptotic target genes, including death receptors (e.g. Fas, DR5) and their ligands (e.g. FasL, TNFα), and proapoptotic Bcl-2-proteins like Bax, Bim and Noxa (Dutta et al., 2006; Inta et al., 2006). Second, NF-κB can repress antiapoptotic target genes instead of inducing them, which was shown e.g. after DNA damage with doxorubicin or UV-C irradiation of fibroblasts (Campbell et al., 2004). Third, the inflammatory environment resulting from NF-κB activation *in vivo* can indirectly impair cell survival, e.g. by increasing oxidative stress (Morgan and Liu, 2011).

These two faces of NF-κB signaling were also demonstrated in the CNS both *in vitro* and *in vivo*. Several studies reported a protective role of NF-κB activation in neurons after treatment with DNA-damaging, excitotoxic or oxidative stress inducing agents and also Aβ (Kaltschmidt and Kaltschmidt, 2009). By contrast, others described conditions in which NF-κB inhibition can be protective, e.g. after stimulation with TWEAK or Aβ or the DNA damaging agent camptothecin (Aleyasin et al., 2004; Potrovita et al., 2004; Valerio et al., 2006).

Because of the more complex situation *in vivo*, with cell-cell- and organ-organ-interactions and global influences of the environment, it is even more difficult to understand or predict the role of NF-κB signaling in a specific *in vivo* condition. For example in MCAO, a well studied stroke model, deletion of p50 or pharmacologic inhibition of NF-κB signaling in a preconditioning stimulation increases infarct size,

suggesting a protective role of NF-κB signaling. By contrast, several other models with blocked NF-κB signaling, e.g. some pharmacological approaches, conditional RelA deletion or neuron specific blockade of IKK signaling reduced infarct size, indicating a cell death promoting function of NF-κB (Kaltschmidt and Kaltschmidt, 2009; Ridder and Schwaninger, 2009). In general, the non cell-autonomous, especially the inflammatory functions of NF-κB are often dominant *in vivo*, therefore *in vivo* a cell death promoting outcome of NF-κB activation seems to occur more frequently than predicted from earlier *in vitro* data.

Beside its functions in inflammation and cell survival, NF-κB signaling also regulates cell proliferation. Among the described NF-κB targets that promote proliferation are intracellular factors like c-Myc and the cyclins D1/2/3, and, maybe more importantly, a number of growth factors and pro-proliferative inflammatory cytokines (Gupta et al., 2010).
As all these three functions in inflammation, proliferation and cell death inhibition are important in the course of tumor initiation and promotion, enhanced NF-κB signaling is frequently found in cancers, although NF-κB can also act as tumor suppressor under certain conditions (Ben-Neriah and Karin, 2011).

1.4 Role of NF-κB signaling in astrocytes

As described above, neuroinflammation is important for the response of the CNS to pathogens and acute and chronic injury, but it also seems to contribute to the pathogenesis of many CNS disorders. Astrocytes are key players in these inflammatory responses, as well as in CNS development and normal CNS homeostasis. The NF-κB signaling pathway is well characterized as a critical regulator of inflammation in many tissues, and has many additional functions in cell proliferation, differentiation and survival. Therefore it is not suprising that NF-κB signaling in astrocytes seems to have important functions in CNS development, homeostasis and disease.
Indeed a large number of NF-κB target genes like proinflammatory cytokines (e.g. TNFα, IL-1β, IL-6, CCL2, CCL5, CXCL10, iNOS, NADPH oxidase, complement proteins) and growth factors (e.g. NGF, BDNF) that are known to be involved in these processes, can be produced by astrocytes (Farina et al., 2007; Glass et al., 2010;

Gupta et al., 2010). Furthermore, also the astroglial glutamate transporters EAAT1/GLAST and EAAT2/GLT-1, which are supposed to be important for the neuroprotective function of astrocytes, are putative NF-κB regulated target genes that, dependent on the context, can be induced or repressed by NF-κB (Kim et al., 2003; Dallas et al., 2007; Boycott et al., 2008).

Because of the widespread functions of NF-κB signaling in various cell types, the dissection of specific NF-κB functions in astrocytes *in vivo* requires genetic approaches that target NF-κB signaling specifically in this cell type. So far, the best characterized model for this purpose is a transgenic mouse line that overexpresses a dominant negative mutant of IκBα (IκBα-dn) under the control of a fragment of the human GFAP promotor, on which many astrocyte targeted transgenic models are based. This IκBα mutant possesses a N-terminal truncation that includes the serine residues that are normally phosphorylated by the IKK complex, thereby preventing IκBα-dn degradation and canonical NF-κB activation (Brenner et al., 1994; Brambilla et al., 2005).
This mouse line (GFAP.IκBα-dn) was generated by the group of John R. Bethea and first described in 2005 in a model of SCI. While under normal conditions these animals with inhibited astroglial NF-κB signaling did not show any overt phenotype, after SCI they displayed reduced expression of proinflammatory chemokines and cytokines and reduced production of TGF-β and proteoglycans involved in glial scar formation. This resulted in a reduced lesion volume, increased white matter preservation and improved functional recovery in this paradigm (Brambilla et al., 2005). Later the group showed that the reduced scar formation in this model indeed improves the regeneration of the dissected spinal chord axons (Brambilla et al., 2009a). Whereas in this model of acute brain damage inhibition of NF-κB activation had a neuroprotective and regeneration promoting function, another group, which in parallel developed a similar mouse model (GFAP.IκBa-SS32/36AA), could not show an effect of astroglial NF-κB in a MCAO stroke model, another model of acute brain injury, (Zhang et al., 2005). In ischemic neuronal injury in the retina, blockade of astrocytic NF-κB signaling by GFAP.IκBα-dn reduced expression of proinflammatory cytokines, chemokines, cell adhesion molecules and inflammatory ROS stress inducers (iNOS and components of the NADPH oxidase). This resulted in reduced

microglia activation and reduced neuronal loss, which seems at least in part due to reduced NADPH oxidase dependent ROS production (Dvoriantchikova et al., 2009; Barakat et al., 2011).

Beside acute brain injury, the role of astroglial NF-κB in CNS autoimmunity was also studied in the EAE model of MS. A study with mice with a Nestin-Cre induced conditional deletion of IKK2 or NEMO in neuroectodermal cells (i.e. neurons, astrocytes and oligodendrocytes), showed reduced infiltration of inflammatory cells and reduced severity of the disease, which was postulated to be due to impaired expression of proinflammatory mediators by astrocytes (van Loo et al., 2006). This was largely confirmed with the GFAP.IκBα-dn model, where EAE severity (paralysis) and expression of proinflammatory mediators was also prominently reduced. In contrast to the model mentioned before, there was even increased infiltration of T-cells observed, mainly of anti-inflammatory regulatory T-cells, in the chronic phase of the disease, when there was a major symptomatic improvement. In addition to the symptomatic improvement, the group also showed reduced neuronal damage in this model (Brambilla et al., 2009b).

The role of astroglial NF-κB signaling in classical chronic neurodegenerative diseases is not studied in detail yet. One group investigated the role of astroglial NF-κB in ALS, in a third independently generated mouse line with a GFAP.IκBα-SS32/36AA transgene, which was crossed to the $SOD1^{G93A}$ model of ALS. Rather unexpectedly they could not detect any effect of astroglial NF-κB on ALS onset or progression (Crosio et al., 2011).

The studies on the GFAP.IκBα-dn model clearly indicate that activation of astroglial NF-κB prominently enhance neuroinflammatory responses to pathological insults, but is dispensable for normal brain development and function.

However, two studies on the behaviour of this mouse line suggest that there might be some subtle alterations of normal CNS function. The first study showed that sensitivity to formalin induced pain, which is associated with inflammatory processes, is reduced by blocking astroglial NF-κB (Fu et al., 2007). The second study found impaired performance of female mice with GFAP.IκBα-dn in certain tests for learning and memory, which might be related to an impairment in long-term potentiation. This finding was explained by a reduced expression of the glutamate receptor mGluR5 and the synaptic scaffolding protein PSD95 in cortex and hippocampus (Bracchi-Ricard et al., 2008).

1.5 Aims of the study

As outlined above, previous studies have shown that a block of canonical NF-κB activation in astrocytes does not interfere with normal brain development and homeostasis, but is critically involved in inflammatory responses and alters the pathological consequences of acute injuries and autoimmune reactions in the CNS. However, these loss-of-function models could only address the consequences of impaired NF-κB activation after pathological insults, but importantly, they are not suitable to study the biological outcome of NF-κB activation under non-pathological conditions.

The present study was designed to investigate the function of astroglial NF-κB activation under normal physiological conditions in the mouse, in particular to address the following questions:

1) Is activation of NF-κB signaling in astrocytes sufficient to drive neuroinflammation under normal physiological conditions or does it only enhance inflammatory responses caused by pathological insults?
2) Does NF-κB activation in astrocytes interfere with normal brain development?
3) What are the consequences of transient and chronic NF-κB activation in astrocytes for brain homeostasis and function?

2 Material and methods

2.1 Transgenic mice

2.1.1 Mouse strains

To activate NF-κB signaling in astrocytes *in vivo*, a constitutively active allele of human IKK2 was expressed conditionally in a doxycycline regulated binary transgene system ("tet-off system") controlled by a fragment of the human GFAP promotor.
To obtain GFAP.tTA/(tetO)7.IKK2-CA double transgenic mice (short GFAP/IKK2-CA), GFAP.tTA mice from a C57BL/6 background (Pascual et al., 2005) were directly crossed with (tetO)7.IKK2-CA mice (founder line A or EE-5) from a NMRI background (Herrmann et al., 2005) to generate a defined C57BL/6:NMRI hybrid background. Mainly (tetO)7.IKK2-CA single transgenic (if not indicated otherwise), but also wildtype and GFAP.tTA or (tetO)7.IKK2-CA single transgenic littermates were used as controls.
To control for the genotype, mice were genotyped by PCR with DNA of tail biopsies (see 2.1.3). In addition transgene expression was controlled by the measurement of the activity of a luciferase reporter gene (see 2.3.2), which is expressed from the same bidirectional promotor as the IKK2-transgene. For this analysis native brain tissue protein extracts of the medulla oblongata were used. Mice with a very low luciferase activity (<1000 RLU/μg protein) were excluded from further analysis.
For certain control experiments, another (tetO)7.IKK2-CA founder line was used, that was backcrossed for 9-10 generations to a C57BL/6 background (founder line B or EE-7). In the same manner, mice with a dominant negative allele of IKK2 ((tetO)7.IKK2-DN) from a NMRI background (founder line KD-12; (Herrmann et al., 2005)) were used for additional control experiments. All animals were housed under SPF conditions with 12 h/12 h light/dark cycles and food and water ad libitum.

2.1.2 Doxycycline administration

To avoid developmental defects and early postnatal lethality, for experiments with adult animals the transgene expression of was inactivated during brain development by the administration of doxycycline (MP Biomedicals, Eschwege). It was provided in the drinking water (0,5 g/l in 1% sucrose) for the whole pregnancy and up to an age

of the offspring of four weeks. Afterwards, doxycycline was withdrawn to reactivate IKK2-CA expression.
To reactivate the transgene at an earlier time point, one experiment was performed in which doxycycline application was stopped at P7.
For a rescue approach in the adult animals that received doxycycline until 4 weeks of age, doxycycline was readministered from 3 months of age until the animals were sacrificed at 5 months of age.
To investigate of postnatal consequences of prenatal transgene expression, the transgene expression was stopped at P0 by intraperitoneal injection of doxycycline (100 μg/g body weight) into the mother and the offspring, followed by doxycycline administration in the drinking water as above.

2.1.3 Genotyping

For genotyping of the transgenic mice, DNA from mouse tail biopsies was obtained as follows: Tail samples were digested by incubation with a SDS and proteinase K containing buffer (733 μl TNES-buffer with 17 μl proteinase K 20 μg/μl) for at least 4 h at 56 °C. Then protein was precipitated by 250 μl 6 M NaCl and centrifugation (13.000 rpm, 10 min). DNA from the supernatant was precipitated with 1 vol. of isopropanol and collected by centrifugation. The pellet was washed once with 70 % ethanol and air-dried. The DNA was dissolved in water and the transgenes were detected by PCR with specific primers (see 2.8.8).

2.2 Primary astrocyte culture

2.2.1 Preparation of astrocyte enriched primary cultures

To study the direct effects of the GFAP/IKK2-CA transgene expression system in astrocytes, primary astrocyte enriched cultures were prepared from pups (P1-P3) of double-transgenic mice and control littermates that were bred in the presence of doxycycline to avoid secondary effects of transgene expression on the composition of the isolated cell populations.
From these pups the cortices were dissected and the meninges removed. The cortical tissue was kept on ice in HBSS until all animals were prepared, washed once

with HBSS and then incubated with trypsin/EDTA (2 ml/brain) for 20 min at RT. At the beginning of incubation brain tissue was minced by pipetting up and down once with a 10 ml glass pipette. At the end of incubation, 1 vol. of culture medium (DMEM + 20 % FBS + 1 % penicillin/streptomycin) was added to stop trypsin activity. The digested tissue was further homogenized 3-5 times with a glass pipette and large tissue remnants were removed with a 100 μm cell strainer, which was washed with additional 5 ml of culture medium. The cell suspension was centrifuged (1000 rpm, 10 min) to pellet the cells and to remove trypsin and cellular debris. Cells were resuspended in fresh culture medium and seeded on laminin coated cell culture dishes (equivalent of about 10 6-wells per brain).

2.2.2 Culture conditions and *ex vivo* transgene reactivation

Astrocyte enriched primary cells were cultured in DMEM medium with 20 % fetal bovine serum (FBS) and 1 % penicilline-streptomycine supplement. This culture medium was exchanged every 2-3 days.

For the first 3-4 days astrocytes were cultured in the presence of doxycycline (1 μg/ml), then doxycycline was removed and the cultures were washed and grown for another 3 days before they were passaged once (dilution 1:3). Luciferase assay of culture samples before the first passage revealed no transgene expression, whereas 3 days after the first passage robust luciferase activity was detected. At this time RNA was prepared for gene expression studies (section 3.2.1.3).

Additional cells were frozen 2 days later in a kryoprotection medium containing 40 % DMEM, 50 % FBS and 10 % DMSO and stored in liquid nitrogen. For experiments with the IKK2 inhibitor BMS-345541 (section 3.1.6), some of these cells were thawed, cultured for 3 days, then passaged and harvested 4 days later for RNA preparation.

2.3 Protein biochemistry

2.3.1 Protein extraction

2.3.1.1 Native protein extracts with Dignam C buffer

After sacrificing the mouse, the brain was rapidly removed and the tissue dissected into the main parts forebrain, midbrain/thalamus/hypothalamus, cerebellum and

medulla oblongata. The brain parts were divided along the middle axis in two pieces that were immediately snap frozen in liquid nitrogen. The frozen tissue was grinded in the frozen state cooled by liquid nitrogen.

To prepare native protein extracts, the frozen powder was taken up in approximately 3-5 volumes of Dignam C buffer supplemented with 1 mM DTT, 1mM PMSF and the Roche complete mini protease inhibitor (1 tablet/10 ml). The suspension was immediately thoroughly mixed, snap frozen in liquid nitrogen and thawed again, which was repeated for 3 freeze/thaw-cycles. Afterwards the crude extract was centrifuged (13000 rpm, 25 min, 4 °C) and the pellet was discarded. Finally the protein concentration of the supernatant was determined with a conventional Bradford assay (Biorad, München) according to manufacturer's instructions.

Similarly native protein extracts were prepared from primary astrocyte cultures. In this case, the cells on the culture dish were washed twice with PBS and then scraped off the plate in Dignam C buffer with the mentioned supplements (70 µl for 6-wells, 300 µl for 10 cm dishes). The further procedure was performed as with tissue preparations.

2.3.1.2 Whole tissue protein extracts with RIPA buffer

For many immunoblotting experiments tissue protein extracts were prepared with the detergent-containing RIPA buffer, which in contrast to the Dignam C buffer yields denatured proteins, but does additionally extract membrane and organelle bound proteins.

For this extraction, tissue powder prepared as above was suspended in 3-5 volumes of RIPA buffer supplemented with 1 mM PMSF and the Roche complete mini protease inhibitor (1 tablet/10 ml). This supension was mixed thoroughly, frozen once and thawed again. The extracts were incubated for 1 h on ice, mixed repeatedly and centrifuged (13000 rpm, 25 min, 4 °C). Protein concentration in the supernatant was determined by a Bradford assay.

2.3.1.3 Cerebellar crude synaptosomal protein extracts

The cerebellum was rapidly dissected and homogenized by 20 strokes with a glass douncer in 1 ml Tris/sucrose buffer (50 mM Tris, 320 mM Sucrose, pH 7,4,

supplemented with Roche “complete mini” protease inhibitors). Large debris was removed by a centrifugation step (10 min, 1000g) and lysed with RIPA buffer for further analysis (P1-fraction). Crude synaptosomes were then sedimented by centrifugation for 20 min at 10000g resuspended in a sodium-free physiological buffer (16,2 mM Tris, 0.38 M sucrose, 10 mM glucose, 5 mM KCl, 1,2 mM CaCl2, 1,2 mM MgSO4, pH 7.4, described by (Tao et al., 2011) and centrifuged again. This fraction (synaptosomes, P2-fraction) was lysed with RIPA buffer for further analysis and protein concentration determined by a Bradford assay (see above).

2.3.2 Luciferase reporter gene activity measurement in native protein extracts

For a quick determination of transgene expression in native protein extracts, luciferase reporter gene activity was measured with the luminometer Lumat LB9507 (Berthold, Bad Wildbad). For this assay, 3 µl of Dignam C protein extract were mixed with 50 µl of luciferin buffer and light emission (RLU) was measured over 15 s. From mean values of two measurements the background activity (Dignam C) was subtracted and the obtained values were normalized to the amount of protein (RLU/µg).

2.3.3 SDS-PAGE and immunoblotting

For SDS-PAGE 5-50 µg of protein were diluted with the extraction buffer to a specific volume and incubated with the appropriate amount of 4x-Laemmli loading buffer (+ 5 % β-mercaptoethanol, freshly added) for 10 min at 95 °C.

In some cases, in particular for the detection of the EAAT1/2 glutamate transporters, an alternative protocol with a 2x-laemmli/urea loading buffer (+ 5 % β-mercaptoethanol, freshly added) was used and the samples were incubated for 30 minutes at 37 °C. This treatment allows better dissociation of protein multimers and reduces protein aggregation.

The protein samples were separated on polyacrylamide gels with a 5 % polyacrylamide stacking gel (with 0.125 M Tris-HCl pH 6.8, 0.1 % SDS) and a resolving gel with 8/10/12.5/15 % polyacrylamide depending on the size of the proteins to detect (with 0.375 M Tris/HCl pH 8.8, 0.1% SDS). The gels were run with

the Mini-PROTEAN system (Biorad, München) in a Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). With the Trans-Blot-Cell system (Biorad, München) the proteins were transferred to an Immobilon-SQ-PVDF membrane (pore size 0.2 μm; Millipore, Billerica, MA, USA), which was activated with methanol and washed in the blotting buffer (25 mM Tris, 192 mM glycine, 0.025 % SDS, 20 % methanol).

After the transfer, the proteins were postfixed with methanol on the membrane, which was then washed three times with TBS/Tween (0.1 %). Unspecific protein binding was blocked by incubation with 5 % dry milk powder in TBS/Tween (0.1 %) for 1 h at RT. The membranes were incubated with primary antibodies (in blocking solution, dilution see 2.8.4) overnight at 4° C or for 2 h at RT. After three washes with TBS/Tween the membranes were incubated with the HRP-coupled secondary antibody (1:5000 in blocking solution) for 1 h at RT. After additional three washes luminescence signals were detected with HRP-juice+ (PJK, Kleinblittersdorf) in the Intelligent Dark Box LAS-1000 (Fuji, Düsseldorf).

Similarly ERK2 was detected on the same membrane as a control for equal loading.

Sometimes the membranes were reprobed with several antibodies. If old bands might mask the new bands to detect, the membrane was stripped by incubation with 0.2 M NaOH for 5 min, followed by thorough washing and new blocking.

2.4 RNA analysis

2.4.1 RNA extraction and cDNA synthesis

For standard qPCR applications RNA was prepared with the PeqGOLD Trifast reagent (Peqlab, Erlangen).

Frozen tissue powder (see 2.3.1.1) with added Trifast reagent or cells scraped off the plate in Trifast (each 1 ml) were lyzed by repeated pipetting and then treated according to the standard protocol. After chloroform mediated phase separation RNA was precipitated with 1 volume isopropanol, centrifuged and the pellet washed twice with 75 % ethanol in DEPC water. Then the RNA was dissolved in 50 μl of DEPC water and the concentration of RNA was measured with the Nanodrop 1000 spectrophotometer (Thermo Scientific). For purity control the ratio of absorption at 260/280 nm and 260/230 nm was measured. To remove remaining traces of phenol,

the RNA was precipitated by adding 1/10 volume of sodium acetate buffer (3M, pH 4.8) and 2.5 volumes of ethanol. After precipitation overnight at –20 °C the pellet was washed with 80 % ethanol in DEPC water and dissolved in an appropriate volume of DEPC to a final concentration of 100 or 200 ng/μl.

The RNA from forebrain tissue at P7, which was also used for the microarray analysis, was prepared with the Ambion mirVana miRNA isolation kit (Invitrogen, Paisly, UK), which is also suitable for the extraction of whole cellular RNA including miRNAs. RNA was extracted with the protocol for total RNA isolation according to manufacturer's instructions. As above, RNA concentration and purity was determined and the RNA was reprecipitated to eliminate traces of phenol.

Of the obtained RNA 1 μg was used for cDNA synthesis of polyA-mRNA with oligo-dT primers with the "Transcriptor High Fidelity cDNA synthesis kit" (Roche, Mannheim) according to manufacturer's instructions. The cDNA samples were used in a dilution of 1:10.

To control for RNA quality and equal amounts of total cDNA, a PCR for β-actin was performed. In addition, a PCR for the 3'-UTR of the transgene ("β-glob-exon") was used to control for transgene expression. Both PCRs (primers see 2.8.8) are intron-spanning, thereby also allowing an approximation of the amount of contaminating genomic DNA.

2.4.2 Microarray analysis

The microarray experiment with forebrain whole tissue RNA was performed with the Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) in the lab of Karlheinz Holzmann (Microarray Core Facility, Ulm University).

The microarray data were evaluated with the "Genesifter"-software (Geospiza, Seattle, WA, USA). A Benjamini-Hochberg corrected 2-tailed t-test with a cutoff of at least 1.5 fold regulation and $p < 0.05$ was used to identify differentially regulated genes.

2.4.3 Quantitative Realtime-PCR (qPCR)

Quantification of gene expression on mRNA/cDNA level was performed by qPCR with the Lightcycler 480 and the Universal Probe Library (UPL) system (Roche,

Mannheim). Each 2.5 µl of cDNA dilution were analyzed in a 10 µl assay volume with the 2x Lightcycler 480 Probes Master mix, primers with a final concentration of 0.2 µM and 0.15 µl of the appropriate Universal Probe (fluorescent nucleotide hydrolysis probe). Every gene was analyzed in duplicates for each cDNA sample. Primers and the appropriate Universal Probes were selected with the "UPL Assay Design Center" on the Roche Applied Science website (specific primers and UPL probes see 2.8.8). Amplification of the specific PCR fragment was performed and measured with the "UPL mono color hydrolysis probe" standard protocol of the Lightcycler 480 and the specific Cp value was calculated with the "2nd derivative maximum"-algorithm. mRNA levels were expressed as relative values versus the housekeeping gene HPRT, calculated as:

Rel. Expr. (x) = $2^{(Cp(HPRT)-Cp(x))}$;

2.5 Histology

2.5.1 Tissue preparation

Histology and immunofluorescence studies were performed with coronal sections of either paraffin embedded PFA fixed or natively frozen brain tissue.

For paraffin sections, the brain removed from the sacrificed animal was fixed for 3-6 hours in 4 % PFA in PBS on ice, followed by washing 2-3 times with 70 % ethanol. Brains were dehydrated in an increasing concentration of ethanol and embedded in paraffin according to standard histological procedures. From the paraffin blocks serial coronal sections with a thickness of 7 µm were cut with a microtome and mounted on slides (7 sections per slide).

For kryosections, the fresh brain tissue was immediately frozen on a copper block that was cooled with liquid nitrogen. The native frozen tissue was cut with a kryotome into 10 µm thick sections (2 sections per slide) and stored at –20 °C.

2.5.2 Nissl staining

For overview histology and selection of the appropriate plane for further analysis Nissl staining with cresyl violet was performed. Usually every 10th slide was stained, i.e. sections with a distance of 0.5 mm in rostro-caudal direction. For this purpose,

the sections were deparaffinized with 2 baths of xylene (each 2 min) and rehydrated with baths with an decreasing isopropanol concentration (100 %, 100 %, 96 %, 70 %, each 2 min), followed by a short dipping into water and an incubation in 0.1 % cresyl violet in 0.1 % acetic acid for 4 minutes. After short dipping into 3 subsequent 96 % isopropanol baths, the slides were incubated each 2 min in 2x 100 % iso-propanol and 2x xylene. Finally the slides were mounted with a coverglass with entellan as anhydrous mounting medium.
Overview images were taken with the Zeiss SteREO Discovery.V20 at magnifications of 10-15x, detail pictures at higher magnifications.

2.5.3 Immunofluorescence staining of paraffin embedded sections

Sections were deparaffinized with 2x xylene, and rehydrated with ethanol (2x 100 %, 90 %, 70 %, water), for each at least 5 min per bath. Then the slides were boiled for antigen retrieval for 15-20 min in a microwave in preheated citric acid buffer (10 mM, pH 6.0, with 0.05 % Tween 20) or TE-buffer (10 mM Tris, 1 mM EDTA, 0.05 % Tween 20).
After cooling for at least 30 min at RT the slides were incubated for 30 min with 0.5 % Triton X-100 in PBS for full permeabilization. Then the slides were washed two times for at least 10 min with PBS. For each slide, 4-6 slices were surrounded with a PAP-pen to create two independent staining compartments per slide.
To prevent unspecific antibody binding, sections were incubated for 1 h at RT with blocking solution containing 5 % BSA in PBS in a dark humidified chamber which was also used for antibody incubations. After removal of the blocking solution the slices were incubated with the primary antibodies in blocking solution, usually overnight at 4 °C. After 2 washes with PBS the slides were incubated with the fluorescently labeled secondary antibodies (dilution 1:500, labeled with Alexa Fluor 488 (green) or 568/594 (red)) in blocking solution for 1 h at RT, with DAPI (250 ng/ml) for nuclear counterstaining. After two additional washes with PBS (in dark), the slide were shortly washed with water and mounted with a coverglass with mowiol as aqueous mounting medium.
For a staining for sialic acid containing glycoproteins, oregon green 488 labeled wheat germ agglutinin (WGA) was applied in a dilution of 1:200 in PBS for 2 h at RT after the secondary antibodies.

2.5.4 Immunofluorescence staining of kryosections

Kryosections were used for stainings for CD45, CD11b, F4/80, Mac-2, CD4 and CD8, which were not working with paraffin-embedded sections. Kryosections were dried at room temperature, fixed for 3-5 min in cold methanol at –20 °C and washed twice with PBS, before they were subjected to blocking and antibody incubations as described above. As the mentioned antigens are located at the cell surface, no permeabilization was performed.

In case of CD11b costaining with CD45, directly PE-labeled rat anti-CD11b antibody in blocking solution was applied for 2 h at RT after two extensive washes with PBS after the secondary antibody solution.

2.5.5 Immunofluorescence staining of primary astrocyte cultures

For immunostaining of primary astrocyte cultures, the cells were seeded on laminin-coated round coverglasses in 24-well dishes. The medium was discarded and the cells were washed twice with PBS, before they were fixed for 15 min in 4 % PFA in PBS and washed again with PBS. Afterwards, the coverglasses were removed from the dish and subjected to permeabilization, blocking and antibody incubation as described in 2.5.3. Incubation with the primary antibodies was performed for 2 h at RT, for GFAP/CD11b costaining PE labeled anti-CD11b was applied with the secondary antibody solution for 2 h at RT.

2.5.6 Immunofluorescence image aquisition and processing

To avoid bleaching of the fluorescent dyes, the slides were stored in the darkness and analyzed within a few days. Because of the rapid bleaching of PE, images of CD11b-stainings were usually taken at the day of staining.

Images were taken with the Zeiss Axiovert 200M, with filters for DAPI, FITC/Alexa Fluor 488, DsRed/PE and Texas Red/Alexa Fluor 568/594. All directly compared images were taken at the same session with the Zeiss Axiovision-software (version 4.8) with the same settings that were separately adjusted for each channel. After export to jpg files the final adjustment of total brightness (equally for all directly compared images) and the cutting of relevant image parts was performed with Adobe Photoshop.

2.5.7 Quantification of nuclear RelA staining

All samples for quantification of DAPI/hIKK2/RelA staining were stained simultaneously and images were taken in one session with the same settings with a 20x objective. The raw images were analyzed with the ImageJ software. For each animal, three fields of the midbrain region with about 400-600 nuclei (or about 40-80 nuclei of transgenic cells) were measured. Nuclei were detected semi-automatically with automatic threshold setting and the “particle analysis” tool in the DAPI layer, with a detection of particle sizes of 200-600 pixel (approx. 20-60 μm^2). These particles (single nuclei) were saved as regions of interest (ROI) in the “ROI manager” and the fluorescence intensity of the corresponding areas of the RelA layer was automatically measured for each nucleus. To measure only nuclei of transgene positive cells, these cells were manually selected in the hIKK2 layer before definition of the nuclei as above. With the Prism software (Graphpad) RelA intensitiy histograms were generated to set a threshold above which cells were regarded as nuclear RelA positive.

2.5.8 Quantification of Purkinje cell numbers

For the quantification of Purkinje cell numbers, images of Nissl stainings of the simple lobule or the paramedian respectively were taken with the Zeiss Axiovert 200M with a 10x objective. A corresponding part of the Purkinje cell layer was labeled in each picture in ImageJ and its length measured, before the number of Purkinje cells (identified by the location at the outer border of the granule layer and their morphology/size) was counted manually. Total counted numbers were normalized to the length of the analyzed segment.

2.6 Electron microscopy

2.6.1 Scanning electron microscopy of the lateral ventricle walls

Brains of sacrificed mice were removed and fixed by immersion for at least 24 h with 2.5% glutaraldehyde in PBS. The brain were washed and cut to 0.5 mm sagital sections with a vibratome to expose parts of the lateral walls of the lateral ventricles.

The sections were then postfixed with OsO_4, critical point dried and vaporized with Au/Pd by technicians of the Central Facility for Electron Microscopy of Ulm University. Images were acquired with the DSM 962 microscope (Zeiss, Oberkochen).

2.6.2 Transmission electron microscopy of cerebellar Purkinje cells

The protocol for the TEM studies on Purkinje cells was adapted from the work of Custer et al. (Custer et al., 2006).

Mice were anesthetized and transcardially perfused with PBS followed by 4 % PFA / 0.5 % glutaraldehyde and then 2 % PFA / 3 % glutaraldehyde in PBS (each approximately 15 ml). After excision the whole brain was postfixed for at least overnight by immersion in 2 % PFA / 3 % glutaraldehyde in PBS. After washing with PBS, the cerebella were cut with a vibratome into 0.5 mm thick coronal sections. Of these sections, pieces of the cerebellum containing the simple lobule were dissected. These pieces were given to the Central Facility for Electron Microscopy of Ulm University for staining, embedding and cutting. Tissue pieces were stained with osmium tetroxide and uranyl acetate and epoxy embedded. Semi-thin sections were prepared and stained with toluidine blue for the selection of Purkinje cell containing areas, which were cut out for ultrathin sectioning. Ultrathin sections were mounted on grids and stained with lead citrate. Images of individual Purkinje cells (about 20 if possible) were taken with the JEM-1400 (JEOL, Tokyo, Japan) at a lower magnification to show the whole cell body and a higher magnification to investigate ER and Golgi structures. Purkinje cells were graded by three criteria, namely darkened cytoplasm, ER/Golgi swelling and irregular cell shape, on a scale of each 0 (normal) to 2 (prominently altered). Cells that had the score 2 in at least 2 criteria were regarded as degenerating.

2.7 Behavioral experiments

2.7.1 String agility test

As a simple test of general motor function, including muscle strength, general agility and movement coordination, a string agility test was performed. Animals were guided

to hold themselves with the forepaws on an elevated streched string (lengths 50 cm, diameter 2.5 mm). Animals were observed for 60 s and scored according to their abilities to move on the string with the following scoring system (Castillo Vega, 2011):
0 - unable to remain on the string
1 - hangs on its forepaws
2 - attempts to climb the string
3 - forepaws and one or both hindpaws around the string
4 - four paws and tail around string with lateral movement
5 - escape to lateral platforms
In addition, for animals that fell of the string, the time until they fall was recorded.

2.7.2 Rotarod

To more specifically address fast movement coordination, a rotarod test was performed with the ENV-575M rotarod apparatus (Med Associates Inc., St. Albans, VT, USA). Animals were placed on a 3 cm thick rotating cylinder. After 1 min of adjustment a 4 rpm, the cylinder accelerated within 5 min to 40 rpm. The latency to fall was recorded with the RotaRod 1.2.0 software (Med Associates Inc., St. Albans, VT, USA). When animals held passively to the cylinder instead of running, the experiment was stopped manually.
To observe motor learning and increase statistical robustness, each animal was subjected to the task 3 times per day for 4 consecutive days.

2.7.3 Beam-walking test

The beam-walking test is used to analyze balance and motor coordination. In this test, the mice are forced to traverse a narrow beam to escape from a small, elevated platform (50 cm above the ground) to a closed dark box. The horizontal wooden beams have a length of 1 m. According to a typical protocol, the mice should cross the beam because of their light avoidance behavior, as there are lamps located at the open platform (Castillo Vega, 2011). As this was not sufficient to reliably motivate the mice to cross the beam, they were encouraged by the hand of the experimenter, especially at the start of the beam, without applying force to push them after they started crossing the beam. One initial trial with increased encouragement was

needed to introduce the mice to the task. Beginning from the second trial for each trial the time was recorded that the mice needed to pass the last 80 cm of the beam.
According to the initial protocol (Castillo Vega, 2011), for the first study 4 training trials per day for 3 days were performed with a 12 mm square beam. On the two following days, probe trials with different beam sizes were done in duplicate (square beams 28 mm, 12 mm, 5 mm; round beams 28 mm, 17 mm, 11 mm).
As a quite stable performance was already reached at the first training day, for a second experiment a short protocol with 4 consecutive trials on one day with a 12 mm square beam was used.

2.7.4 Open field

To assess general activity and anxiety/exploratory behavior, the mice were put for 30 min in an open field arena with a size of 50 x 50 cm (Castillo Vega, 2011). The mice were observed with a video camera with the Tracking System Viewer 2 software (Biobserve, Fort Lee, NJ, USA), which records several parameters. Of these parameters, the overall velocity and activity were analyzed for their general behavior. In addition, the time and track length in a 20 x 20 cm center zone was analyzed as a measure for their avoidance of free open areas, which indicates anxiety and exploratory behavior.

2.7.5 Elevated plus maze

As an additional experiment to study anxiety and exploratory behavior, the mice were subjected to an elevated plus maze test. This maze is an elevated plus-shaped platform with four arms of equal size (30 x 5 cm), of which two are surrounded by 16 cm high walls (Braunstein, 2008). Animals were put in the center of the maze facing an open arm and video-tracked for 5 min with the Tracking System Viewer 2 software (Biobserve, Fort Lee, NJ, USA). As measure for the balance of anxiety and exploratory behavior the time, track length and number of visits in the open arms were analyzed.

2.7.6 Morris water maze test

The Morris water maze is a paradigm to assess hippocampal learning and memory, in which mice have to remember the location of a hidden platform in a water filled pool.

In the test a circular pool with a diameter of 1.2 m was used, which was filled with water of a temperature of 19-22 °C that was colored with 2 l of milk to enhance the contrast for the video-tracking system. A platform with a diameter of 10 cm that was wrapped with white paper and a plastic foil was placed in the pool, either above the water surface, labeled with a 100 ml graded cylinder as an easily visible visual cue (visible platform), or unlabeled and submerged about 0.5 – 1.5 cm below the water surface (hidden platform). The platform was placed 35 cm from the border of the pool at positions indicated below, which were defined by an arbitrary division of the pool in compass direction quadrants. Four different simple geometric motifs were placed around the pool (N-S-E-W position, distance approx. 2m, diameter approx. 50 cm) to allow visual orientation for the mice.

2.7.6.1 Visible platform

The visible platform test is used to check the ability of the mice to swim in a directed manner towards the platform, thereby helping to discriminate between learning and memory alterations and a potential influence of motor impairments on the swimming performance in the actual hippocampal learning task. This is important to clarify the reliability of the Morris water maze test data for animals with motor deficits.

For the visible platform test 8 trials per mouse were performed on two consecutive days. Before the first trial, the animal was placed on the platform for 15 s, then it was placed carefully into the water at a different position at the border of the pool. The time to reach the platform was recorded by hand with a timer.

To avoid a preference of the animals for a certain quadrant, platform and starting point positions were changed for each trial according to the following scheme (Vorhees and Williams, 2006): N (start position) - SE (platform position), E – NE, S – SW, W – SE, S – NE, N – NW, W – NE, E – SE.

2.7.6.2 Training trials with hidden platform

For the actual spatial learning paradigm, the platform was used without direct cue and submerged below the water surface, at a fixed position in the SW quadrant. Before the first trial, the mouse was again placed for 15 s on the platform, allowing its spatial orientation with the distant visual cues.

Then the mouse was placed in the water at the pool border at the first starting position (N) and the track of the mouse towards the platform was followed by video-tracking with the Tracking System Viewer 2 software (Biobserve) until it found the platform, for a maximum of 60 s. At the end of the trial, the mouse was left again for 15 s on the platform, or it was placed there, when it was unable to find the platform.

These trials were repeated for each mouse 4 times per day for 5 consecutive days, and the time to reach the platform (mean value for each day) was analyzed as a measure for learning progress. The starting position was changed for each trial to avoid acquisition of a specific swimming path memory. The starting points were chosen as follows according to a published protocol (Vorhees and Williams, 2006): N-E-SE-NW (trial no. 1-2-3-4, day 1)/ SE-N-NW-E (day 2)/ NW-SE-E-N (day 3)/ E-NW-N-SE (day 4)/ N-SE-E-NW (day 5)

2.7.6.3 Probe trials

To assess spatial memory formation, an additional trial was performed without the platform 1 and 10 days after the last training session. In this trial, the animal track (from the NE starting position) was followed for 30 s before the animals were removed from the pool. As a measure for the memory for the platform position the time spent in the former platform quadrant was analyzed.

2.8 Reagents and Materials

2.8.1 General chemicals

General chemicals (anorganic chemicals, solvents, standard organic salts, acids,...) were purchased from Applichem (Darmstadt), Roth (Karlsruhe), Merck (Darmstadt) and Sigma (Taufkirchen).

2.8.2 Buffers and solutions

TNES-buffer: 50 mM Tris-HCl (stock 1M pH 7.5), 0.1 M EDTA (stock 0.5 M, pH 8.0), 0.1 M NaCl, 1 % SDS; (+ Proteinase K (Applichem, Darmstadt) 0.45 μg/μl)

PBS (10x): 87.65 g/l NaCl, 2 g/l KCl, 11.7 g/l Na_2HPO_4, 2.4 g/l NaH_2PO_4, pH 7.3

TBS (10x): 24.2 g/l Tris, 80g/l NaCl, pH 7.6

TBE (10x): 121.1 g/l Tris, 61.8 g/l hydroboric acid, 7,4 g/l EDTA

Dignam C buffer: 20 mM Hepes (pH 7.9), 25 % glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA (+ 1 mM PMSF, 1 mM DTT, 1 tablet/10 ml complete mini protease inhibitor (Roche, Mannheim))

RIPA buffer: 50 mM Tris-HCl (stock 1M pH 7.4), 150 mM NaCl, 1 % Triton X-100; 0.5 % sodium desoxycholate, 0.1 % SDS (+ 1 mM PMSF, 1 tablet/10 ml complete mini protease inhibitor (Roche, Mannheim))

Luciferin buffer: 20 mM tricin, 1.07 mM magnesiumcarbonatehydroxyde, 2.67 mM magnesiumsulfate, 0.1 mM EDTA, 33.3 mM DTT, 0.27 mM coenzyme A, 0.47 mM luciferin, 0.53 mM ATP

4x Laemmli loading buffer: 125 mM Tris-Hcl (pH 6.8), 20 % SDS, 25 % glycerol, bromphenol blue for coloring; (+ 5 % β-mercaptoethanol)

2x Laemmli/urea loading buffer (for 80 ml): glycerol 15 ml, 22.5 ml SDS 20%, 9.4 ml Tris (2M, pH 6.8), 28.83 g urea (final concentration 6 M), bromphenol blue for coloring; (+ 5 % β-mercaptoethanol)

2.8.3 Cell culture

HBSS buffer (PAA, Pasching, Austria)

Trypsin(0.05%)/EDTA (Gibco, Life Technologies, Paisley, UK)

DMEM medium (Gibco, Life Technologies, Paisley, UK)

Penicilline-Streptomycine, 100x (Gibco/ Life Technologies, Paisley, UK)

FBS (PAA, Pasching, Austria)

Laminin (Sigma, Taufkirchen)

BMS-345541 (Axon Medchem, Groningen, Netherlands)

10 cm tissue culture dishes (Greiner Bio-One, Frickenhausen)

6-well tissue culture plates (Greiner Bio-One, Frickenhausen)

24-well tissue culture plates (BD Falcon, San Diego, CA, USA)

13 mm coverglasses (VWR, Ulm)

cell strainer 100 μm (BD Falcon, San Diego, CA, USA)

sterilization filters (Whatman, GE Healthcare, Maidstone, UK)

2.8.4 Antibodies

Primary antibodies:

antigen	source	company	cat. no.	IB dil.	IF dil.
Aldh1l1	Rb	Abcam	Ab56777		1:100-1:200
Aqp4	Rb	Santa Cruz	Sc-20812		1:200
β-catenin	Ms	BD	610154		1:200-1:500
Calbindin	Ms	Sigma	C9848		1:500-1:2000
Cleaved Casp3	Rb	Cell signaling	#9661		1:100
CD11b	Rt	eBioscience	12-0112(PE-lab.)		1:100
CD4	Rt	BD	550278		1:50
CD8	Rt	BD	550281		1:50
CD45	Rt	BD	550539		1:100
EAAT1/GLAST	Rb	Santa Cruz	Sc-15316	1:500-1:1000	
EAAT2/GLT-1	Rb	Cell signaling	#3838	1:1000	
ERK2	Rb	Santa Cruz	Sc-154	1:1000	
F4/80	Rt	eBioscience	Clone BM8		1:100
Fibronectin	Rb	Abcam	Ab23750		1:500
GFAP	Rb	Abcam	Ab7779	1:1000	1:200-1:500
GFAP	Ms	Santa Cruz	Sc-33673	1:1000	1:200-1:500
hIKK2/transgene	G	Santa Cruz	Sc-7329	1:1000	1:100-1:200
IKK1/2	Rb	Santa Cruz	Sc-7607	1:1000	
Ki67	Rb	Thermo Sci.	RM-9106-S		1:100-1:200
Laminin	Rb	Abcam	Ab11575		1:500
Lcn2	G	R&D	AF1857	1:2000	
Mac-2/galectin-3	Rb	Santa Cruz	Sc-20157		1:100-1:200
NeuN	Ms	Millipore	MAB 377	1:1000	
RelA	Rb	Santa Cruz	Sc-372		1:100-1:200
Ac.-α-Tub	Ms	Sigma	T7451		1:500

Abbreviations: IB Immunoblot, IF immunofluorescence, dil. dilution, Rb rabbit, Rt rat, Ms mouse, G goat, D donkey, PE Phycoerythrin

Secondary Antibodies for immunoblotting (HRP-coupled goat anti-rabbit, goat anti-mouse, donkey anti-goat, goat anti-rat) were purchased from Santa Cruz Biotechnologies and applied in a dilution of 1:5000.
Alexa Fluor-coupled secondary antibodies for immunofluorescence (goat anti-rabbit A488/A568, donkey anti-rabbit A594, goat anti-mouse A488/A568, donkey anti-mouse A594, donkey anti-goat A488, goat anti-rat A488) were obtained from Life Technologies (Molecular Probes, Paisley, UK) and used in a dilution of 1:500.

2.8.5 Protein biochemistry materials

5x Bradford reagent (Biorad, München)
HRP-juice+ (J.P.K., Kleinblittersdorf)
PageRuler prestained protein ladder (Fermentas, St. Leon-Rot)
MagicMarkXP Western Protein Standard (Invitrogen, Life Technologies, Paisley, UK)
Whatman paper (Whatman, GE Healthcare, Maidstone, UK)

2.8.6 Histology and microscopy materials

Cresyl violet (Merck, Darmstadt)
DAPI (Merck, Darmstadt)
Wheat germ agglutinin (WGA), oregon green labeled 488 (Invitrogen, Life Technologies, Paisley, UK)
Entellan (Merck, Darmstadt)
Mowiol (Merck, Darmstadt)
Paraformaldehyde/PFA (Sigma, Taufkirchen)

PAP-Pen (Sigma, Taufkirchen)
Peel-A-Way disposable embedding molds (Polysciences, Warrington, PA, USA)
Microscopy slides “Superfrost Ultra Plus” (Menzel, Braunschweig)
Coverglasses 24x50 mm (VWR, Ulm)

2.8.7 Molecular biology materials

1kb plus DNA ladder (Invitrogen, Life Technologies, Paisley, UK)
Agarose Ultrapure, Electrophoresis Grade (Invitrogen, Life Technologies, Paisley, UK)
Ethidium bromide (Sigma, Taufkirchen)
Taq Poymerase and 5x Green GoTaq Reaction buffer (Promega, Mannheim)
dNTPs (Genaxxon, Ulm)

PCR tubes 0.2 ml (Brand, Wertheim)
Lightcycler 480 Multiwell Plate 96 (Roche, Mannheim)

2.8.8 Primer sequences and PCR programs

2.8.8.1 Conventional PCR (genotyping + cDNA control)

Reaction mix:	DNA sample	2 µl
	dNTPs 2mM	3 µl
	5x GoTaq buffer	6 µl
	primers 10 µM	each 1 µl
	water	16.8 µl
	Taq 5000 U/ml	0.2 µl

Primers (5' - 3'):
GFAP.tTA: cca acc aac cct ttc ttg acc cac c / cga ttc cga cct cat taa gca gct c
(tetO)7.IKK2-CA/DN: cga ttc cga cct cat taa gca gct c / ggt cac tgt gta ctt ctg ctg ctc cag
β-Actin: ggt cag aag gac tcc tat gtg / aga gca aca tag cac agc ttc
β-glob-exon (transgene expr.): cag cct gca cct gag gag tga att c / ggc gct gga ctg ttg ccc aag

Programs:

PCR	GFAP.tTA	(tetO)7.IKK2-CA /DN	β-Actin	β-glob-exon
Start	3 min / 94 °C	3 min / 94 °C	3 min / 94 °C	3 min / 94 °C
Cycles	35x	32x	25x	30x
Annealing	40 s / 62 °C	45 s / 63 °C	45 s / 60 °C	40 s / 62 °C
Synthesis	70 s / 72 °C	85 s / 72 °C	60 s / 72 °C	70 s / 72 °C
Denaturation	40 s / 94 °C	45 s / 94 °C	45 s / 94 °C	40 s / 94 °C
End	60 s / 62 °C 10 min / 72 °C	60 s / 63 °C 10 min / 72 °C	60 s / 60 °C 10 min / 72 °C	60 s / 62 °C 10 min / 72 °C

2.8.8.2 qPCR

Gene	Primer 1 (5'- 3')	Primer 2 (5'- 3')	UPL #
HPRT1	gga gcg gta gca cct cct	ctg gtt cat cat cgc taa tca c	69
CCL2	cat cca cgt gtt ggc tca	gat cat ctt gct ggt gaa tga gt	62
CCL5	tgcagaggactctgagacagc	gagtggtgtccgagccata	110
CXCL10	gct gcc gtc att ttc tgc	tct cac tgg ccc gtc atc	3
TNF-α	tgcctatgtctcagcctcttc	gaggccatttgggaacttct	49
IL-1β	tgt aat gaa aga cgg cac acc	tct tct ttg ggt att gct tgg	78
IL-6	gct acc aaa ctg gat ata atc agg a	cca ggt agc tat ggt act cca gaa	6
ICAM-1	ccc acg cta cct ctg ctc	gat gga tac ctg agc atc acc	81
Madcam-1	gggcaggtgaccaatctgta	ataggacgacggtggagga	72
Lcn2	ccatctatgagctacaagagaacaat	tctgatccagtagcgacagc	58
C3	accttacctcggcaagtttct	ttgtagagctgctggtcagg	76
C4b	tctcacaaacccctcgacat	agcatcctggaacacctgaa	10
C1s	tggatacttctgctcctgtcc	cagggcagtgaacacatctc	69
CD74	gccctagagagccagaaagg	tggtacaggaagtaagcagtgg	21
H2-Aa	tggaggtgaagacgacattg	ctcatcaccatcaaattcaaatg	80
Gbp2	tgtagaccaaaagttccagacaga	gataaaggcatctcgcttgg	62
Cp	cctgatgaggaacatcttgga	aaaggtgactttaatggtgtctcc	29

All primers were purchased from Biomers, Ulm.

2.8.9 General laboratory materials

Reaction tubes 1.5/2 ml (Eppendorf, Wesseling-Berzdorf)
15/50 ml High-Clarity Polypropylen Conical Tubes (BD Falcon, San Diego, CA, USA)
5 ml Polystyrene Round Bottom tubes GLKL (Greiner Bio-One, Frickenhausen)
Pipette tips 1-1000 µl (Hightech lab, Warsaw, Poland and Axygen, Union City, CA, USA)
5/10/25 ml glass pipettes (Brand, Wertheim)
1 ml Sub-Q syringes (BD, Franklin Lakes, NJ, USA)

2.9 Laboratory equipment

2.9.1 General laboratory equipment

Pipettes 0.1-2/2-20/20-200/200-1000 µl (Abimed, Gilson, Brand)
Pipette controller Pipetus (Hirschmann, Eberstadt)
Surgical forcipes and scissors (FST, Heidelberg)
Biofuge Pico 17 (Thermo Scientific, Waltham, MA, USA)
Megafuge 1.0R (Thermo Scientific, Waltham, MA, USA)
Digital Heat Block (VWR, Ulm)
pH-meter Calimatic 761 (Knick, Berlin)
Scale Scaltec (Scaltec, Heiligenstadt)
Scale BP-210-S (Sartorius, Göttingen)
Spectrophotometer Genesys 10S UV/VIS (Thermo Scientific, Waltham, MA, USA)
Thermocycler Primus 96 plus (MWG, Ebersberg)
Gel documentation Genosmart (VWR, Ulm)
Cell culture incubator Hera-Safe (Thermo Scientific, Waltham, MA, USA)
Laminar flow sterile bank (Thermo Scientific, Waltham, MA, USA)
Shaker Polymax 1040 (Heidolph, Schwabach)
Power supplies for electrophoresis Desatronic 500/400 (Desaga, Heidelberg)
Agarose gel equipment and behavioral test equipment was constructed by the scientific workshop facilities of Ulm University

2.9.2 Histology and microscopy equipment

Microtome Microm HM355S (Thermo Scientific, Waltham, MA, USA)

Cryotome CM1900 (Leica, Wetzlar)

Vibratome VT1200S (Leica, Wetzlar)

Microscope Axiovert 200M (Zeiss, Oberkochen):

- b/w camera AxioCam MRm
- objectives Plan-Apochromat 2.5x, 5x, 10x, 20x, 40x, 63x
- UV-lamp X-Cite 120 EXFO (Lumen dynamics, Mississauga, Canada)
- Excitation/emission filters for DAPI, FITC/Alexa Fluor 488, DsRed/PE and Texasred/Alexa Fluor 568/594 (AHF Analysentechnik)

Stereomicroscope SteREO Discovery.V20 (Zeiss, Oberkochen):

- RGB camera AxioCam MRc
- Objective Achromat S 0.63x

2.9.3 Software

Microsoft Office X

Adobe Photoshop CS2 V9.0

Adobe Illustrator CS2 V12.0.0

Graphpad Prism V5.01

ImageJ 1.41o

Zeiss Axiovision 4.8

Biobserve Tracking System Viewer 2

3 Results

3.1 Enhanced NF-κB activation in astrocytes by conditional expression of constitutively active IKK2

The present study was designed to investigate the physiological and pathophysiological functions of astroglial NF-κB signaling *in vivo* during development and in the adult CNS. For this purpose transgenic mouse models were generated that overexpress mutant (constitutively active or dominant negative) IKK2 alleles specifically in astrocytes with an inducible transgene expression system to conditionally modulate IKK2 dependent NF-κB activity in astrocytes. Others already showed that suppression of NF-κB signaling in astrocytes has no prominent effect on brain development and normal brain homeostasis, but reduces inflammatory responses under pathological conditions (see 1.4). By contrast, so far no gain-of-function mouse model is available that specifically activates NF-κB in astrocytes, thus the specific consequences of NF-κB activation in astrocytes under normal physiological conditions are unknown. Therefore this study mainly focuses on the functional characterization of a mouse model that conditionally expresses a constitutively active allele of IKK2 (IKK2-CA) to enhance NF-κB activity in astrocytes.

3.1.1 Tetracycline repressible expression of a constitutively active IKK2 allele driven by the GFAP promotor

To activate NF-κB signaling in astrocytes a transgenic construct was used in which a constitutively active allele of human IKK2 (IKK2-CA) and a luciferase reporter gene are controlled by a bidirectional promotor ((tetO)7.IKK2-CA) that can be regulated by the tetracycline dependent transactivator (tTA), an artificial transcription factor that can be inactivated by tetracyclines like doxycycline (Figure 7). The IKK2-CA mutant contains two phosphomimetic mutations, which replace the key serine residues for the activation of IKK2 by phosphorylation by glutamate (S177E, S181E), keeping the kinase permanently in an activated conformation.

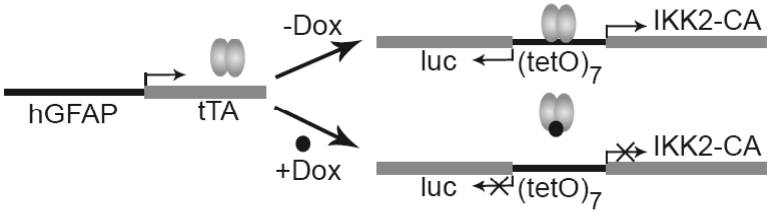

Figure 7: Tetracycline regulated ("tet-off") expression system for the astrocyte specific expression of IKK2-CA and a luciferase reporter gene

The artificial transcription factor tetracycline-regulated transactivator (tTA) is expressed under the control of the astrocyte-specific human GFAP promotor in the driver transgene. tTA binds then to tet-operator elements (tetO) in a bidirectional promotor in the responder transgene and induces transcription of the IKK2-CA transgene and a luciferase reporter gene. Binding of doxycyline can block transgene expression induced by tTA.

In addition, a similar construct with a dominant negative allele of human IKK2 (IKK2-DN), which contains an inactivating mutation in the kinase domain (D145N), was used to block IKK2 signaling (Herrmann et al., 2005). To distinguish between kinase activity dependent phenotypes and kinase independent effects of IKK2 overexpression, this construct was used for some control experiments.

To induce expression of these transgenes, a second transgene was crossed in these mouse lines, which was described previously (Pascual et al., 2005). This transgene expresses tTA in specifically astrocytes by the use of a fragment of the human GFAP promotor (GFAP.tTA).

To control the genomic presence of the two transgenes, DNA from mouse-tail biopsies was prepared and the mice were genotyped as described in the "Materials and Methods" section. Offspring derived from such breedings (GFAP.tTA x (tetO)7.IKK2-CA) which possess both transgenes (GFAP/IKK2-CA animals) should express IKK2-CA in a doxycycline-dependent manner, whereas single transgenic or wild type littermates should not express IKK2-CA and could be used as controls (Co animals).

With this binary approach ("tet-off"-system) it is possible to induce expression of the IKK2 mutants specifically in astrocytes, and to reversibly repress these transgenes by doxycycline administration to the mice (e.g. in the drinking water), to induce or block NF-κB activation at a specific time point (Figure 7).

3.1.2 Analysis of the IKK2-CA regional and cellular expression pattern

To confirm specificity of the transgene expression system, the transgene was detected by immunoblotting with an antibody specifically recognizing human IKK2 (the transgene), but not endogenous mouse IKK2. As expected, at postnatal day 7 (P7) IKK2-CA was expressed in all parts of the brain of GFAP/IKK2-CA animals, but not in kidney and spleen (Figure 8A), organs that do not express GFAP (Brenner et al., 1994). In control animals that possess the (tetO)7.IKK2-CA transgene, but not the GFAP.tTA transgene, no leak expression of IKK2-CA was detected (Figure 8A).

To show the specificity of transgene expression on a cellular level, the hIKK2 specific antibody was used to detect IKK2-CA expression by immunofluorescence costaining with Aldh1l1 in brain sections. Aldh1l1 is a novel marker discovered in a systematic screening approach, which labels a broad range of astrocyte subpopulations, but with a less variable expression than GFAP (Cahoy et al., 2008). The staining shows a colocalisation of the transgene with the astrocyte marker, indicating that IKK2-CA expression is indeed targeted to astrocytes (Figure 8B). In addition, some cells in the ependymal and subependymal zone of the lateral ventricles display transgene expression, suggesting that also certain subpopulations of ependymal cells and/or neural stem cells might express the transgene (Figure 8C). This is expected since NSC and ependymal cells are closely related to astrocytes and are described to express GFAP (see 1.2.1). As we found that not all GFAP/IKK2-CA animals express the transgene with the same strength and kinetics (especially during early postnatal development), in later experiments we routinely used enzyme activity measurements for the luciferase reporter gene as a simple and rapid method for the semi-quantitative detection of transgene expression. Animals with very low luciferase activities in native proteins extracts of the medulla oblongata and spinal chord (<1000 RLU/μg protein) were excluded from further analysis.

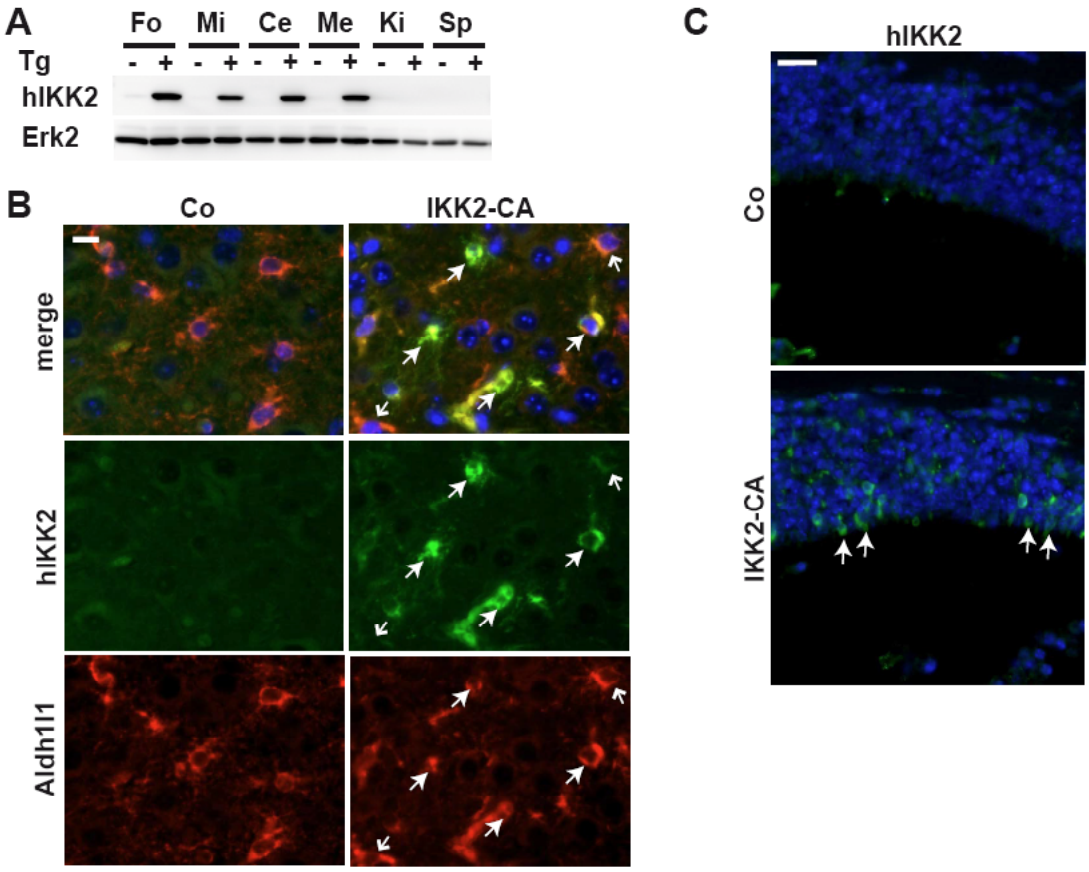

Figure 8: The IKK2-CA transgene is expressed *in vivo* in the CNS in astrocytes and a subpopulation of ependymal/subependymal cells

(A) IKK2-CA is expressed in all brain parts of GFAP/IKK2-CA animals (Tg+) at P7, but not in kidney and spleen, shown by immunoblot for human IKK2 (transgene); No promotor leakiness is observed in (tetO)7.IKK2-CA single transgenic mice (Tg-); Erk2 is detected as loading control; Fo: forebrain, Mi: midbrain/thalamus/hypothalamus, Ce: cerebellum, Me: medulla oblongata, Ki: kidney, Sp: spleen;

(B) Variable transgene expression in astrocytes (Aldh1l1 positive); hIKK2/Aldh1l1 double staining at P7; filled arrows show IKK2-CA expressing astrocytes, open arrows shows IKK2-CA negative astrocytes.

(C) The transgene is also expressed in a subpopulation of cells in the ependymal layer (arrows) and subventricular zone (P2); hIKK2 staining.

Scale bars: (B): 10 μm (C): 20 μm; All merged images shown with DAPI costaining.

3.1.3 NF-κB activation by IKK2-CA in astrocytes *in vivo*

To confirm the functionality of the IKK2-CA transgene in astrocytes *in vivo*, the intracellular localization of RelA was assessed by RelA/hIKK2 immunofluorescence costainings, which showed a prominent nuclear RelA staining in most transgene positive cells (Figure 9A). To quantify this finding, nuclear RelA staining intensity was measured in images of the thalamus. A histogram shows a slight shift of the nuclear RelA intensity distribution to higher intensities in IKK2-CA expressing animals, when all cells within the images are considered, and a clearly higher staining intensity of those cells that express the transgene was detected (Figure 9B).

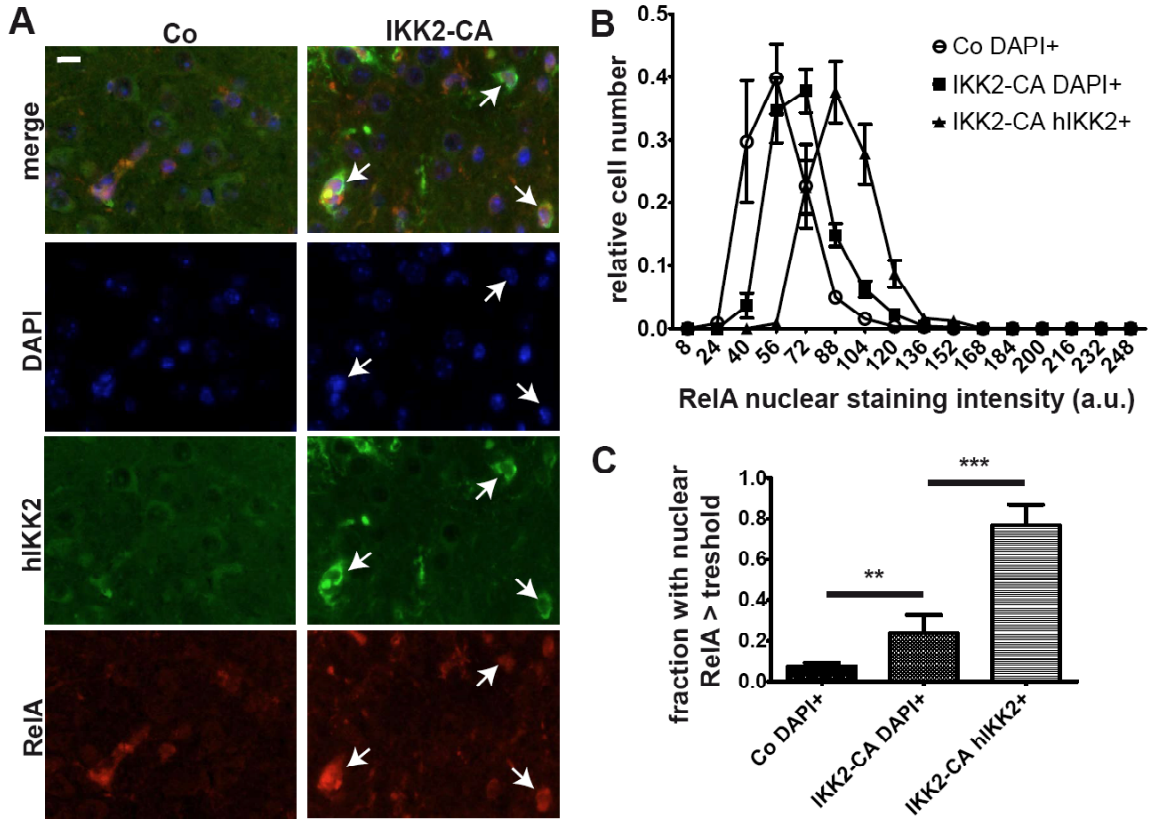

Figure 9: RelA nuclear translocation is induced by IKK2-CA in astrocytes *in vivo*

(A) IKK2-CA induces nuclear localization of RelA in most transgene expressing cells (DAPI/hIKK2/RelA costaining, arrows);

(B) Distribution of nuclear fluorescence intensity for RelA staining; in total 400-600 nuclei were measured per animal, including 40-80 nuclei of hIKK2 positive cells in GFAP/IKK2-CA animals

(C) Fraction of nuclei with a RelA staining intensity above a threshold for all nuclei (DAPI+) in control and transgenic animals and nuclei of transgene positive cells (hIKK2+); values show mean +/- SD; statistical analysis: t-test (2-tailed, n = 5-6 animals); ** $p < 0.01$, *** $p < 0.001$

For a different evaluation, a threshold was defined, above which the cells were counted as nuclear RelA positive. This population included around 10% of the cells in control animals. In GFAP/IKK2-CA animals, the total number of nuclear RelA positive cells was about 3-fold increased, and most strikingly, around 80% of the transgene expressing cells had nuclear localization of RelA (Figure 9C). This clearly demonstrates that IKK2-CA expression induces NF-κB activation in astrocytes as expected.

3.1.4 IKK2-CA expression in development

When IKK2-CA is expressed during the complete development, unexpectedly the animals show spontaneous hydrocephalus formation and variable growth retardation in the early postnatal phase (Figure 10A). Finally, these animals usually die before weaning, except for some animals with a low or absent transgene expression.

Hydrocephalus formation was also found in a second founder line for the (tetO)7.IKK2-CA transgene ((tetO)7.IKK2-CA(B)), but not in single transgenic GFAP.tTA or (tetO)7.IKK2-CA control animals, showing that IKK2-CA overexpression and not the disruption of another gene by transgene integration is responsible for the development of the phenotype (Figure 10B/C). The more severe phenotype in line A correlates with a higher transgene expression (Figure 10C).

To further confirm that the phenotype is indeed dependent on IKK2-CA expression and that the transgene expression system can be regulated by doxycycline, animals were treated with doxycycline in the drinking water during pregnancy until postnatal day 7 (P7), which both abolished transgene expression and hydrocephalus formation (Figure 11A/B). Additionally, to exclude that hydrocephalus formation is an artifact of overexpression of IKK2 protein independent of its kinase function, the kinase inactive allele of IKK2 (IKK2-DN) was expressed instead of the constitutively active allele in the same system. Despite a higher expression, IKK2-DN could not induce hydrocephalus formation (Figure 11A/B).

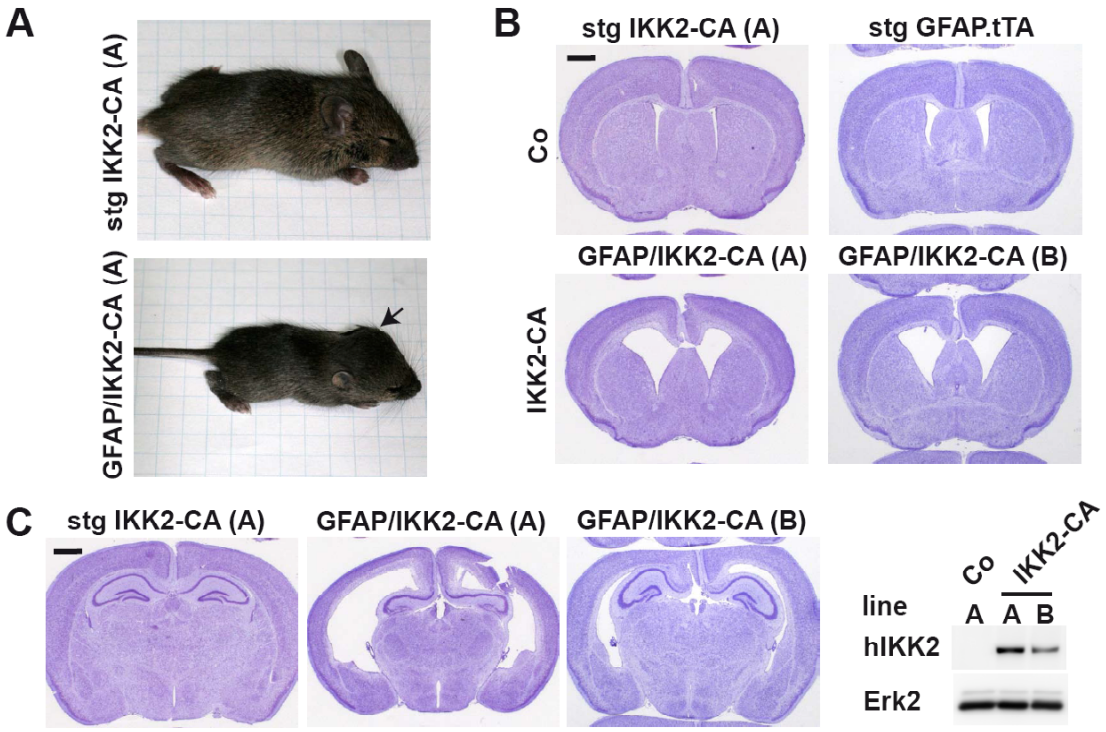

Figure 10: Expression of GFAP/IKK2-CA results in early postnatal hydrocephalus formation and growth retardation

(A) A case of severe hydrocephalus (arrow: enlarged skull) and reduced growth at postnatal day 16 (P16) and a control littermate (only (tetO)7.IKK2-CA transgenic).

(B) Nissl staining shows massive enlargement of the lateral ventricles at P16 in IKK2-CA expressing mice from two different (tetO)7.IKK2-CA founder lines (IKK2-CA A/B), but not in either GFAP.tTA or (tetO)7.IKK2-CA single transgenic control animals; scale bar 1 mm.

(C) Ventricular enlargement is most pronounced in the caudal part of the lateral ventricles and is associated with a hippocampal malformation; line A shows a more severe hydrocephalus correlating with stronger transgene expression; Nissl staining (scale bar 1 mm) and anti-hIKK2 immunoblot for IKK2-CA in the medulla oblongata; Erk2 is detected as loading control.

To characterize transgene expression in this system in more detail, the developmental onset of IKK2-CA expression was investigated. Immunoblotting revealed a perinatal onset of transgene expression around P2 (Figure 12A). To identify a potential prenatal expression in small populations of GFAP expressing NPC, immunofluorescence staining for the trangene at the embryonal stage E18.5 was performed, which did not detect any prenatal transgene expression (Figure 12B). Taken together, expression of GFAP/IKK2-CA starts perinatally and is repressible by doxycycline. It induces rapid hydrocephalus formation, which requires IKK2 kinase activity, as this phenotype is not induced by the kinase inactive IKK2-DN allele.

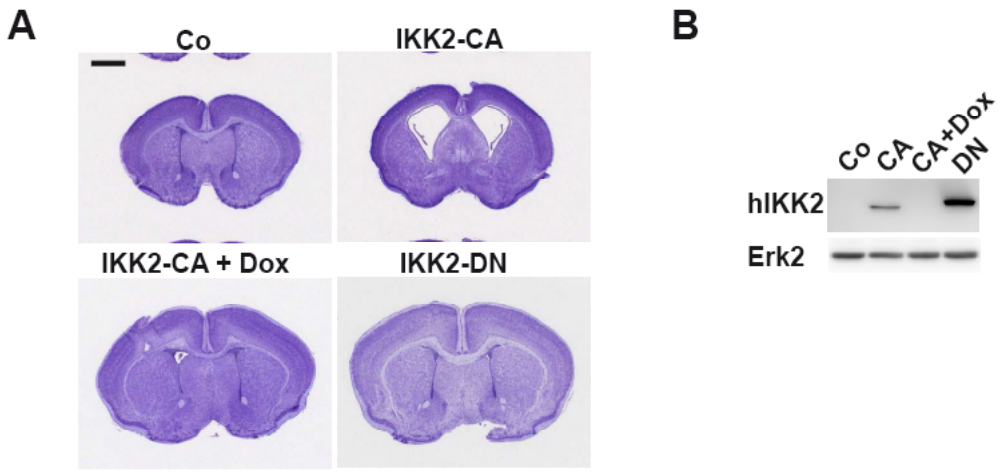

Figure 11: The GFAP/IKK2-CA system can be regulated by doxycycline; hydrocephalus formation requires kinase activity of IKK2

(A) Hydrocephalus is not induced in the presence of doxycycline or by overexpression of an inactive IKK2 allele (IKK2-DN); Nissl staining (scale bar 1 mm);

(B) IKK2-CA expression can be abolished by doxycycline, and is lower than IKK2-DN expression; immunoblot for human IKK2 (transgene) in medulla oblongata; Erk2 is detected as loading control.

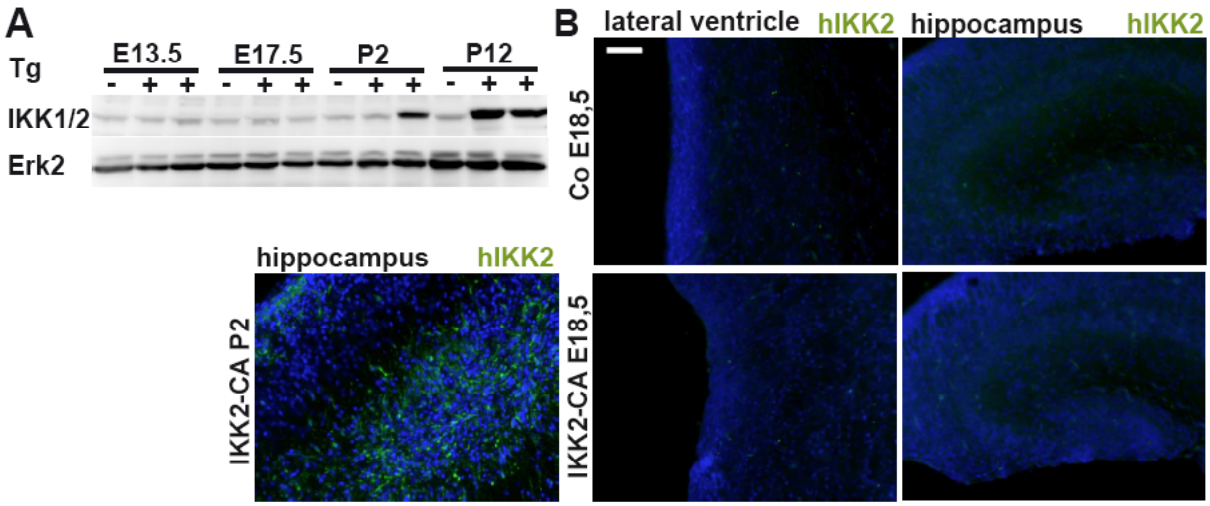

Figure 12: IKK2-CA expression starts perinatally

(A) Expression of the IKK2-CA transgene becomes detectable by immunoblotting around birth in GFAP/IKK2-CA transgenic animals (Tg+); the transgene is detected with an IKK1/2 antibody, which also shows a weaker band of endogenous IKK; Erk2 is used as loading control.

(B) No expression of the transgene is detectable by immunofluorescence at embryonic stage E18.5, shown for hippocampus and lateral ventricle/subventricular zone; hIKK2 staining; an image of a hippocampus/dentate gyrus section at P2 was taken with same exposition time as a positive control, DAPI costaining was performed for the visualization of nuclei.

3.1.5 Transgene reactivation in adult animals

As depicted above, transgene expression can be repressed by administration of doxycycline in the drinking water. In principle, this repression should be reversible by doxycycline withdrawal, allowing the reactivation of IKK2-CA expression at any designated time point. To study the consequences of NF-κB activation in the fully developed brain, doxycyline was administered until the age of 4 weeks, when brain development is completed.

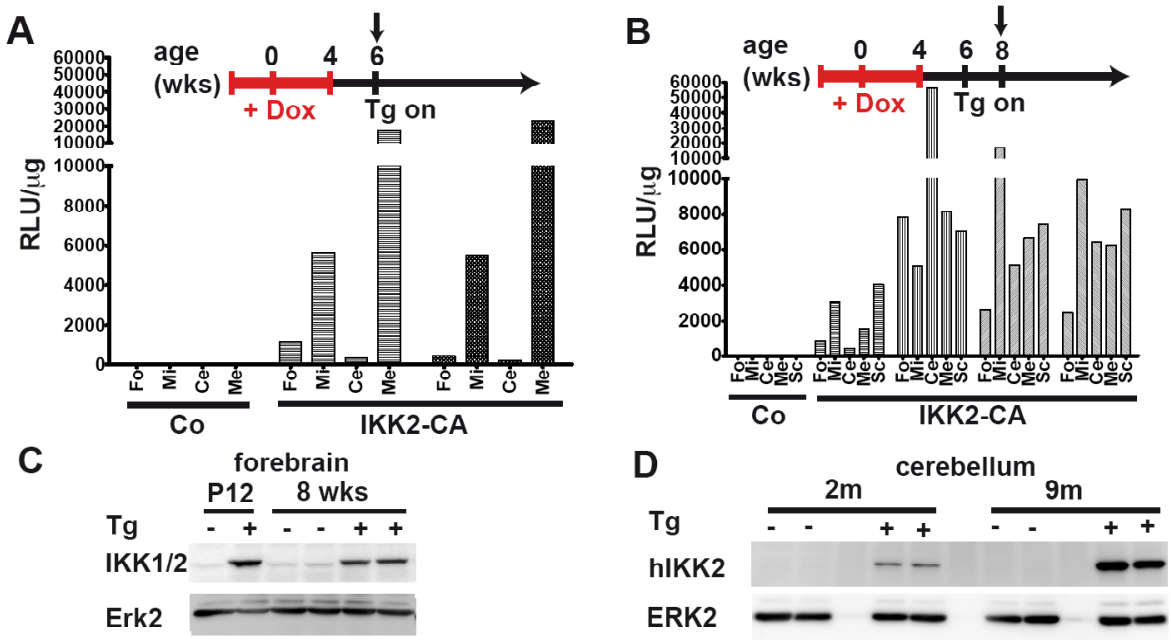

Figure 13: IKK2-CA is re-expressed in the fully developed brain after doxycycline withdrawal at 4 weeks of age

(A) Activity of the luciferase reporter gene in native protein extracts of different brain parts of one control animal and two GFAP/IKK2-CA animals at 6 weeks of age shows prominent transgene expression in medulla oblongata and midbrain/thalamus/hypothalmus;

(B) Activity of the luciferase reporter gene of one control and 4 GFAP/IKK2-CA animals at 8 weeks of age shows prominent transgene expression in all parts of the CNS in 3 of 4 transgenic animals;

(C) Transgene expression in forebrain tissue at 8 weeks of age is similar to the expression in young animals (P12) without doxycycline treatment; immunoblot for IKK1/2 and Erk2 (loading control).

(D) IKK2-CA is persistently expressed, starting around 2 months of age till at least 9 months of age, shown by immunoblotting of cerebellar tissue extracts; the human IKK2 derived transgene is detected with an antibody against human IKK2, which does not recognize endogenous IKK2; ERK2 was detected as loading control.

Fo: forebrain, Mi: midbrain/thalamus/hypothalamus, Ce: cerebellum, Me: medulla oblongata, Sc: spinal chord

To confirm reversibility of transgene repression by doxycycline, activity of the luciferase reporter gene was measured at 6 and 8 weeks of age, i.e. 2 and 4 weeks after doxycycline withdrawal, in native protein extracts of different brain parts. Whereas in the medulla oblongata and the midbrain/thalamus/hypothalamus prominent luciferase activity was present 2 weeks after doxycycline withdrawal, transgene expression in the forebrain and cerebellum was very low at this time (Figure 13A). Despite a high inter-individual variation, at 8 weeks of age most animals show a luciferase activity in the range of 2000-60000 RLU/μg protein in all brain parts and the spinal cord (Figure 13B).

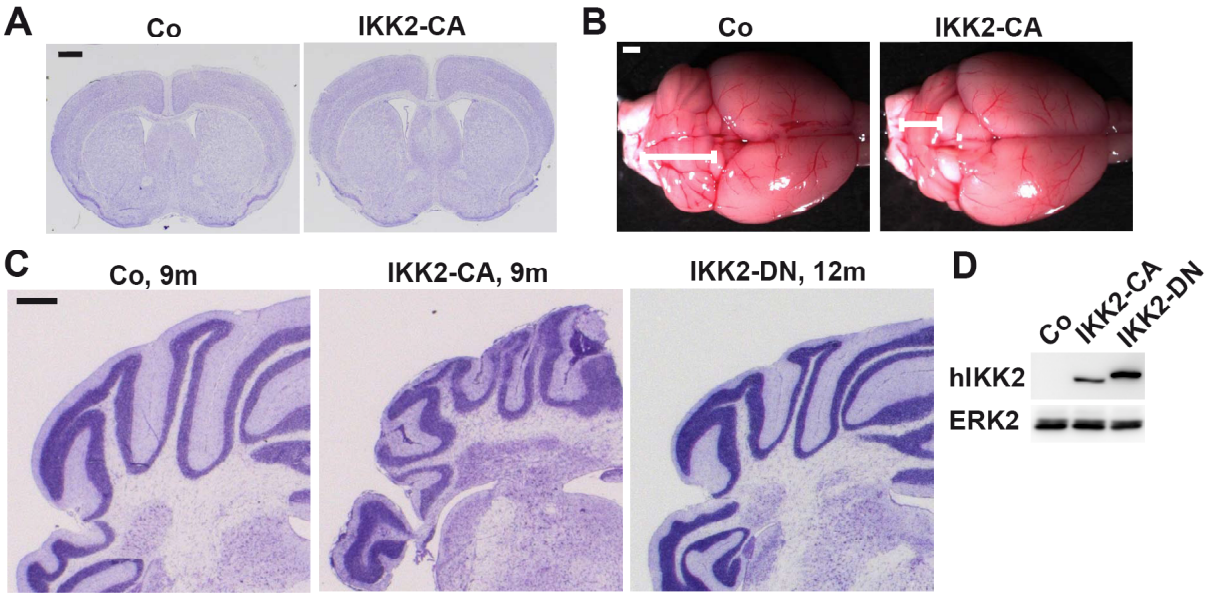

Figure 14: GFAP/IKK2-CA re-expression in the fully developed brain at 4 weeks of age does not result in hydrocephalus formation, but causes delayed cerebellar atrophy, which is not found in GFAP/IKK2-DN animals

(A) Animals do not show prominent dilatation of the lateral ventricles at the age of 8 weeks (Nissl-staining, scale bar 1 mm).

(B) Long-term expression of IKK2-CA causes subacute cerebellar atrophy, shown at 9 months of age; scale bar 1 mm;

(C) Nissl staining shows a compressed overall structure of the cerebellar cortex in the rostral part of the cerebellum at 9 months in GFAP/IKK2-CA animals, but not in GFAP/IKK2-DN animals at 12 months of age; scale bar: 500 μm

(D) Transgene expression in adult GFAP/IKK2-DN animals (age 14 months) is higher than in GFAP/IKK2-CA animals (age 9 months); immunoblot for the transgene (hIKK2) and ERK2 as loading control;

Expression of the transgene in the forebrain 4 weeks after doxycycline withdrawal was comparable with the expression in P12 animals that never received doxycycline, determined by immunoblotting, indicating a full reversibility of transgene expression (Figure 13C).

The observed transgene expression level seems to be stable over a long time. In case of the cerebellum, which possesses a relatively late onset of transgene expression, expression levels at 9 months are even higher than at the age of 2 months (Figure 13D).

Of note, when transgene expression is induced after brain development is completed, the animals do not develop a hydrocephalus (Figure 14A) or other signs of severe sickness as the young animals with permanent IKK2-CA expression. However, older animals begin to display variable motor impairments and an atrophy of the cerebellum, which is obvious in macroscopic images and Nissl stained sections at 9 months of age (Figure 14B/C). This phenotype is also a result of increased IKK2 activity, and not a protein overexpression artifact, as mice that express the kinase inactive IKK2 allele at an even higher level do not develop this atrophy (Figure 14C/D).

3.1.6 *Ex vivo* transgene reactivation in primary astrocyte cultures

For the investigation of the direct effects of IKK2-CA expression on astrocytes, primary astrocytes were prepared from GFAP/IKK2-CA mice and control littermates and cultured *ex vivo*. To avoid indirect effects of the transgene on the composition and physiologic state of the cellular isolates, the primary cultures were established from P2 pups that received doxycycline during pregnancy until preparation, and then further until the first passage. Before the first passage doxycycline was washed out and 3 days later the cultures were analyzed for their cellular composition and transgene expression.

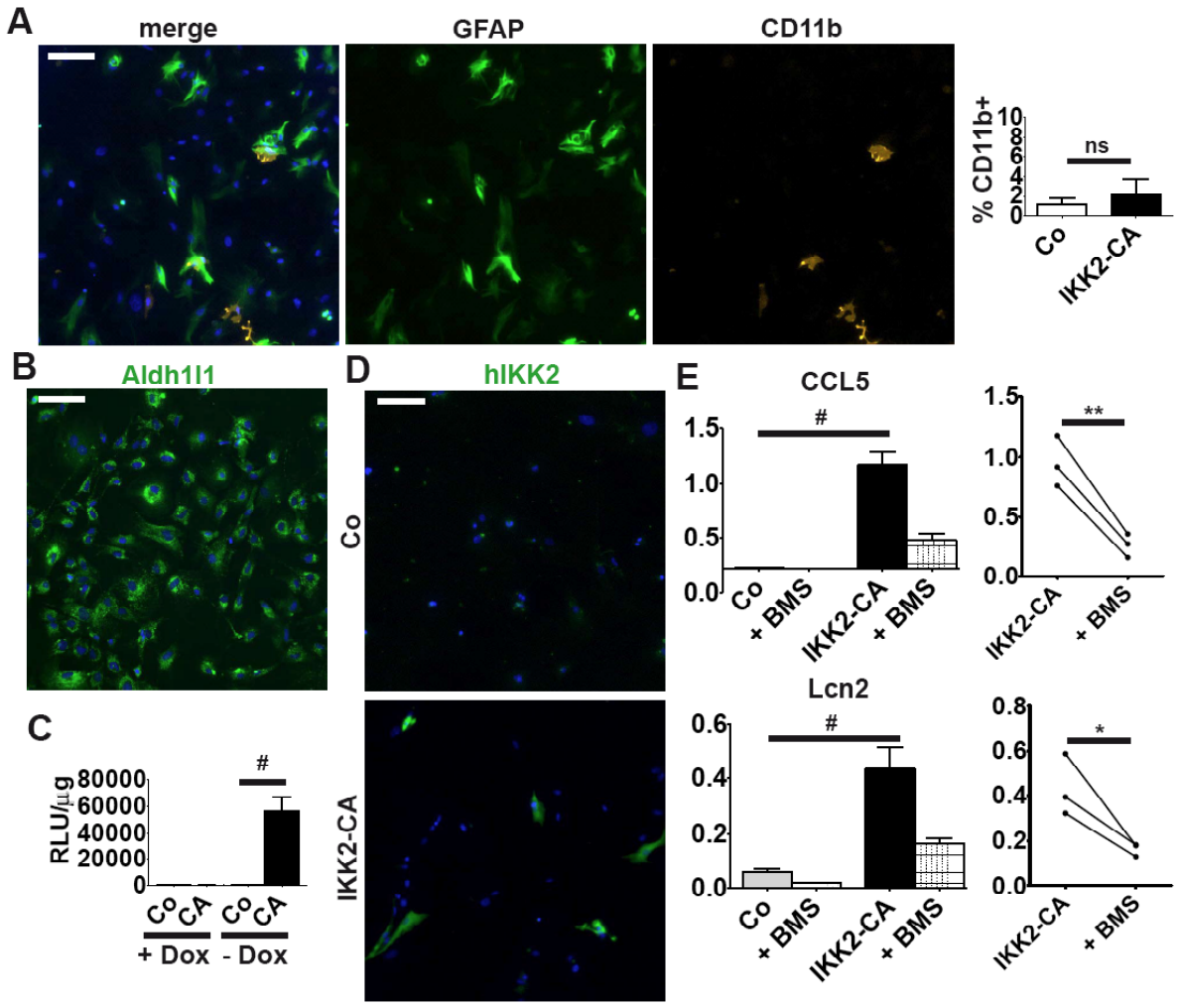

Figure 15: Primary astrocytes from GFAP/IKK2-CA animals show mosaic transgene expression and upregulation of NF-κB target genes

(A) Primary astrocyte cultures show variable GFAP expression and low microglia contamination (CD11b positive); quantification shows no significant difference in microglia content 3 days after transgene reactivation (statistical analysis: unpaired 2-tailed t-test, n = 5-6)

(B) Staining for the astrocyte marker Aldh1l1 confirms high astrocyte enrichment in the cultures

(C) Measurement of luciferase activity demonstrates complete repression of the transgene before (passage 1) and strong reexpression three days after doxycycline withdrawal (passage 2); statistical analysis: unpaired 2-tailed t-test, n = 3

(D) Immunostaining for IKK2-CA (hIKK2) reveals mosaic pattern of transgene expression;

(E) IKK2-CA induces the NF-κB the target genes CCL5 and Lcn2 in primary astrocytes from GFAP/IKK2-CA mice (qPCR), which can be largely reduced by the pharmacological IKK2 inhibitor BMS-345541 (5 µM, 24 h); each n = 3 preparations of IKK2-CA and control animals, untreated and treated with BMS-345541; statistical analysis: Co vs. IKK2-CA unpaired 2-tailed t-test (# $p<0.05$), +/- BMS treatment paired 1-tailed t-test (* $p<0.05$, ** $p<0.01$);

All merged immunofluorescence images show nuclear DAPI costaining; scale bars: 100 µm; all bar diagrams display mean +/- s.e.m.;

Regardless of the genotype, the cultures had a low contamination with microglia (below 5%), shown by immunoflurescence staining for the myeloid cell marker CD11b (Figure 15A). This is important to distinguish direct astroglial specific effects of the transgene from possible secondary effects caused by activated microglia that secrete high levels of cytokines and prominently alter the properties of primary astrocyte cultures (Saura, 2007). The GFAP expression in the cultured astrocytes was highly variable. This was observed before both *in vivo* and in primary cultures, which inspired the search for new astrocyte specific markers, and resulted in the discovery of Aldh1l1 as an alternative marker, which is more broadly and less variably expressed (Cahoy et al., 2008).

According to Aldh1l1 staining, the vast majority of cells in these primary cultures seem to be astrocytes (Figure 15B). To control for IKK2-CA re-expression under *ex vivo* conditions, luciferase activity in native protein extracts was measured before and 3 days after docycycline withdrawal, showing a complete repression of luciferase before passage 1 and strong reexpression 3 days thereafter (Figure 15C). As *in vivo* (see 3.1.2), not all astrocytes express the transgene, indeed in culture only about 10-20 % of all cells are positive for human IKK2 (the transgene) in immunofluorescence staining (Figure 15D). To test whether this is sufficient to drive NF-κB dependent transcription, expression of the two NF-κB target genes CCL5 and Lcn2 was assessed by qPCR. Both genes are dramatically upregulated in primary astrocytes from GFAP/IKK2-CA animals, which is significantly reduced by the pharmacological IKK2-inhibitor BMS-345541, confirming that canonical NF-κB signaling is responsible for this upregulation (Figure 15E).

Taken together, these experiments show that the GFAP/IKK2-CA transgene system can also be used to study IKK2/NF-κB signaling in astrocytes *ex vivo*, at least for experiments that use whole cell populations, e.g. gene expression analysis with RNA or protein extracts.

3.2 Characterization of neuroinflammation induced by NF-κB activation in astrocytes in the GFAP/IKK2-CA model

3.2.1 Transcriptional alterations induced by enhanced IKK2 activation in astrocytes

Astrocytes are critical cellular regulators of neuroinflammation, a process for which NF-κB signaling is a critical mediator, suggesting that astroglial NF-κB activation might induce or at least enhance neuroinflammatory responses. NF-κB is known to regulate hundreds of target genes that are involved not only in neuroinflammation, like chemokines, cytokines and cell adhesion molecules, but also in various other processes like cell proliferation, differentiation and survival. Therefore a systematic approach was made to characterize the global gene expression profile induced by canonical NF-κB activation in astrocytes in the GFAP/IKK2-CA model.

3.2.1.1 Global transcriptional profiling of early postnatal forebrain of animals with IKK2-CA expression in astrocytes

To get a broad and physiologically relevant global overview over the genes and pathways that are directly and indirectly transcriptionally regulated by astroglial NF-κB signaling in *in vivo* conditions, a microarray analysis of mRNA from P7 forebrain tissue of GFAP/IKK2-CA animals and (tetO)7.IKK2-CA single transgenic littermate controls was performed.

This screening approach revealed a total of 399 deregulated genes with a threshold of 1.5-fold regulation and a significance level of $p < 0.05$ (Benjamini-Hochberg corrected t-test). Of those 388 were upregulated, whereas only 11 were downregulated (no more than 2-fold).

Comparative analysis of these genes with the KEGG-database revealed that the vast majority of annotated deregulated genes is implicated in inflammatory and immune response networks (Table 1). These included networks involved in the recognition of potential dangers like genes involved in antigen presentation or TLR signaling.

Table 1: KEGG-pathway analysis of microarray data from forebrain of GFAP/IKK2-CA animals vs. controls at P7

KEGG Pathway	regulated	total	z-score
general immune system/inflammation			
Antigen processing and presentation	18	88	9,92
Toll-like receptor signaling pathway	17	99	8,56
Cell adhesion molecules (CAMs)	21	143	8,53
Hematopoietic cell lineage	15	82	8,39
NOD-like receptor signaling pathway	12	61	7,87
Complement and coagulation cascades	13	72	7,73
Cytokine-cytokine receptor interaction	27	251	7,61
B cell receptor signaling pathway	13	76	7,45
Fc gamma R-mediated phagocytosis	14	87	7,41
Chemokine signaling pathway	21	174	7,34
Intestinal immune network for IgA production	9	44	6,98
Natural killer cell mediated cytotoxicity	17	135	6,81
Leukocyte transendothelial migration	14	115	6,01
Fc epsilon RI signaling pathway	11	78	5,95
Cytosolic DNA-sensing pathway	7	55	4,38
Jak-STAT signaling pathway	13	150	4,28
Primary immunodeficiency	5	35	4,04
RIG-I-like receptor signaling pathway	7	68	3,67
Autoimmunity			
Graft-versus-host disease	12	49	9,07
Type I diabetes mellitus	12	54	8,52
Allograft rejection	11	48	8,32
Systemic lupus erythematosus	17	115	7,69
Autoimmune thyroid disease	11	64	6,87
Asthma	6	26	6,16
Infectious disease			
Leishmaniasis	21	64	14,37
Viral myocarditis	15	83	8,32
Chagas disease	16	103	7,73
Prion diseases	6	35	5,05
Malaria	7	50	4,71
Other pathways			
Apoptosis	9	85	4,27
Lysosome	10	118	3,66
Ether lipid metabolism	4	33	3,18
Arachidonic acid metabolism	6	76	2,62
Other glycan degradation	2	15	2,42
Pathways in cancer	16	316	2,37
alpha-Linolenic acid metabolism	2	16	2,3
Pancreatic cancer	5	70	2,14
Regulation of actin cytoskeleton	11	208	2,11
VEGF signaling pathway	5	73	2,03

Other factors are important for the recruitment of immune cells like cell adhesion molecules, chemokine and cytokine signaling networks. Finally, also immune effector pathways like complement cascade and phagocytosis genes are induced. Many of these genes are also related to autoimmune and infectious diseases.

Beside inflammation and immune signaling pathways, other less prominently altered gene networks found in the KEGG pathway analysis are involved in apoptosis, lysosomal function, lipid metabolism and others (Table 1).

This analysis indicates that the main function of astroglial NF-κB signaling seems to be the coordinated activation of the inflammatory and immune defense programs of the CNS, which is in line with the anti-inflammatory role of astroglial NF-κB inhibition found in previous studies (see 1.4).

The inflammation and immune response associated genes are not only the most numerous genes upregulated, but many of them are also very highly upregulated. Indeed among the 30 most prominently upregulated genes are almost exclusively genes involved in these processes (Table 2).

The most highly induced gene found in this screen is lipocalin 2 (Lcn2, also known as neutrophil gelatinase associated lipocalin, NGAL or 24p3). This protein was recently described as an important mediator of neuroinflammation, which is involved in astrogliosis, microgliosis and neural development (Lee et al., 2007; Lee et al., 2009; Lee et al., 2011b; Lee et al., 2011a; Zamanian et al., 2012). Three other genes within the 10 most highly upregulated are the major proinflammatory chemokines CCL5/RANTES, CXCL10/IP-10 and CCL2/MCP-1, which are important chemoattractants for immune cells (Szczucinski and Losy, 2007). Another group of genes very prominently induced by IKK2-CA are several components of both MHC classes I and II. Of these, the three MHC class II factors CD74, H2-Aa and H2-Ab1 are among the 10 most highly upregulated genes. This indicates the activation of professional antigen presenting cells, which is a prerequisite for the induction of the adaptive immune system. In addition, many acute phase proteins and other effectors of the innate immune response are highly induced, like the complement component C3, the antibacterial enzyme lysozyme 2 and several guanylate binding proteins (Gbp2/5/6), a group of cytokine induced proteins with antiviral, antimicrobial and anti-proliferative functions (Vestal and Jeyaratnam, 2011).

Together, these findings indicate that astroglial NF-κB activation initiates an activation of the immune system by the production of proinflammatory mediators and acute phase proteins.

Table 2: The 30 most highly upregulated genes in microarray analysis from forebrain P7 GFAP/IKK2-CA animals vs. controls

Fold upregulation IKK2-CA vs. Co	Gene ID	Gene Title
59,48	Lcn2	Lipocalin 2
24,44	Ccl5	Chemokine (C-C motif) ligand 5 (RANTES)
20,84	Cxcl10	Chemokine (C-X-C motif) ligand 10 (IP-10)
19,04	C3	Complement component 3 (C3)
14,96	Cd74	CD74 antigen (invariant polypeptide of MHC class II)
10,23	H2-Aa	Histocompatibility 2, class II antigen A, alpha
8,04	H2-Ab1	Histocompatibility 2, class II antigen A, beta 1
7,60	Gbp2	Guanylate binding protein 2
7,52	Ccl2	Chemokine (C-C motif) ligand 2 (MCP-1)
7,23	Cp	Ceruloplasmin (Cp)
5,85	AW112010	Small secreted protein interferon-induced
4,81	H2-K1	MRNA similar to histocompatibility 2, D region locus 1
4,63	Steap4	STEAP family member 4
4,58	Ifitm3	Interferon induced transmembrane protein 3
4,40	H2-K1	Histocompatibility 2, K1, K region, mRNA
4,36	Mpa2l	Guanylate-binding protein 10
4,14	Tnfaip2	Tumor necrosis factor, alpha-induced protein 2
4,07	H2-Q8	Histocompatibility 2, Q region locus 8 (H2-Q8)
4,01	Gbp5	Guanylate binding protein 5
3,90	A2m	Alpha-2-macroglobulin
3,84	Madcam1	Mucosal vascular addressin cell adhesion molecule 1
3,70	Lyz2	Lysozyme 2
3,64	Mpeg1	MPS1 gene and mRNA, 3'end
3,57	Tslp	Thymic stromal lymphopoietin
3,55	AI607873	Expressed sequence AI607873
3,51	Gbp6	Guanylate binding protein 6
3,51	Ctss	Cathepsin S
3,48	Ms4a6d	Membrane-spanning 4-domains, subfamily A, member 6D
3,39	Spp1	Osteopontin (OPN)
3,29	Oasl2	2'-5' oligoadenylate synthetase-like 2

3.2.1.2 Comparison of transcriptional alterations in the developing and adult forebrain and the adult cerebellum

To validate the data obtained from the microarray screen, expression of several deregulated genes was analyzed by qPCR. Samples of different developmental stages and brain parts were analyzed to characterize developmental and regional differences in target gene regulation. In particular, the developing forebrain at P7, the adult forebrain at 2 months of age (4 weeks after transgene reactivation), and the adult cerebellum at 2 and 9 months of age were studied, i.e. before onset and in a late stage of cerebellar degeneration.

Two of the chemokines, CCL2/MCP-1 and CCL5/RANTES that were highly upregulated in the microarray screen show a even more prominent induction in qPCR assays in P7 forebrain (around 50 fold or 100 fold respectively), and are also highly upregulated in the other analyzed samples. Both show a similar expression pattern in the GFAP/IKK2-CA animals, with a tendency of lower expression in the adult forebrain, but a higher expression in the cerebellum, which is highest in the degenerated cerebellum (Figure 16A).

A comparable expression profile is also found for lipocalin 2, complement C3 and the MHC class II component CD74, although the absolute expression values vary (Figure 16B/C/D). This indicates that all these highly deregulated genes share a common mechanism of regulation, i.e. they might be direct NF-κB target genes in astrocytes.

This is different for the complement factor C4b, the cell adhesion molecule Madcam-1 and the key proinflammatory cytokine IL-1β (Figure 16C/E/F), indicating that these factors are also subjected to also other regulatory mechanisms. Expression of C4b is very low in the adult cerebellum at 2 and 9 months of age, but is highly upregulated in the late phase of cerebellar degeneration, whereas it is only mildly induced in the other conditions (Figure 16C).

The only incompletely characterized cell adhesion molecule Madcam-1, which was detected in the microarray screen as the most highly upregulated member of the immunoglobulin superfamily of cell adhesion molecules, also showed an different expression pattern (Figure 16E).

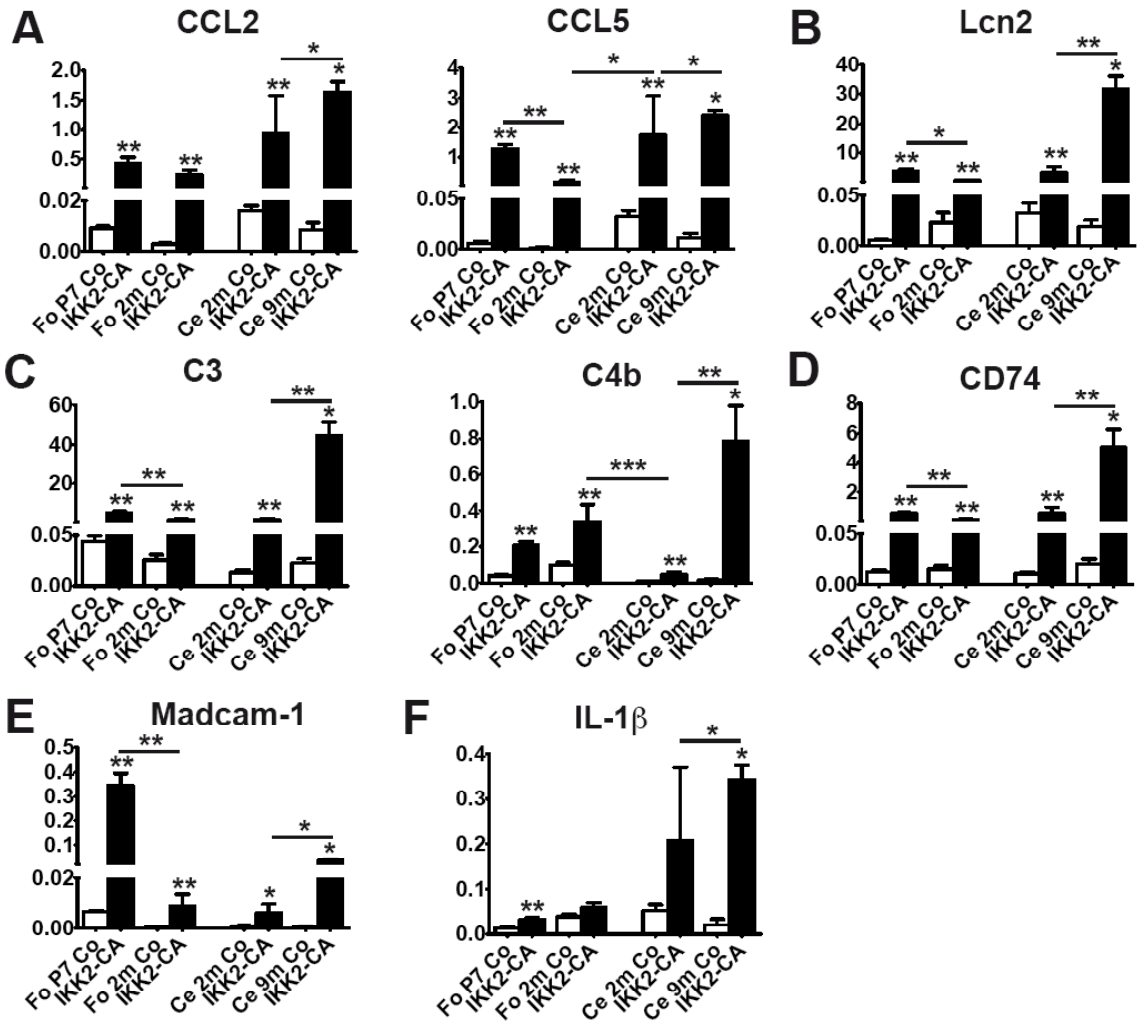

Figure 16: Proinflammatory genes are induced by GFAP/IKK2-CA in the developing and adult forebrain and in the cerebellum before onset and in late stages of degeneration (qPCR)

(A) The chemokines CCL2/MCP-1 and CCL5/RANTES
(B) Lipocalin 2, a major inducer of glial activation
(C) The complement factors C3 and C4b
(D) CD74, a constant chain of the MHC class II complex
(E) The cell adhesion molecule Madcam-1
(F) The major proinflammatory cytokine IL-1β

displayed expression values are relative to HPRT as reference housekeeping gene; statistical analysis: two-tailed Mann-Whitney-test (n=4-7): *p < 0.05, ** p < 0.01, *** p < 0.001;

Remarkably, both the basal expression level and the IKK2-CA induced levels are highest in the developing forebrain, whereas the basal expression level is at the detection limit in the adult brain, indicating that this gene is specifically expressed

during brain development. Induction by IKK2-CA is observed in all conditions, but reaches only low expression levels in the adult brain.

IL-1β was studied as a prototypical proinflammatory cytokine which was not found upregulated in the microarray (Figure 16F). Although by qPCR a mild upregulation was detected in forebrain P7, it seems not prominently induced by IKK2 activation in astrocytes. However, as for C4b, a strong upregulation in the late stage of cerebellar degeneration was found, indicating that both genes might be upregulated as an indirect response to long-term IKK2 activation in astrocytes.

3.2.1.3 Primary alterations of gene transcription induced by IKK2 activation in *ex vivo* cultivated astrocytes

To distinguish between genes that are induced by NF-κB activation directly in astrocytes and genes that are indirectly upregulated by the pathological consequences of GFAP/IKK2-CA expression, we analyzed expression of several genes found in the microarray screen in primary astrocytes. To avoid indirect effects of the transgene, its expression was induced *ex vivo* for only three days before transcriptional alterations were assessed (see also 3.1.6).

Of the 10 highest upregulated genes in the microarray screen, 7 were also massively induced in primary astrocytes. These include the major proinflammatory chemokines CCL2/MCP-1, CCL5/RANTES and CXCL10/IP-10 and the astrogliosis inducer Lcn2 (Figure 17A/B).

These also include the acute phase response genes C3 and Gbp2, and remarkably also CD74, a component of the MHC class II complex, which is characteristic for professional antigen presenting cells (Figure 17C/D/F). Indeed all of these genes are described to be regulated by NF-κB (Wei et al., 2008; Gupta et al., 2010), further supporting the hypothesis that these genes are direct NF-κB-targets in astrocytes.

An important class of proinflammatory NF-κB target genes are cell adhesion molecules, which are less prominently upregulated in the screen. Madcam-1, the cell adhesion molecule with the most pronounced upregulation in the microarray experiment, is also highly induced in primary astrocytes (Figure 17E). Icam-1, which was upregulated only around 2-fold in the screen, is a well-characterized and important proinflammatory factor regulated by NF-κB.

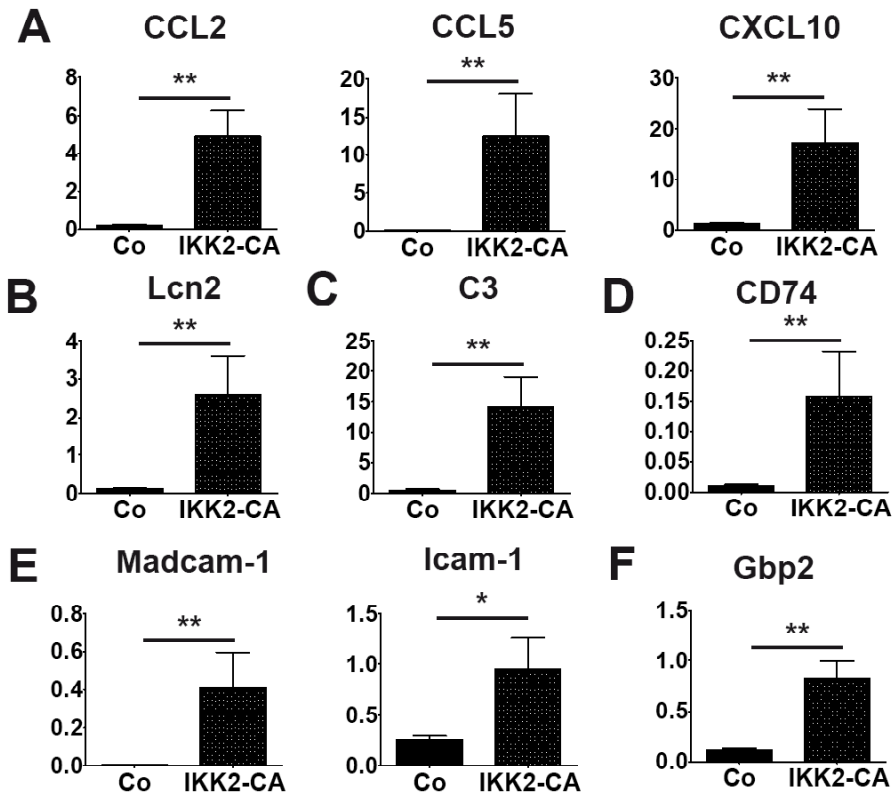

Figure 17: Inflammatory NF-κB target genes are upregulated in primary astrocytes 3 days after *ex vivo* IKK2-CA induction (qPCR)

(A) The chemokines CCL2/MCP-1, CCL5/RANTES and CXCL10/IP-10
(B) Lipocalin 2
(C) Complement factor C3
(D) Constant chain of MHC class II complex, CD74
(E) Cell adhesion molecules Madcam-1 and Icam-1
(F) Antimicrobial factor guanylate binding protein 2 (Gbp2)

displayed expression values are relative to HPRT as reference housekeeping gene; statistical analysis: two-tailed Mann-Whitney-test (n=5-7): *p < 0.05, ** p < 0.01;

This gene is also upregulated in primary astrocytes, but as in the microarray it is only relatively moderately induced (around 3-fold).

In contrast to these genes, several others that are upregulated *in vivo* are not induced in primary astrocytes, i.e. they are induced secondarily by a kind of paracrine action of astrocytes with activated NF-κB (Figure 18). Among these genes are the complement factors C4b and C1s, which in contrast to C3 only show a moderate upregulation in forebrain tissue at P7 (Figure 16C, Figure 18B).

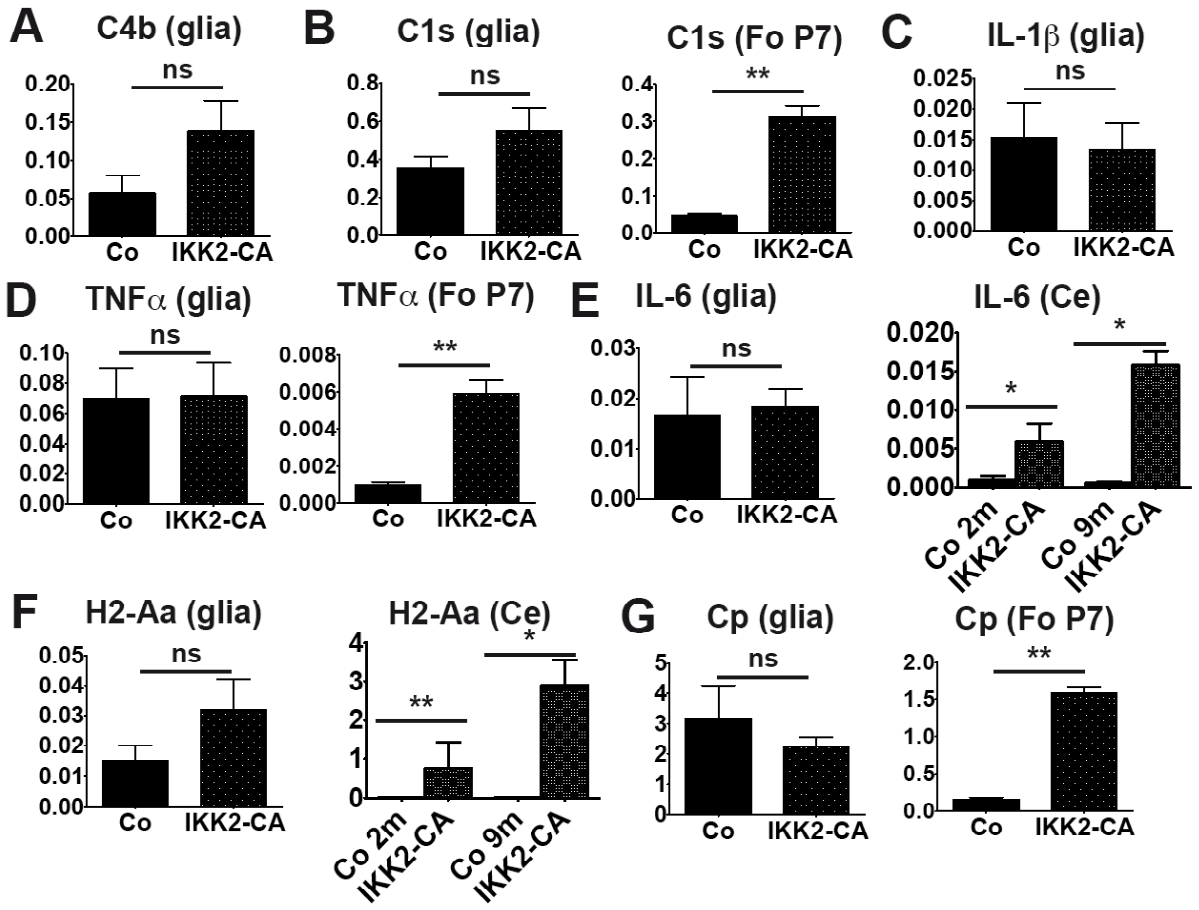

Figure 18: Several genes are indirectly induced by GFAP/IKK2-CA in brain tissue, but not in primary astroglial cultures (see also Figure 16)

(A) Complement factor C4b
(B) Complement factor C1s
(C) Interleukin-1β
(D) TNFα
(E) Interleukin-6
(F) MHC class II gene H2-Aa
(G) Ceruloplasmin (ferroxidase)

displayed expression values are relative to HPRT as reference housekeeping gene; statistical analysis: two-tailed Mann-Whitney-test (n=5-7): ns $p > 0.05$, *$p < 0.05$, ** $p < 0.01$;

Also the major proinflammatory cytokines IL-1β, TNFα and IL-6 are upregulated in brain tissue, but are not directly induced in astrocytes (Figure 16F, Figure 18C-E). In contrast to CD74, another gene of the MHC class II complex, H2-Aa, which was among the most highly upregulated genes in the microarray screen, was not induced in astrocytes. This argues against the inducible expression of a functional MHC class II complex on astrocytes (Figure 18F).

The only gene among the 10 highest upregulated genes in the screen, which is not directly involved in inflammation and immune response functions, is ceruloplasmin, a ferroxidase which is important for redox and iron homeostasis of the CNS (Texel et al., 2008). We could confirm upregulation in brain tissue by qPCR, but not in astrocytes (Figure 18G), although it is described to be regulated by NF-κB and produced by astrocytes (Texel et al., 2008; Persichini et al., 2010).

3.2.2 Activation and recruitment of immune cells induced by enhanced IKK2 activation in astrocytes

As shown in the last section, IKK2 activitation in astrocytes induces a prominent proinflammatory gene expression profile. Among the highly upregulated genes are cell adhesion molecules and especially several chemokines which are important for the recruitment of immune cells to sites of inflammation. Additionally, the upregulation of some complement factors and MHC class II molecules in brain tissue, but not in primary astrocytes indicates that astroglial IKK2-CA expression might result in an activation of innate immune cells in the CNS. Therefore, a potential infiltration and activation of immune cells in the brain of GFAP/IKK2-CA animals was analyzed in tissue sections by immunofluorescence studies in both the developing and adult brain.

3.2.2.1 Activation of the innate immune system in developmental neuroinflammation

Indeed already at the earliest time point with detectable transgene expression (P2), an innate immune response can be detected on the cellular level. Immunofluorescence staining with the general hematopoietic cell marker CD45 and the myeloid cell marker CD11b demonstrates an accumulation of immune cells in the not yet dilated lateral ventricles (Figure 19A/B). The complete colocalization of CD45 and CD11b staining indicates that all or at least the vast majority of these immune cells are myeloid cells, whereas there is no major infiltration of lymphoid cells. To further characterize the myeloid cells accumulating in the ventricles, a costaining of CD11b and the macrophage specific marker F4/80 was performed, which demonstrated that these cells are macrophages (Figure 19C).

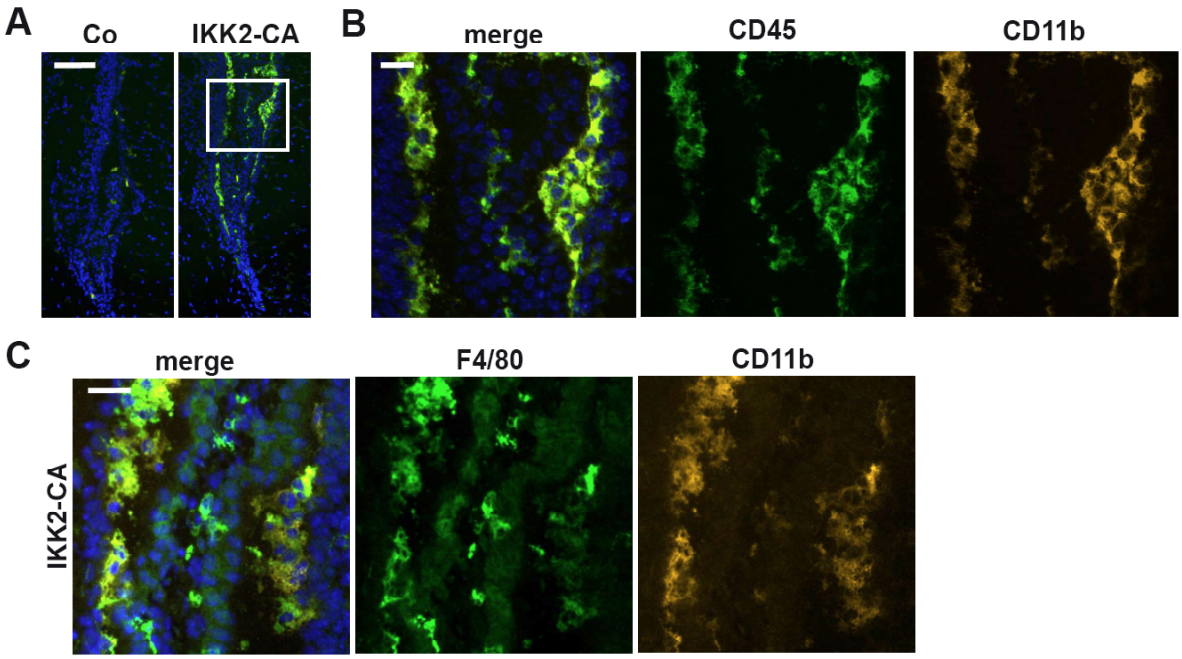

Figure 19: GFAP/IKK2-CA expression during brain development results in accumulation of macrophages in the lateral ventricles at P2

(A) Massive accumulation of myeloid cells in the posterior part of the lateral ventricles (CD45/CD11b costaining);

(B) A magnification of the marked area in (A) shows complete colocalization of the general hematopoetic cell marker CD45 and the myeloid cell marker CD11b, i.e. only myeloid cells are found in the ventricles of the GFAP/IKK2-CA animals;

(C) The intraventricularly accumulating myeloid cells (CD11b+) are mainly macrophages, as shown by costaining with the macrophage marker F4/80;

Scale bars: (A) 100 μm; (B, C) 20 μm; All merged fluorescent images show also DAPI costaining.

While at this point in time an accumulation of macrophages is only found in the lateral ventricles, at later stages an inflammatory infiltration and/or activation of immune cells is found in various brain areas, depicting a global neuroinflammatory response (Figure 20). Both at P2 and at P7 the infiltrating immune cells are mainly myeloid cells that show a coexpression of CD45 and CD11b. The most prominent areas of immune cell activation beside the lateral ventricles are the dentate gyrus and the cerebellum, but also in the cerebral cortex myeloid cells with high CD11b expression are found.

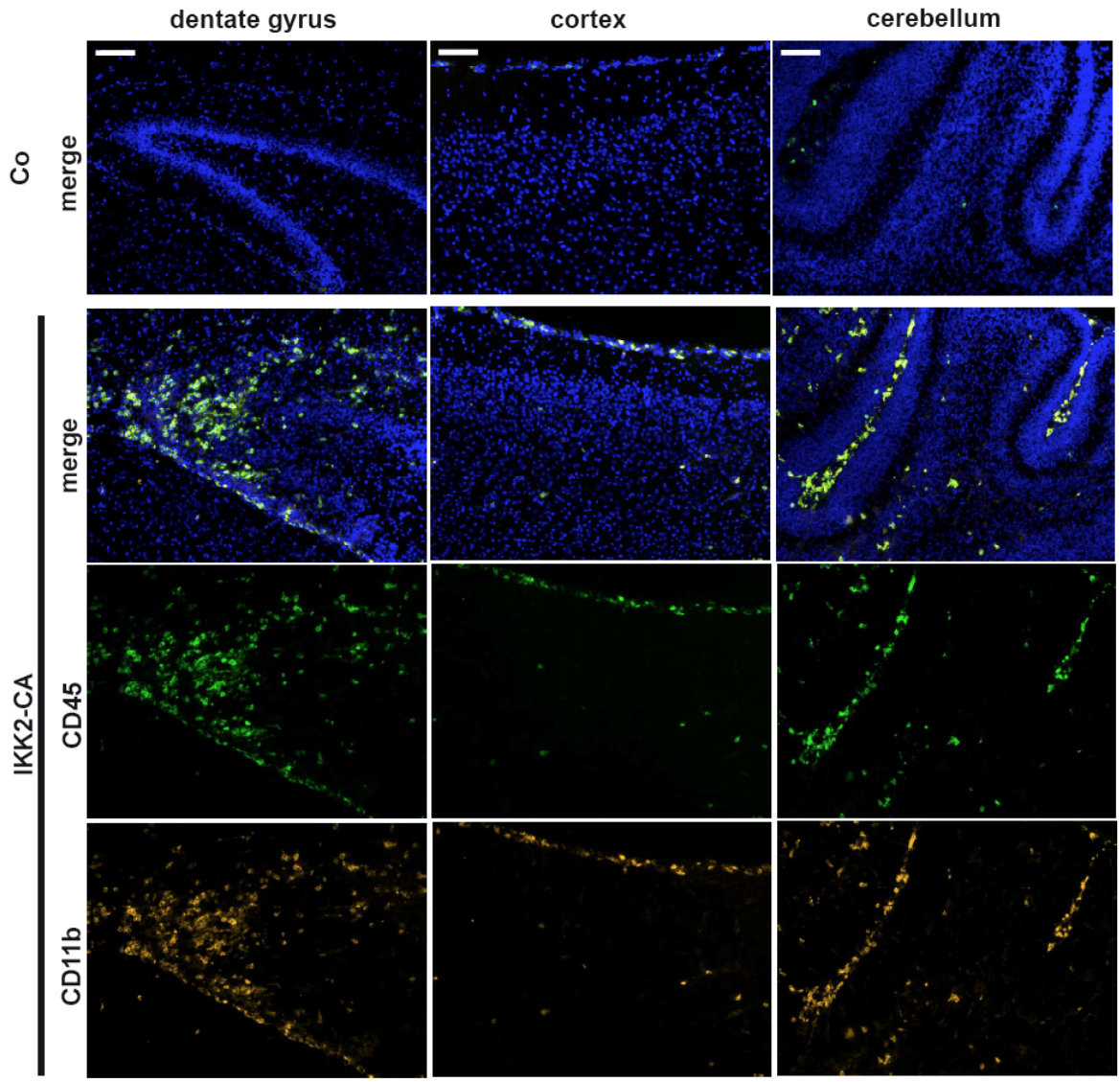

Figure 20: GFAP/IKK-CA induces a global neuroinflammatory response

Infiltration/activation of myeloid cells is found in various brain regions at P7, shown by CD45/CD11b/DAPI costaining for the dentate gyrus, the cortex and the cerebellum; scale bars 100 μm

Remarkably, most CD11b positive cells are found in areas with specialized properties of the BBB, which are important entry gates for the infiltration of peripheral immune cells, in particular the ventricles and the menigeal zones of the cortex and the cerebellum (Wilson et al., 2010). By contrast relatively few cells with high CD11b expression are found within the brain parenchyma. This suggests that not tissue resident microglia are activated, but peripheral macrophages are recruited from the blood stream at the BBB, but have not (yet) migrated into the brain parenchyma (Figure 20).

3.2.2.2 Microglia activation and immune cell infiltration in adult IKK2-CA expressing animals

In the adult brain, the pattern of immune cell infiltration and activation is different and the composition of the infiltrates is more complex.

One month after transgene reactivation (2 months of age), an increase of CD45 and CD11b staining was found mainly in the brainstem, e.g. in the thalamus and the medulla oblongata, indicating an infiltration of immune cells and/or an activation of microglia in these brain parts (Figure 21A/B).

In contrast to the developing brain, no accumulation of macrophages in the lateral ventricles and no prominent hippocampal neuroinflammation was observed at 2 months of age (Figure 21A/B). Also in the cerebellum no inflammatory infiltration is observed at that stage (Figure 22A).

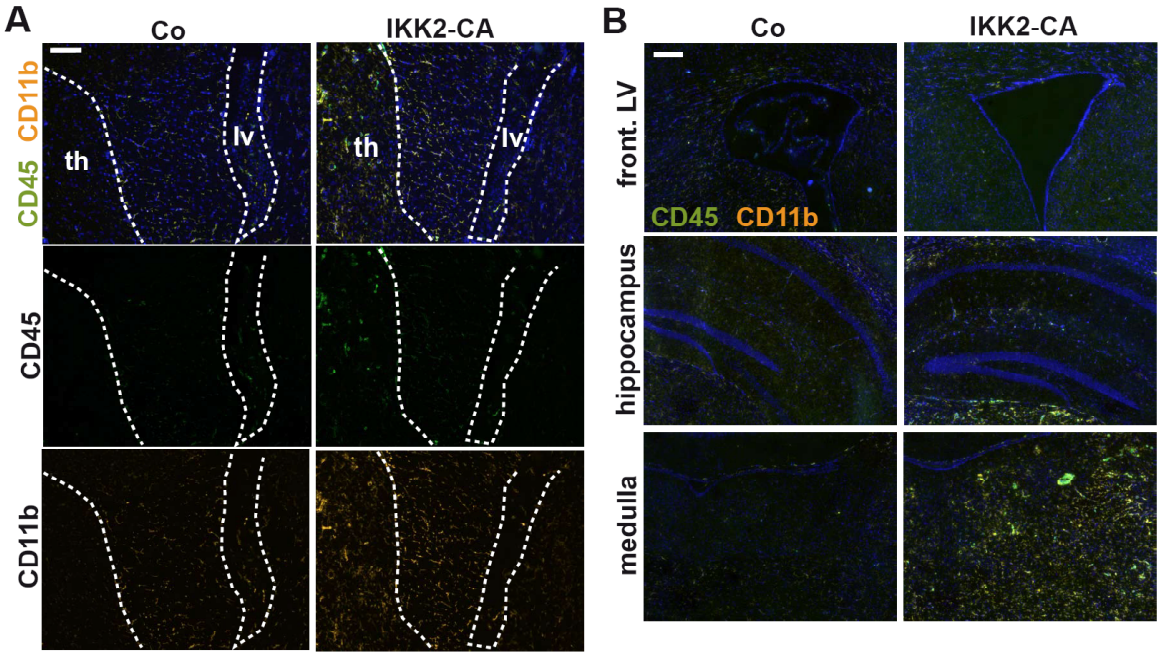

Figure 21: Early neuroinflammation in the brain of adult GFAP/IKK2-CA animals at 2 months of age (1 month after transgene activation) mainly affects the brainstem and results in prominent microglia activation

(A) Microglia activation in the thalamus shown by increased CD11b staining in CD45 low cells; moderate infiltration with peripheral immune cells (CD45 high) is visible in the thalamus (th), but no accumulation of CD45/CD11b positive macrophages in the lateral ventricles (lv)

(B) Increase of immune cell marker expression (CD45/CD11b) in the medulla oblongata, but not in the hippocampus and in the forebrain surrounding the frontal part of the lateral ventricles

Scale bars: (A) 100 μm, (B) 200 μm; all merged images show nuclear DAPI costaining

At later points in time, the neuroinflammation induced by IKK2-CA in astrocytes spreads to other parts of the CNS, persisting for a long time. In the cerebellum, inflammatory infiltrates are present beginning between 2 and 3 months until at least 9 months of age (Figure 22).

These findings clearly show that enhanced IKK2 activity in astrocytes is sufficient to induce neuroinflammation, resulting in a global chronic neuroinflammation in adult GFAP/IKK2-CA animals.

To characterize the immune cells that are found in the brains of the IKK2-CA expressing mice, several immunofluorescence stainings were performed. The prominent increase in immunoreactivity for the myeloid cell marker CD11b and the macrophage/microglia marker Mac-2 indicates a strong microglia activation and/or macrophage infiltration in the cerebellum at 3 months of age, most prominently in the meningeal zone and the white matter (Figure 23A/B). The majority of strongly stained cells show an arborized (ramnified) glial morphology, suggesting that a large fraction of these cells are partially activated microglia. In addition, also rather rounded (amoeboid) cells positive for CD11b or Mac-2 are found, representing fully activated microglia or infiltrating peripheral macrophages.

Beside microglia/macrophages as part of the innate immune system, also cells of the adaptive immunity are infiltrating into the CNS of GFAP/IKK2-CA animals. As shown for the cerebellum at 3 months of age, both CD4 positive Th-cells and cytotoxic (CD8 positive) T-cells are recruited by IKK2-CA expressing astrocytes (Figure 23C/D).

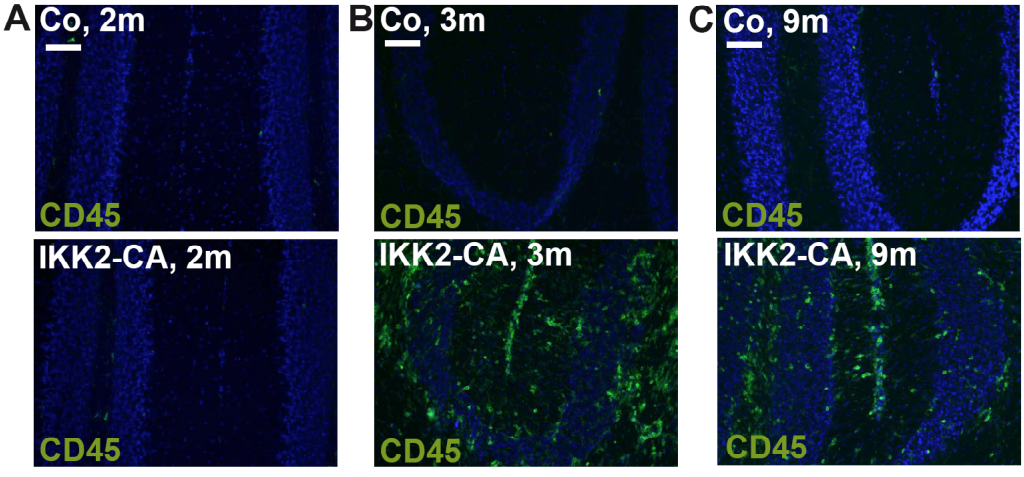

Figure 22: GFAP/IKK2-CA expression causes chronic neuroinflammation

Infiltration of immune cells into the cerebellum starts between 2 months (A) and 3 months of age (B) and persists at least till 9 months of age (C), shown by CD45 staining; DAPI costaining for the visualization of nuclei, scale bars 100 μm

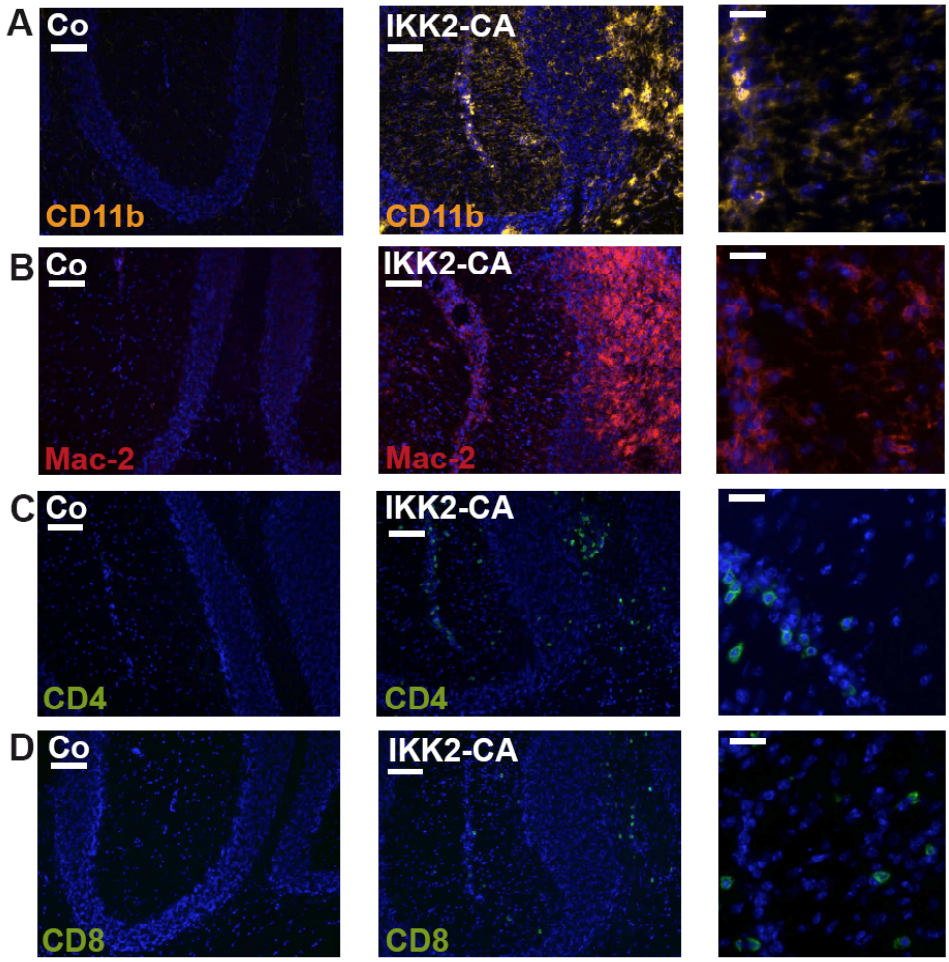

Figure 23: GFAP/IKK2-CA expression results in microglia activation and infiltration of T-cells (cerebellum, age 3 months)

(A) Strong and widespread increase of staining for the macrophage marker the myeloid cell marker CD11b indicates massive activation of microglia induced by IKK2-CA; higher magnification (right panel) shows both rounded (amoeboid) and arborized morphology of CD11b positive cells

(B) The macrophage/microglia marker Mac-2 shows a similar staining pattern and celular morpholgy as CD11b, confirming microglial identity of the stained cells

(C) Infiltration of Th-cells, shown by CD4 staining; higher magnification (right panel) shows small round CD4 positive cells

(D) Infiltration of CD8 positive cytotoxic T-cells; higher magnification (right panel) shows small round CD8 positive cells

All images show DAPI co-staining (blue); scale bars: 100 μm (right panels 25 μm);

These results demonstrate that NF-κB activation in astrocytes is sufficient to activate the innate immunity of the CNS, and also to recruit immune cells from the periphery, including cells of the adaptive immune system.

3.3 Consequences of enhanced NF-κB activation in astrocytes for brain development

Pathological conditions that are associated with neuroinflammation often result in CNS malformations, as many inflammatory mediators also regulate different steps of neural development (see 1.1.5.1). NF-κB activation in astrocytes induces various inflammatory mediators (see 3.2), and might therefore interfere with normal brain development.

As already briefly described above (see section 3.1.4), enhanced NF-κB activation in IKK2-CA expressing astrocytes indeed results in hydrocephalus formation, variable reduction of weight gain, and usually death within the first three postnatal weeks. By contrast, animals that express the transgene only in the fully developed brain are viable, without prominent signs of sickness despite a chronic neuroinflammatory phenotype (see 3.1.5 and 3.2.2.2). This indicates that astroglial IKK2 activation specifically interferes with processes that are important for normal brain development.

3.3.1 Characterization of IKK2-induced hydrocephalus

The most prominent anatomical alteration found in GFAP/IKK2-CA mice is the massive dilatation of the lateral ventricles (see section 3.1.4). This phenotype resembles the neuropathology of hydrocephalus, which is a relatively frequent and devastating complication of several inflammation associated CNS disorders (see 1.1.5.1). Interestingly, this suggests a novel and unexpected link of astroglial NF-κB signaling and inflammation-associated hydrocephalus formation.

Causative for the dilatation of the ventricles is an increased pressure of the CSF that fills the ventricular system. This increased pressure can be a consequence of increased CSF production, decreased resorption, or impaired flow through the ventricular system (Zhang et al., 2006). Several experiments were performed to characterize this phenotype of the GFAP/IKK2-CA mice more closely, to elucidate which of these mechanisms is responsible for hydrocephalus formation in this model and to investigate to what extent this phenotype resembles the pathology of hydrocephalus in other animal models and in human cases.

3.3.1.1 Histological classification of hydrocephalus in the GFAP/IKK2-CA model

As a first step towards a mechanistic understanding of the hydrocephalus formation in this mouse model, it was investigated whether the hydrocephalus is caused by a defect of the aqueduct of Sylvius, the narrowest part of the ventricular system, which is often obstructed or fused in non-communicating hydrocephalus (Rekate, 2008). At P7, i.e. after onset of hydrocephalus, Nissl staining revealed no obvious anatomical alteration of the aqueduct in the GFAP/IKK2-CA model, thus classifying this phenotype as communicating hydrocephalus (Figure 24A).

Another structure of the ventricular system that is often malformed or absent in hydrocephalus models is the subcomissural organ (Huh et al., 2009). Also this structure is unaltered at the gross morphological level, excluding its malformation as a simple anatomical explanation for hydrocephalus formation (Figure 24A).

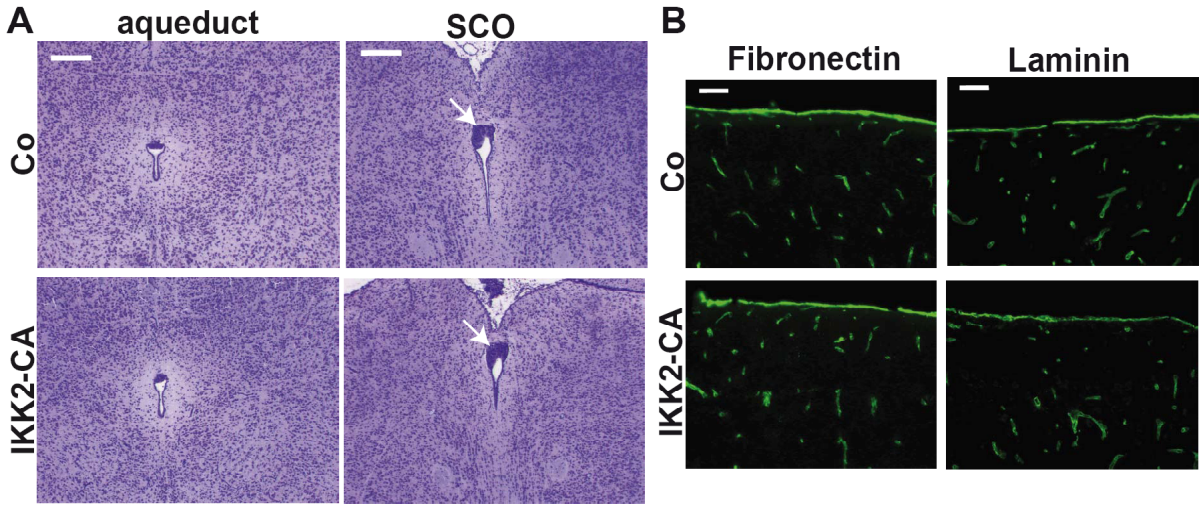

Figure 24: Hydrocephalus in the GFAP/IKK2-CA model is a communicating hydrocephalus that is not caused by fibrosis of the subarachnoid space

(A) No obvious anatomical explanation for hydrocephalus formation: the aqueduct of Sylvius is not obstructed or malformed (left panels), the subcommissural organ is present and appears morphologically normal (right panels, arrow); Nissl staining, scale bars 200 μm

(B) Deposition of fibronectin and laminin in the subarachnoid space/meninges is not increased; immunofluorescence staining, scale bars 50 μm

As inflammation can cause fibrosis and IKK2-CA expression in astrocytes causes neuroinflammation, another probable mechanism for hydrocephalus formation is an impaired resorption of CSF due to fibrotic alterations of the subarachnoid space. However, investigation of the extracellular matrix (ECM) structure by immunofluorescence stainings of the ECM proteins fibronectin and laminin revealed no alterations in ECM deposition (Figure 24B).

3.3.1.2 Alterations of the ependyma of the lateral ventricles

Another potential cause of hydrocephalus formation is a defect of the ependyma, the epithelium that covers the walls of the ventricular system. In various models of hydrocephalus a loss of ependymal cells or the agenesis or dysfunction of their motile cilia was shown to cause hydrocephalus (Del Bigio, 2010). Cilia have a microtubuli backbone that is stabilized by acetylation of α-tubulin, which can be recognized by specific antibodies to label cilia.

An immunofluorescence costaining for acetylated α-tubulin and the ependymal cell and astrocyte marker aquaporin 4 indicated a reduced density of cilia bundles at the walls of the lateral ventricles at P12 (Figure 25A). As the resolution of light microscopy is too low, it is difficult to certainly distinguish morphologically between cilia and other structures that show acetylated α-tubulin staining. Therefore scanning electron microscopy (SEM) was used to confirm this finding (Figure 25B). Indeed SEM revealed a nearly complete absence of ependymal cilia at the lateral walls of the lateral ventricles in GFAP/IKK2-CA animals at P12, whereas in control animals the ventricle walls were completely covered with cilia bundles. Instead, many irregularly shaped cells were found attached to the ventricle walls of IKK2-CA expressing mice, most likely resembling the ventricular macrophages described above (see 3.2.2.1). Of note, the lack of ependymal cilia is already found at P2, i.e. before onset of hydrocephalus, when the control ependyma is covered with immature cilia. This indicates that this defect is not a secondary damage of the ependyma due to hydrocephalus formation, but might indeed be the cause for the hydrocephalus.

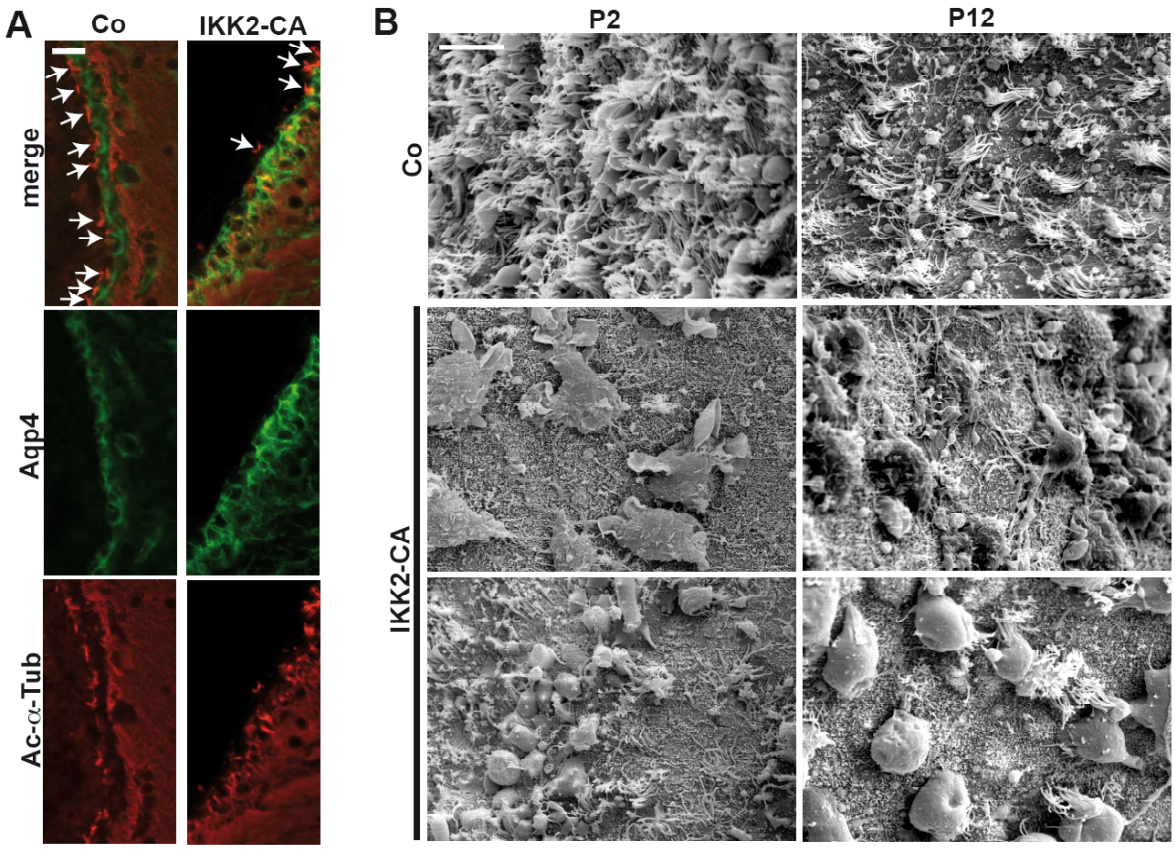

Figure 25: IKK2-CA causes a lack of ependymal cilia of the lateral ventricles

(A) Costaining of the ependyma/astrocyte marker aquaporin 4 and acetylated α-tubulin indicates reduced density of cilia bundles (arrows) on the ependyma at P12; scale bar 20 μm

(B) Scanning electron microscopy of the surface of the lateral walls of the lateral ventricles reveals lack of ependymal cilia in GFAP/IKK2-CA animals. The ependyma of control animals is covered with immature cilia bundles at P2, which are mature at P12 (upper panels), while the ependyma of transgenic animals is largely devoid of cilia and covered with irregularly shaped cells that most likely resemble macrophages (middle and lower panel); only small areas show a few cilia bundles (lower panel); scale bar 10 μm

To clarify whether the lack of ependymal cilia is due to the loss of the whole ciliated ependymal cells, or whether this is a result of a specific loss or agenesis of the cilia, the structural integrity of the ependymal cell layer was studied. Immunostaining for β-catenin did not reveal any alteration of the epithelial like structure of the ependymal cell layer (Figure 26A).

This finding indicates that there is not a general deficit in ependymal differentiation or a loss of ependymal integrity. In line with this, the sialic acid rich glycocalix of the ependyma, visualized by staining with fluorescently labeled wheat germ agglutinin (WGA), seems to be intact (Figure 26B).

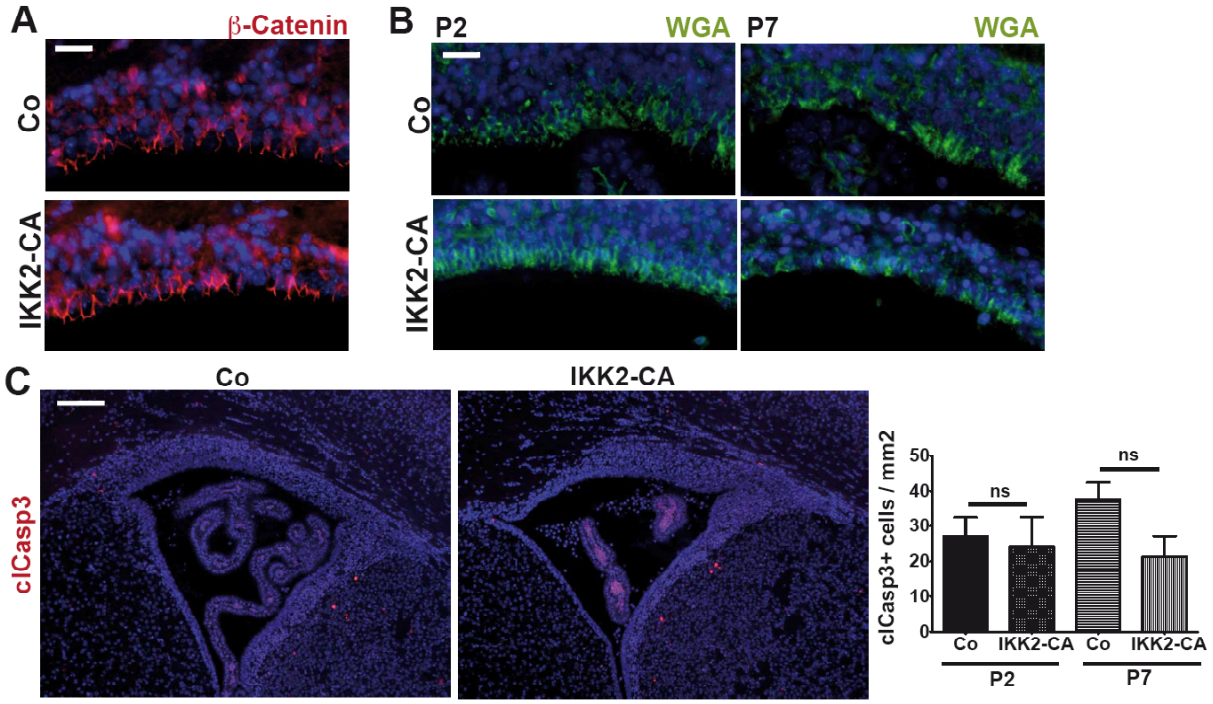

Figure 26: General structural integrity of the ependyma is not disrupted by IKK2-CA

(A) Lack of cilia is not due to loss of ependyma integrity as shown by β-catenin staining.

(B) Sialic acid rich glycocalix of the ependyma appears normal before and after onset of hydrocephalus (P2, P7), shown by wheat germ agglutinin (WGA) staining.

(C) No increase of apoptosis is found in the ependyma/subventricular zone before and after onset of hydrocephalus (P2, P7), shown by staining for cleaved caspase 3 (P7 images not shown); Quantification does not show a significant difference in the number of cleaved caspase 3 positive cells per area; statistical analysis: unpaired t-test (n=3-6); values are shown as mean +/- s.e.m.;

Scale bars: (A,B) 20 μm; (C) 200 μm; All images show also DAPI costaining

This ECM structure was shown to be disrupted in the *hyh* hydrocephalus model, which is caused by a detachment of ependymal cells, further arguing for an intact ependymal cell layer in the GFAP/IKK2-CA model (Wagner et al., 2003). Finally, no increase of apoptotic cell death was found in the ependymal and subventricular zone (Figure 26C).

Altogether these experiments suggest that there is not a general defect of ependymal integrity, but rather a specific loss or agenesis of ependymal cilia.

3.3.1.3 Time course of hydrocephalus formation

The absence of hydrocephalus in animals with IKK2-CA expression in the adult brain (see 3.1.5) suggests that this type of hydrocephalus is actually a defect of brain development. The likely cause of hydrocephalus, the lack of ependymal cilia, might be explained by a defect of ependymal cilia formation, which was described to take place within the first postnatal week (Spassky et al., 2005). Indeed this time window correlates with the perinatal onset of transgene expression (see 3.1.4) and the onset of the phenotype between P2 and P7. Whereas the animals seem normal at P2, the reduced weight gain/growth and the ventricle dilation are both evident at P7 (see 3.1.4 and Figure 27A/B).

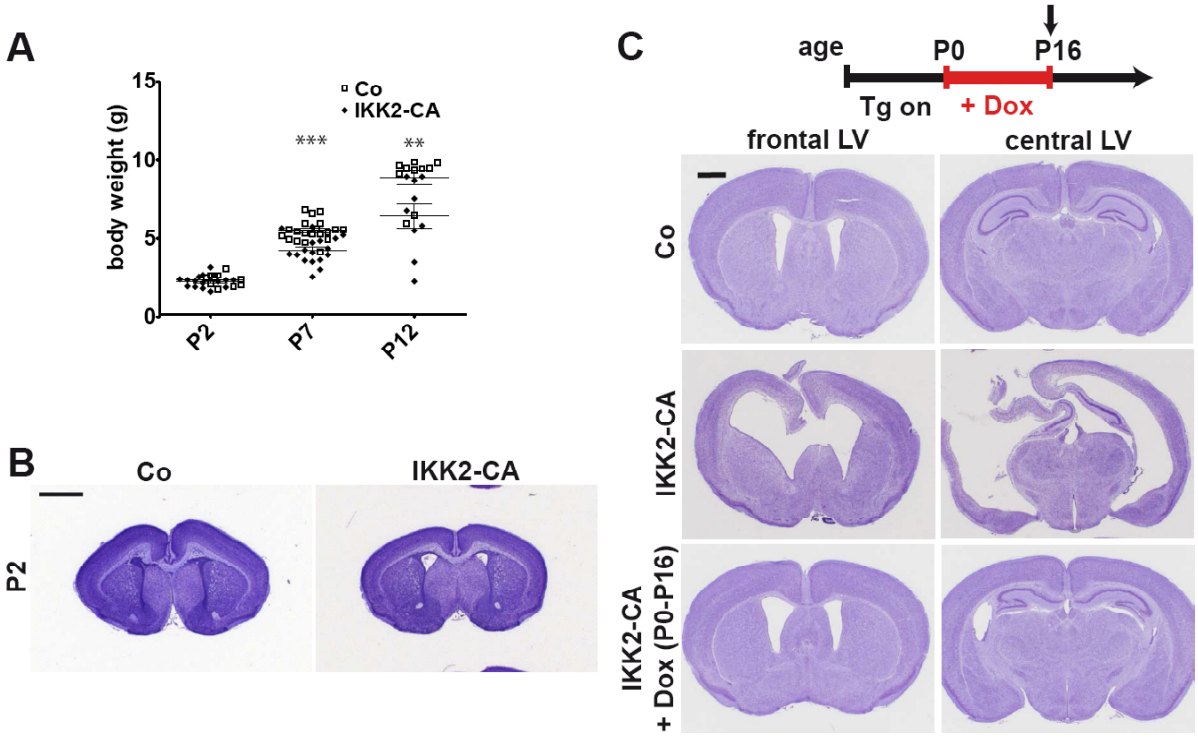

Figure 27: Growth retardation and hydrocephalus become apparent between P2 and P7 (see also 3.1.4) and requires postnatal transgene expression

(A) Postnatal growth is impaired in GFAP/IKK2-CA animals of founder line A, shown by body weight gain between P2 and P12; the diagram displays single values for each animal, showing the variability of growth impairment, and mean +/- s.e.m.; statistical analysis: Mann-Whitney-test (**: p<0.01, *** p<0.001)

(B) No prominent dilatation of the lateral ventricles in GFAP/IKK2-CA mice at P2; Nissl staining;

(C) Repression of IKK2-CA by doxycycline from P0 largely prevents hydrocephalus formation and major hippocampal defects, shown by Nissl staining at P16; doxycycline was administered by a single injection to the mother and then continuously in the drinking water; Nissl staining;

Scale bars: 1mm

To exclude that low levels of prenatal transgene expression, which might not have been detected in immunoblots and immunostainings (see 3.1.4), contribute to hydrocephalus formation, transgene expression was repressed by doxycycline during early postnatal development (P0 to P16). This largely prevented hydrocephalus formation, demonstrating that postnatal IKK2-CA expression is critical for this phenotype (Figure 27C).

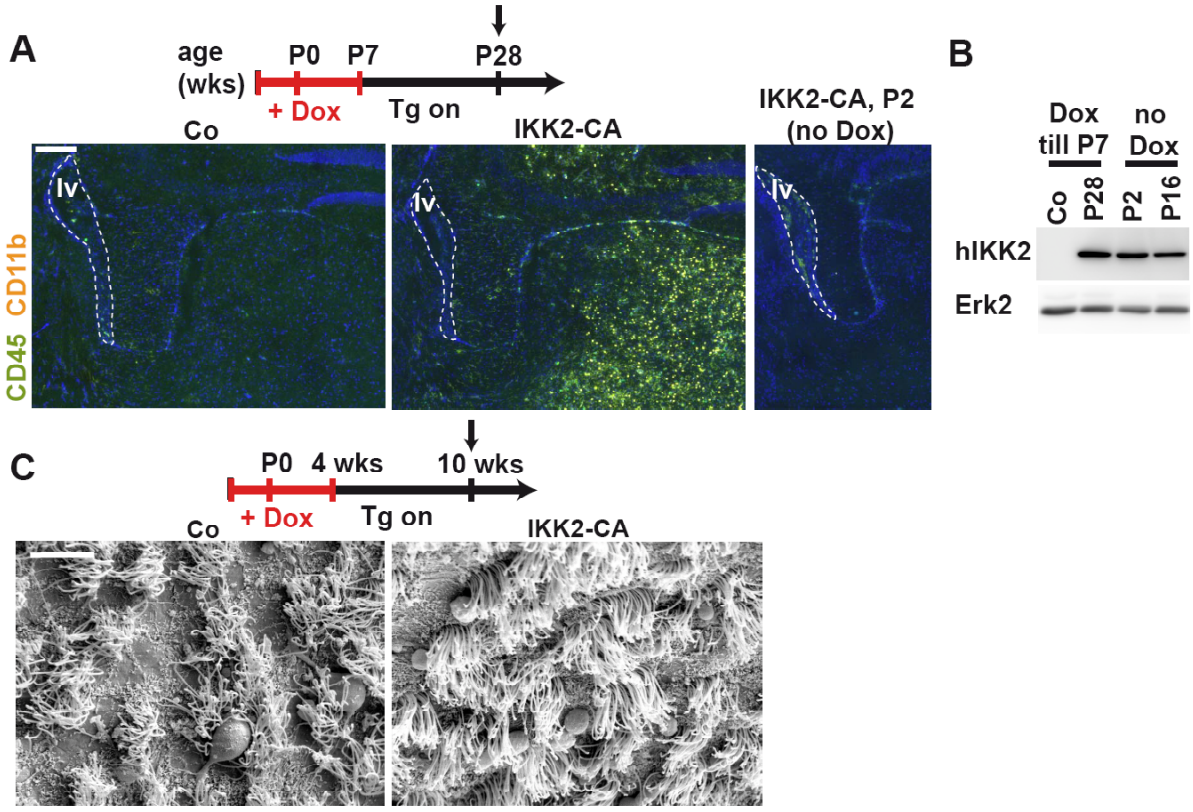

Figure 28: Expression of IKK2-CA after finished ciliogenesis does not cause hydrocephalus and does not affect ependymal cilia

(A) Early transgene reexpression from P7 induces neuroinflammation, but no ventricle dilatation; CD45/CD11b/DAPI costaining shows strong myeloid cell activation in thalamus and hippocampus at 4 weeks of age, but not in the lateral ventricles (lv), as seen in animals with constitutive transgene expression at P2; scale bar 200 μm.

(B) Transgene expression at 4 weeks of age (P28) after doxycycline withdrawal at P7 is comparable to transgene expression in young animals without doxycycline treatment; Immunoblot for transgene (hIKK2) and Erk2 as loading control.

(C) Ependymal cilia are normally developed at the age of 10 weeks, when IKK2-CA expression is induced at 4 weeks of age, shown by scanning electron microscopy; scale bar 10 μm.

On the other hand, when transgene expression is induced shortly after the end of ependymal ciliogenesis at P7, this is also sufficient to rescue hydrocephalus formation, as these animals do show transgene expression and neuroinflammation, but no hydrocephalus at P28 (Figure 28A).
The transgene expression is comparable in this paradigm to pups at P2 that did not receive doxycycline, and overall CD45 and CD11b staining is even stronger (Figure 28B), excluding that the lack of hydrocephalus formation is due to a reduced transgene expression or reduced inflammation. Because of the lack of ventricular macrophage accumulation, this experiment cannot exclude a contribution of these cells to the agenesis or loss of the immature cilia. Nevertheless, investigation of the ventricle walls of animals with adult trangene reactivation shows intact ependymal cilia in SEM (Figure 28C).
In conclusion, the experiments provide evidence that IKK2-CA does not induce a loss of mature cilia in the adult state, but indeed impairs the development of ependymal cilia in a critical period, probably between P0 and P7, either by interfering with ependymal cell intrinsic ciliogenesis programs or by macrophage mediated destruction of the nascent cilia.

3.3.2 Other developmental alterations caused by enhanced IKK2 activity in astrocytes

In most brain regions, key steps of brain development, including major neurogenesis, occur during embryonic development. As there is no detectable prenatal transgene expression in the GFAP/IKK2-CA model, prominent anatomical malformations are not expected in most brain regions. Indeed overall brain anatomy shows only restricted defects, which might partially be secondary to hydrocephalus formation, like a thinning of the cortex in cases of severe hydrocephalus (see 3.1.4).

3.3.2.1 Postnatal hippocampal development

Another region with prominent morphological alterations is the hippocampus, which shows a striking disorganization of the dentate gyrus (DG), the CA3 region and the fimbria (see 3.1.4 and Figure 29A). The increased pressure of the CSF in the adjacent lateral ventricles might cause these defects indirectly, but the IKK2-CA

induced neuroinflammation might also directly be responsible for this malformation. Indeed chemokine signaling, which is prominently deregulated in the GFAP/IKK2-CA model, is critical for the development of the DG. Chemokine signaling regulates the migration of NPCs into the DG, and it also seems to be critical for the postnatal reorganization of the dentate gyrus (Berger et al., 2007; Li et al., 2009).

The diffuse structure of the DG granule layer suggests a failure of the proper postnatal relocalization of the cells within the developing DG (Figure 29A). This is supported by the finding that the localization of proliferating, Ki67 positive cells in control animals is focused to the subgranular zone, one of the neurogenic niches that persist into adulthood. In contrast, proliferating cells stay dispersed throughout the DG in IKK2-CA expressing animals, indicating a defect in the formation of a proper neurogenic niche in the subgranular zone (Figure 29B/C).

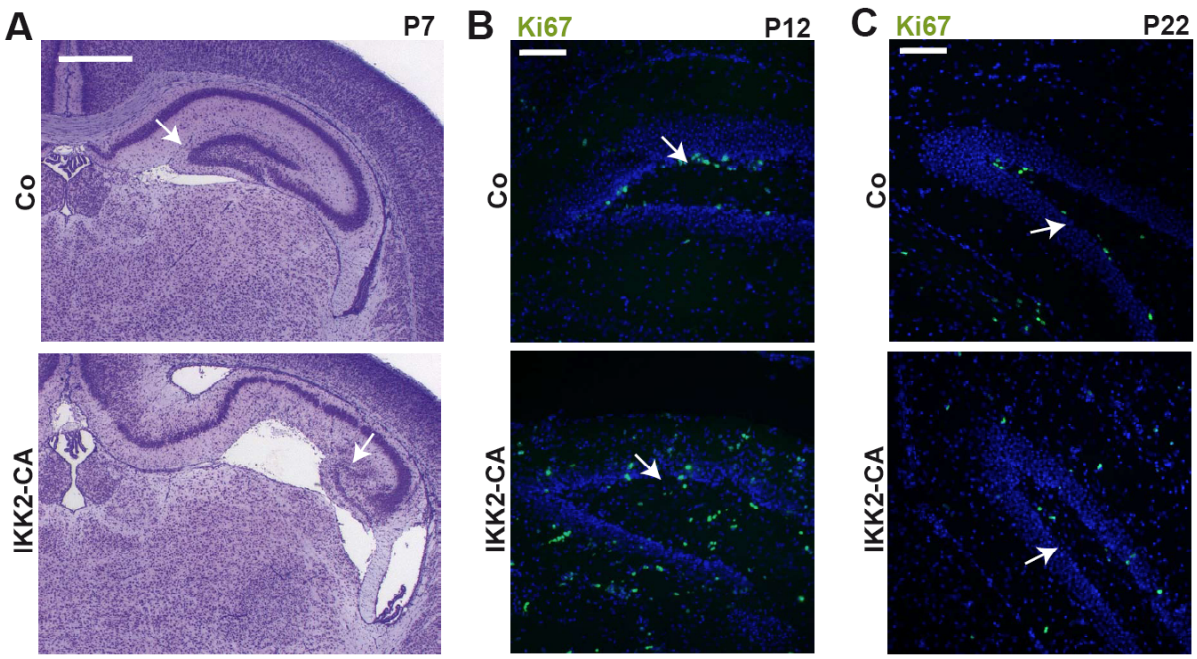

Figure 29: The hippocampus is disorganized in GFAP/IKK2-CA animals

(A) Malformation of the hippocampus with severe distortion of dentate gyrus granule layer (arrow).

(B) Localization of Ki67 expressing proliferating NPCs to the subgranular zone (arrow) is distorted at P12;

(C) Lasting mislocalization of proliferating NPC (P22);

Scale bars: (A) 500 μm, (B,C) 100 μm;

3.3.2.2 Postnatal cerebellar development

As in the hippocampus, major neurogenesis and structural development is ongoing in the cerebellum in the early postnatal phase. This stage of development is dependent on chemokine signaling, in particular on the CXCL12/CXCR4 axis (Deverman and Patterson, 2009).

Also in the cerebellum the normal development is affected by IKK2-CA expression (Figure 30). The maturation of the cerebellar cortex is delayed, depicted by a retarded loss of the proliferating (Ki67 positive) NPCs of the external granule layer (EGL). These cells migrate through the molecular layer into the internal granule layer, where they stop proliferation and differentiate into cerebellar granule neurons. CXCL12 produced by the meninges during early postnatal development counteracts this emigration from the EGL (Deverman and Patterson, 2009). Similarly, the chemokines that are deregulated in the GFAP/IKK2-CA model might delay the migration of NPCs out of the EGL.

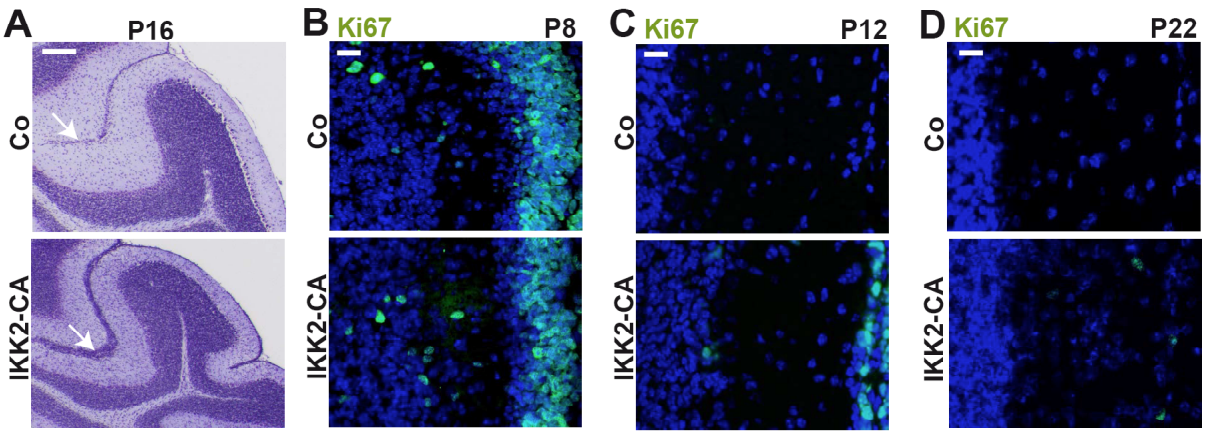

Figure 30: GFAP/IKK2-CA impairs maturation of the cerebellum indicated by the delayed loss of proliferating neural progenitors of the external granule layer

(A) Nissl staining shows a thickened EGL (arrow) at P16

(B) Ki67 staining shows normal proliferation in the EGL at P8

(C) Proliferation in the EGL of IKK2-CA expressing animals continues at P12, but has already ceased in controls

(D) At P22, proliferation in the EGL has stopped also in IKK2-CA expressing animals

scale bars: (A) 200 μm, (B-D) 20 μm; (B-D) shows DAPI costaining;

3.4 Consequences of enhanced IKK2 activity in astrocytes for CNS function and homeostasis

Neuroinflammation does not only interfere with normal brain development, but it also alters CNS homeostasis by various mechanisms, and it is therefore implicated in a large number of neurological diseases. The inflammatory activation of astrocytes is supposed to prominently affect CNS homeostasis and function by altering BBB permeability and impairing their neurotrophic support functions (see 1.2.3). In the GFAP/IKK2-CA model, the most prominent consequence of neuroinflammation and the altered astrocyte function by activation of astroglial NF-κB signaling is an atrophy of the cerebellum (see 3.1.5).

3.4.1 Behavior and motor function of mice with long term activation of IKK2 signaling in astrocytes

To investigate the consequences of the cerebellar atrophy and potential other functional deficits caused by chronic neuroinflammation and long-term astroglial NF-κB activation, a series of behavioural experiments were performed. In particular, motor function, fear and exploratory behaviour and hippocampal learning and memory were studied at 7-8 months of age.

3.4.1.1 Analysis of motor function in GFAP/IKK2-CA mice

The cerebellum is the main brain part responsible for the fine-tuning and coordination of muscle contractions in deliberate movements. As the cerebellum of old GFAP/IKK2-CA mice showed signs of atrophy, first the motor capabilities of these mice were investigated.

Surprisingly, in their normal cage environment, only a few animals showed obvious motor deficits. In a simple test for motor function, the string agility test, where the animals are scored according to their capability to hold themselves on an elevated string, consistently only a part of the animals showed impaired performance (Figure 31A). Nevertheless, this assay measures to a lesser extent the fine-tuning of movement programs, but rather general agility and muscle strength.

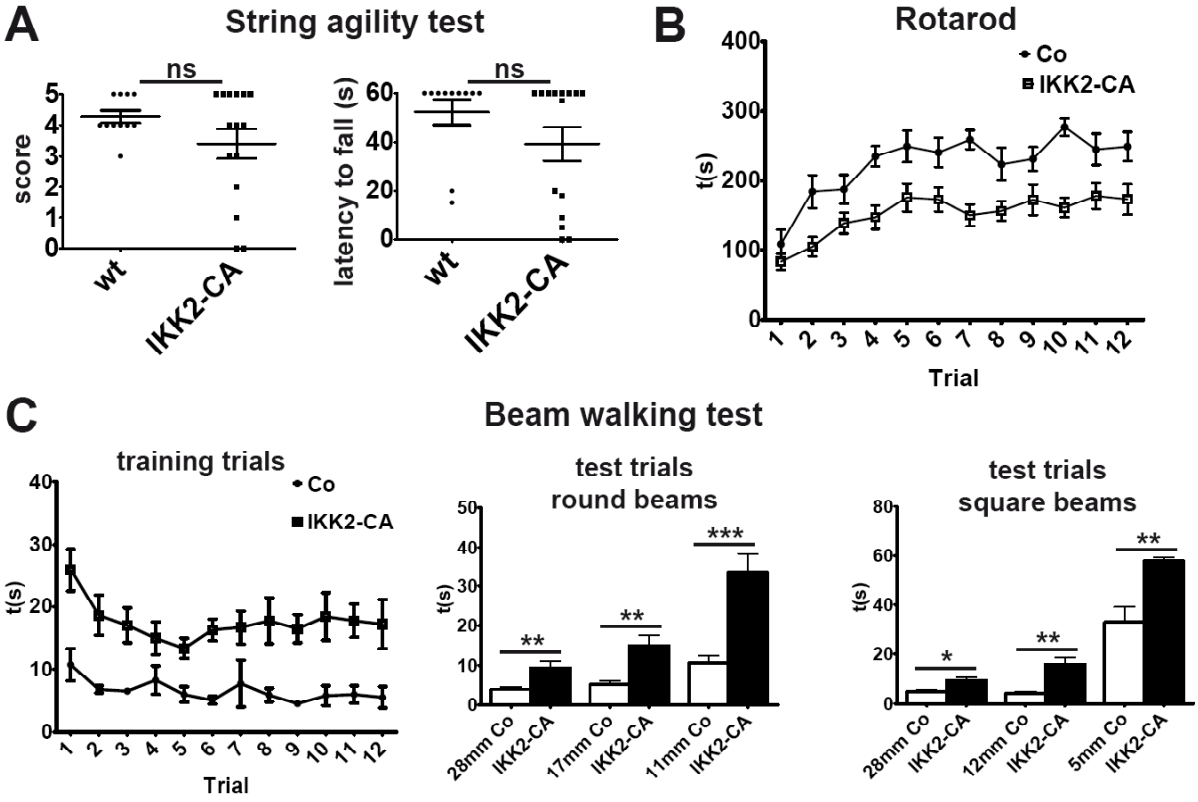

Figure 31: Motor function is impaired in GFAP/IKK2-CA mice at the age of 7-8 months

(A) Only some animals show an impaired ability to hold themselves on an elevated string; the diagram shows single animals and mean +/- s.e.m.; scoring see Material and Methods section; maximal observation time 60s; statistical evaluation: two-tailed Mann-Whitney-test (n=11-15), group differences are not significant ($p > 0.05$);

(B) Motor coordination is impaired in IKK2-CA mice, demonstrated in a rotarod experiment; latency to fall off an accelerating rod is reduced compared to control animals, which cannot be overcome by extended training (3 trials per day for 4 days); diagram shows mean +/- s.e.m.; statistical analysis: 2-way-ANOVA (n=11-15), $p < 0.0001$;

(C) Balance and movement precision is impaired in IKK2-CA mice; time to cross a narrow beam is increased compared to control animals; after 12 training trials with a 12 mm square beam (left diagram) animals were challenged with round and square beam of different sizes (middle and right diagramms); deficits increase with the difficulty of the task, demonstrated by different beam diameters; maximal time of observation 60s, many animals were unable to cross the 5 mm square beam; diagram shows mean +/- s.e.m.; statistical analysis (n=11-15): training trials: 2-way-ANOVA ($p < 0.0001$), test trials: unpaired two-tailed t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$;

To assess more specifically cerebellar function, two more sophisticated tests were performed, the rotarod test and the beam walking test.
In the rotarod test animals are put on an accelerating rotating cylinder (rod), and the time is recorded that the animals are able to stay on this cylinder, as a measure for their ability to coordinate fast movements (running). In this paradigm, the GFAP/IKK2-CA mice show a markedly impaired performance, demonstrating that the cerebellar atrophy indeed has functional consequences (Figure 31B). In the beam-walking test, the motor deficits are even more prominent (Figure 31C). In this paradigm, the mice are forced by an adverse stimulus (bright light) to traverse a narrow beam, first in a number of training sessions, then with beams of different diameter and type, as described previously (Castillo Vega, 2011). The time to cross the beam is increased for all IKK2-CA expressing animals in both training and test trials, indicating an impairment of their balance and walking coordination. With increasing difficulty the deficits become more obvious, culminating in the complete inability of IKK2-CA expressing animals to cross the narrowest beam (5 mm square beam).
Altogether, these results clearly demonstrate that the atrophy results in an impaired function of the cerebellum, as seen in different cerebellar degenerative disease models, e.g. in SCA28 (Maltecca et al., 2009).

3.4.1.2 Exploratory and fear behavior in GFAP/IKK2-CA mice

Neuroinflammation is implicated not only in neurodegenerative disorders, but also in psychiatric diseases such as depression (see 1.1.5.5). These disorders are often characterized by prominent alterations in general activity, fear, and interest in the environment. To investigate if there is any sign for such behavioural alterations, the GFAP/IKK2-CA mice were studied in the open field and the elevated plus maze tests.
The open field test (Figure 32A), where the animals are observed for a certain time in an open square box, revealed a reduced average running speed of the IKK2-CA expressing mice, which might be explained at least in part by the motor impairments caused by cerebellar atrophy. A mild reduction is also seen in the overall activity (percentage of time moving), although it is not clear, whether this is due to the motor problems or if it indicates a kind of depressive or sickness behavior. Against the latter hypothesis argues the unaltered tendency to stay in the center zone of the open field,

which is a measure for exploratory and fear behavior, as the mice are vulnerable to potential predators when moving into an open area without shelter. Neither the duration of stay in the center zone is altered, nor the track length, when corrected for the reduced velocity/total track length of the GFAP/IKK2-CA mice.

The unchanged fear and curiosity behavior of the mice expressing IKK2-CA was confirmed in the elevated plus maze, where the mice explore a cross-shaped maze with two protected arms with side walls and two unprotected open arms without side walls (Walf and Frye, 2007). In this paradigm again no differences are observed, measured by the time, the corrected relative track length and the number of visits in the open arms (Figure 32B).

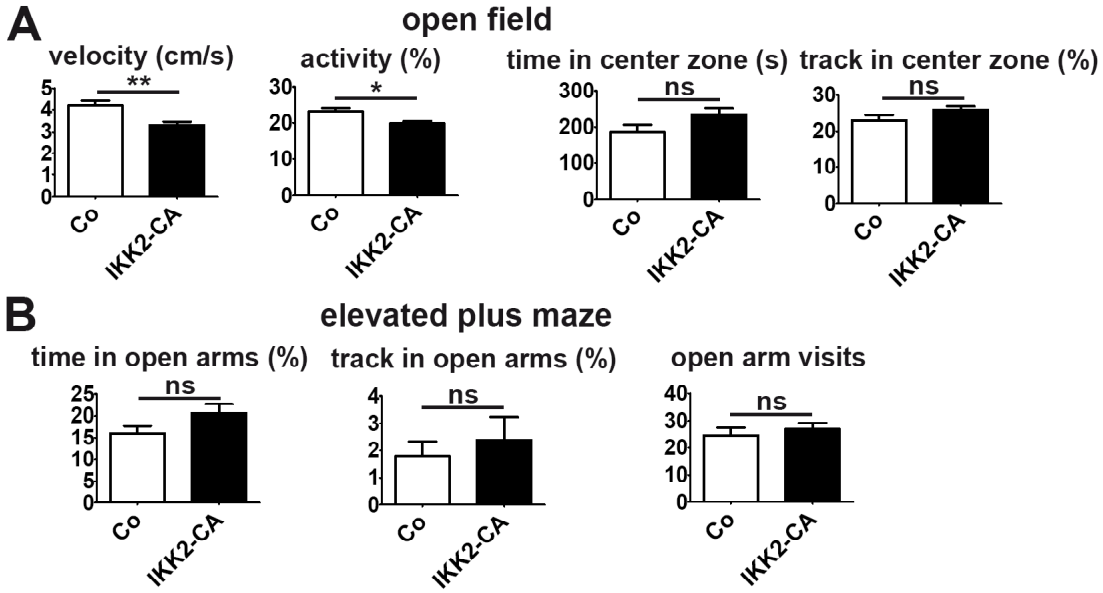

Figure 32: IKK2-CA expressing mice display a moderate reduction in velocity and activity, but no specific alterations in fear and exploratory behavior are detected in open field and elevated plus maze

(A) Open field test reveals reduced running speed and activity, but no change in avoidance of the open center zone, measured by time and fraction of total track length spent in the center zone

(B) No alteration in the exploration of the open arms of an elevated plus maze, measured by the time and fraction of track length in the open arms and the number of visits to the open arms

Data plotted as mean +/- s.e.m.; statistical analysis: unpaired two-tailed t-test (n = 11-15), ns not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$

3.4.1.3 Learning and memory in GFAP/IKK2-CA mice

Another cognitive function that is affected by neuroinflammation in several models, and also by deregulated neuronal NF-κB signaling, is hippocampal learning and memory. A potential influence of enhanced NF-κB signaling in astrocytes and the resulting neuroinflammation on hippocampal function was assessed by the Morris water maze, the most accepted and widely used paradigm for this purpose. In this experimental setup mice have to learn to find a hidden platform in a pool of water and remember its position by orientation with distant visual cues (Vorhees and Williams, 2006).

As swimming is a task that also requires a certain extent of motor coordination and therefore depends on cerebellar function, first the general ability of GFAP/IKK2-CA mice to reach a visible platform by directed swimming was tested (Figure 33A). Although the transgenic animals usually needed more time than controls to reach the platform, after 8 trials most animals reached the platform in 1-2 s, with some of the IKK2-CA expressing animals requiring up to 5-7 s. This proofs that in principle the GFAP/IKK2-CA animals can perform this task, although there are some differences in swimming capabilities that could complicate the interpretation of the results.

Next, the animals were trained with 4 trials per day for 5 days to find a platform with a fixed position, which is hidden about 1 cm under the water surface, with the help of distant visual cues. In this paradigm of spatial learning, the IKK2-CA expressing animals also needed more time to reach the platform, but this time decreased during the learning phase to similar extent as in the control animals (Figure 33B).

This suggests that it is actually not learning that is affected by enhanced astroglial IKK2 activation, but rather the impaired swimming that leads to this difference in the time to reach the platform. Indeed the average swimming speed is reduced by about 25 % (Figure 33C). The absolute distance that is covered to reach the platform is much longer in the hidden platform paradigm than in the visible platform experiment, therefore the altered swimming speed might explain the more pronounced differences in the hidden platform test.

Swimming speed was measured in the first probe trial, which is used to assess memory consolidation, 1 day after the end of the training phase. In this probe trial the platform is removed and the preference of the mice for the area, in which the platform was located before, is analyzed. In particular, the time is measured that the mouse spends in the former platform quadrant, of a total of 30 s.

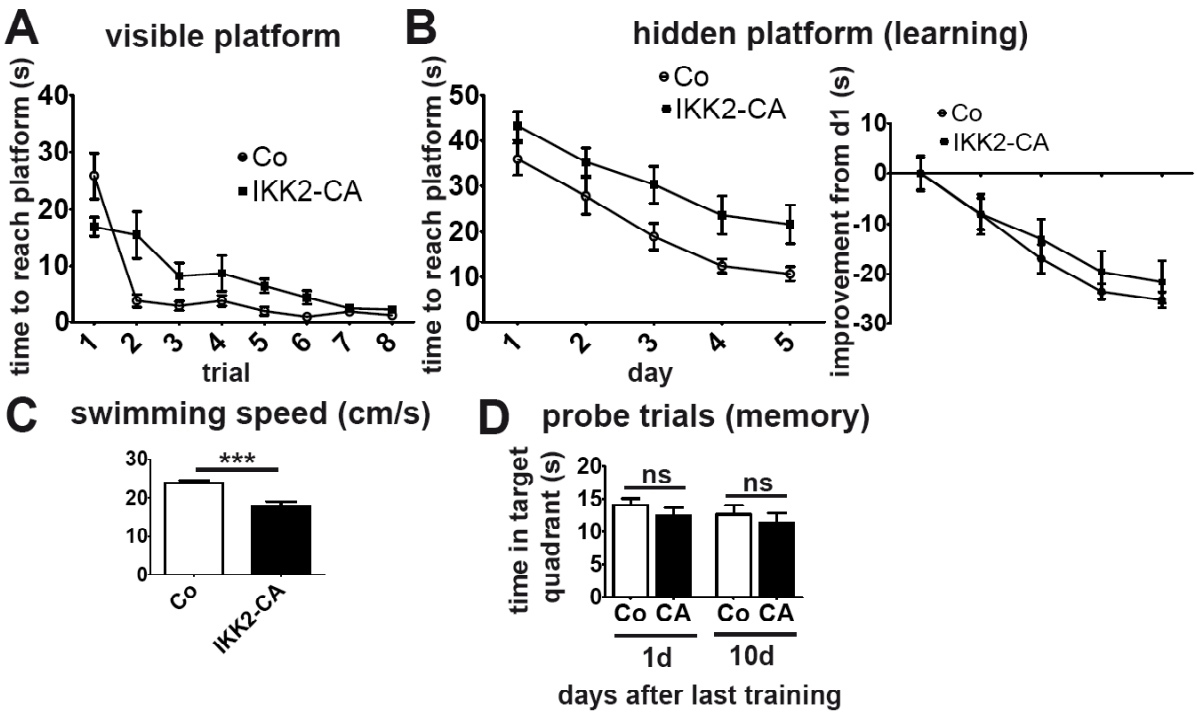

Figure 33: No prominent deficit in hippocampal learning and memory in GFAP/IKK2-CA mice is detectable in the Morris water maze; swimming impairment might mask subtle effects

(A) IKK2-CA expressing animals are capable of learning to reach a visible, labeled platform by swimming in a water filled basin, but are generally slower than controls; animals were trained with 4 trials/day for 2 days

(B) GFAP/IKK2-CA mice are able to learn to find a platform hidden under the water surface by orientation with visual cues; they are generally slower than control animals, but show a similar improvement during training trials (4 trials/day for 5 days); statistical analysis by 2-way-ANOVA ($n = 11\text{-}13$) shows significant difference in total time ($p < 0.0001$), but not in improvement from day 1 to 5 ($p > 0.05$);

(C) Reduced swimming speed of mice expressing IKK2-CA might explain the shift in the learning curves; speed measured during the first probe trial without plattform, 1 day after last training session; statistical analysis: two-tailed unpaired t-test ($n = 11\text{-}13$; $p < 0.001$)

(D) Memory for the platform position after removal is not altered 1 and 10 days after the last training trial, measured by the searching time in the quadrant, where the platform was located before (of total 30 s); statistical analysis: two-tailed unpaired t-test ($n = 11\text{-}13$; $p > 0.05$)

This analysis is much less dependent on the swimming speed, and indeed shows no difference between control and GFAP/IKK2-CA mice (Figure 33D). The same result is also found 10 days after the last training session, further supporting the view that there is no memory impairment in the IKK2-CA expressing mice (Figure 33D).

3.4.2 Characterization of cerebellar degeneration induced by IKK2 hyperactivation in astrocytes

The only clearly detectable functional/behavioral alterations in the animals that express the IKK2-CA transgene in astrocytes in the adult brain for several months are the defects in motor coordination (see 3.4.1) that correlate with the observed prominent cerebellar atrophy (see 3.1.5). Despite the widespread neuroinflammation (see 3.2), pathological consequences of enhanced NF-κB signaling in astrocytes seem to be restricted to this brain part. This suggests a specific sensitivity of the cerebellum for an inflammation-induced disturbance of homeostasis. Interestingly, a relatively rare (prevalence about 15-20 of 100 000), but diverse group of inherited and acquired human diseases shows comparable motor impairments associated with cerebellar defects (Manto and Marmolino, 2009; Klockgether, 2011). Among these cerebellar ataxias are several autoimmune/inflammatory diseases, suggesting that inflammatory mechanisms might play a role in their pathogenesis. Therefore the cerebellar phenotype of the adult GFAP/IKK2-CA mice was investigated in more detail.

3.4.2.1 Neuronal loss in the degenerating cerebellum

Histological analysis of Nissl-stained brain sections showed several prominent alterations of the cerebellum of 9 months old GFAP/IKK2-CA animals, which were studied in detail in the simple lobule in the rostral part of the cerebellum (Figure 34A). There is an accumulation of cells in the meningeal area (probably mainly inflammatory infiltrates, see 3.2.2.2), and also in the molecular layer the density of cells is increased. By contrast, there is a massive loss of Purkinje cells, the major output cells of the cerebellum. In addition, in severely degenerated cerebella also the density of cells in the internal granule layer seems to be reduced.

Non of these morphological alterations was found in even older animals that express a dominant negative allele of IKK2 instead of IKK2-CA, indicating that the observed alterations are indeed dependent on IKK2 activity (Figure 34A). In other regions of the cerebellum similar alterations in the IKK2-CA expressing animals were also found, as shown for the paramedian lobule in a more caudal part of the cerebellum (Figure 34B/C). Quantification of Purkinje cell numbers showed a similar reduction of around 65-80 % in both the simple lobule and the paramedian lobule (Figure 34D).

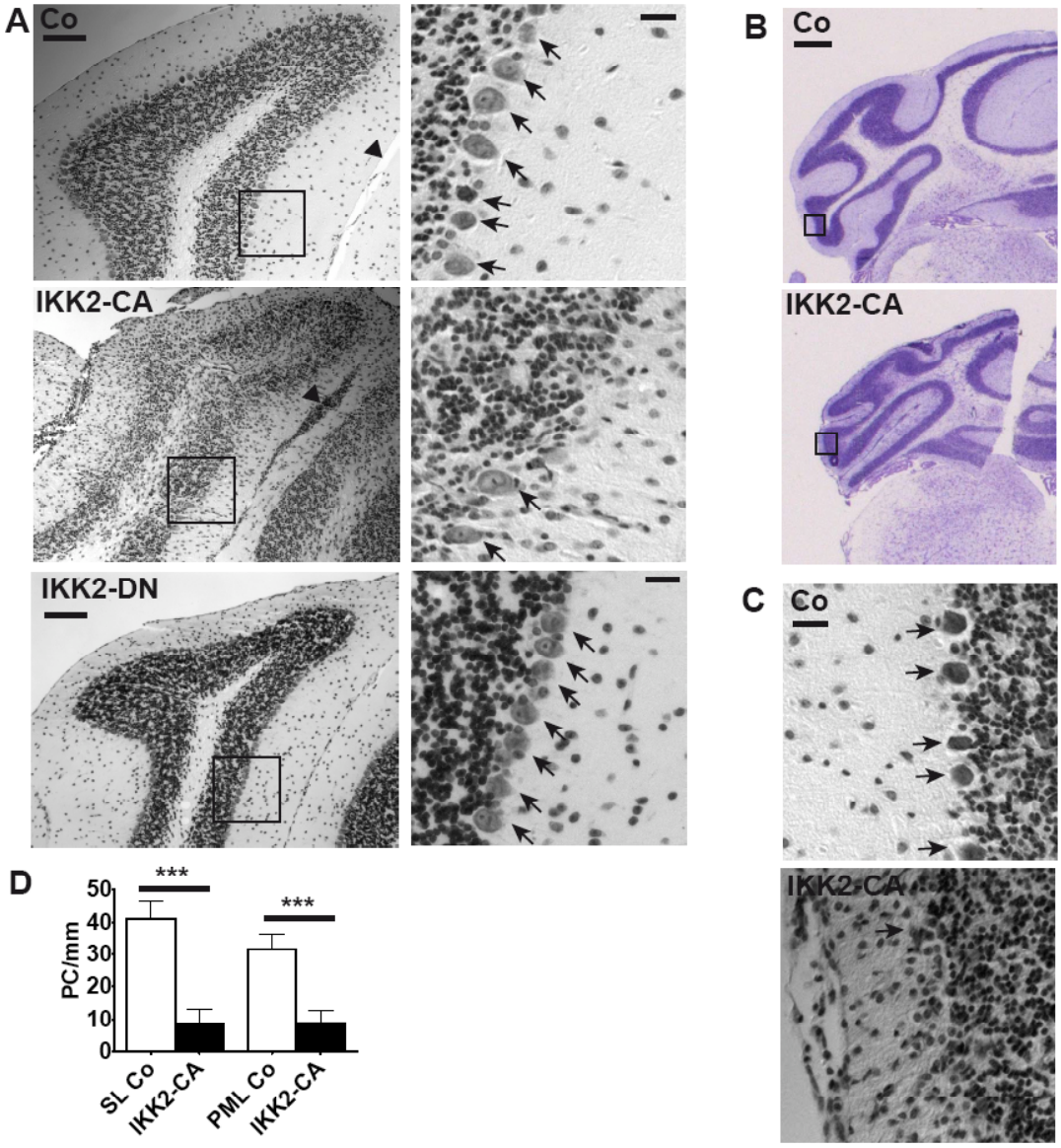

Figure 34: Cerebellar atrophy is associated with a massive global loss of Purkine cells at 9 months of age

(A) Nissl staining of the simple lobule reveals massive loss of Purkinje cells (arrows in enlargements (right panels)), a distortion of the granule layer and accumulation of cells in meningeal foldings (arrowhead, left panels). No changes are observed in animals overexpressing a dominant negative IKK2 allele. Scale bars: 100 μm (left panels); 20 μm (enlargements, right panels)

(B) Nissl staining shows a compressed overall structure of the cerebellar cortex also in the caudal part of the cerebellum; scale bar: 500 μm

(C) Higher magnification images display a massive loss of Purkinje cells (arrows) in the paramedian lobule (boxes in (B)); scale bar: 20 μm

(D) Quantification of Purkinje cells in the simple lobule (SL) and the paramedian lobule (PML) show a loss of 65-80 % of Purkinje cells; statistical analysis: 2-tailed unpaired t-test (n=6-8), *** $p < 0.001$

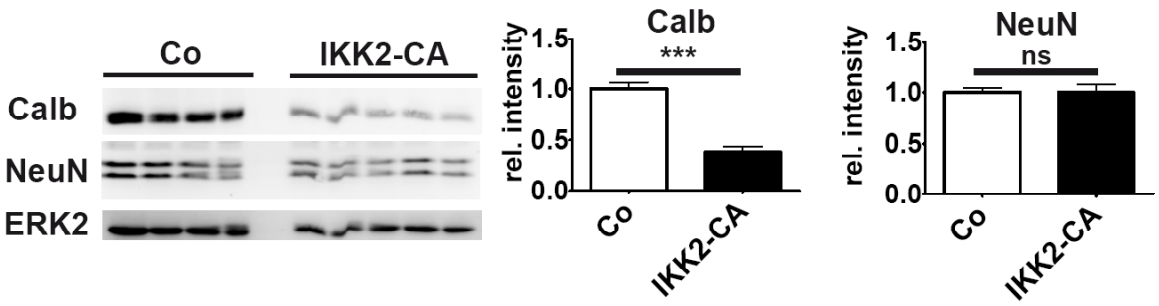

Figure 35: Immunoblotting confirms a major loss of Purkinje cells, but not of other neurons, at 9 months of age

Immunoblotting of cerebellar tissue protein extracts at 9 months reveals a 60% loss of expression of the Purkinje cell marker calbindin in IKK2-CA animals, showing the loss of Purkinje cells; there is no specific loss of other neurons, as indicated by unaltered NeuN immunoreactivity; for densitometric quantification, all values (for NeuN combined two bands) were normalized to the loading control ERK2 and then to the mean of the control samples; statistical analysis: 2-tailed unpaired t-test (n = 4-5), *** $p < 0.001$

This prominent loss of Purkinje cells was confirmed on the whole tissue level by immunoblotting for the Purkinje cell marker calbindin, which showed a reduction of around 60 %, whereas there was no detectable reduction of other neurons, measured by expression of the general neuronal marker NeuN, which is expressed in most neurons except Purkinje cells (Figure 35).

Taken together, the cerebellar atrophy in the GFAP/IKK2-CA mice is associated with a predominant loss of Purkinje neurons, a feature that this model shares with most types of cerebellar ataxias (Manto and Marmolino, 2009).

3.4.2.2 Time course of cerebellar degeneration

To characterize the onset and progression of the cerebellar atrophy and Purkinje cell degeneration, the rostro-caudal length of the cerebellum was measured at different ages (Figure 36A, see also 3.1.5). While until 3 months of age no macroscopic atrophy is visible, at 5 months of age an intermediate stage with highly variable degeneration is reached. Whereas some animals show a normal cerebellar length at this age, others already show the maximal length reduction (around 30-50%) that is reached at later stages (9 and 13 months). This illustrates that once the degenerative process is initiated (at a variable point in time), it progresses relatively rapidly within 2 months to a final endpoint.

The macroscopic atrophy corresponds well with the time course of Purkinje cell loss, which was quantified in Nissl stained sections in the simple lobule (Figure 36B). In this quantification, 2 of 6 IKK2-CA expressing animals at 3 months already show a reduced Purkinje cell number, indicating that the loss of Purkinje cells might precede the macroscopic atrophy. As in the cerebellar length quantification, at 5 months the progression of Purkinje cell loss is heterogeneous, whereas at 9 months of age all analyzed IKK2-CA expressing animals show only few residual Purkinje cells.

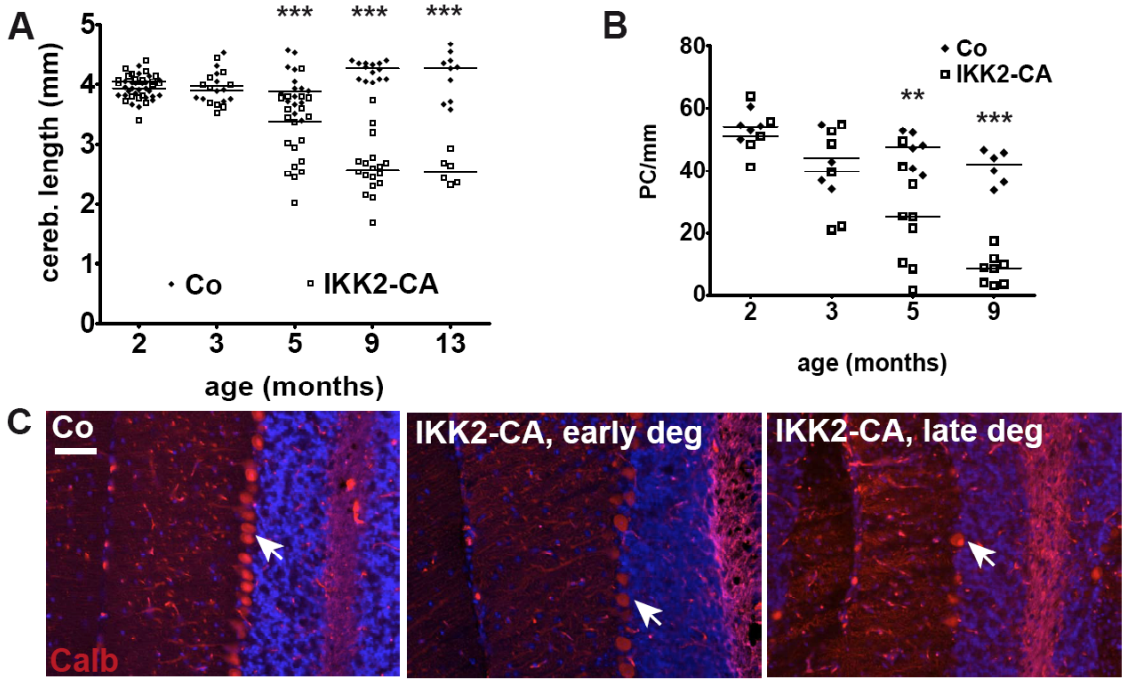

Figure 36: Cerebellar atrophy and Purkinje cell degeneration progress rapidly with a variable onset around 3-5 months of age

(A) Long-term expression of IKK2-CA causes cerebellar atrophy, measured by the maximal rostro-caudal length of the cerebellum; no atrophy is observed at 3 months, whereas some animals already at 5 months have the maximal atrophy reached later by all animals; diagram shows single animals and group median;

(B) Quantification of Purkinje cell numbers in the simple lobule from Nissl stained sections shows a time course correlating to macroscopic cerebellar atrophy;

(C) Fluorescence immunostaining for the specific marker calbindin shows a variable loss of Purkinje cells in GFAP/IKK2-CA animals at 5 months, which correlates with macroscopic cerebellar degeneration in the individual animal; co-staining with DAPI; scale bar: 50 μm

statistical analysis: 2-tailed unpaired t-test, ** $p < 0.01$, *** $p < 0.001$;

The variable loss of Purkinje cells at 5 months of age is also shown by immunofluorescence stainings for the Purkinje cell marker calbindin, and correlates with macroscopic degeneration on the level of the individual animal (Figure 36C).

3.4.2.3 Astrogliosis and Bergmann glia defects

The specific loss of Purkinje cells in the GFAP/IKK2-CA model raises the question why these cells are especially sensitive to degeneration induced by constitutively enhanced NF-κB activation in astrocytes and the resulting chronic neuroinflammation. Remarkably, Purkinje cells are closely associated to a specialized subpopulation of radial glia-like astrocyte related cells, the Bergmann glia. These glial cells are crucial for the maintenance of Purkinje cell homeostasis, as disruption of Bergmann glia function was shown to result in non cell-autonomous Purkinje cell degeneration in genetic mouse models (see 1.2.4). Bergmann glia express GFAP and might therefore express the IKK2-CA transgene. Furthermore, astroglial cells are well known to alter their function in inflammatory conditions. Therefore the structural and functional properties of the Bergmann glia were analyzed in the GFAP/IKK2-CA mice.

IKK2-CA expression in Bergmann glia was assessed by costaining for human IKK2 (the transgene) with the astroglia marker Aldh1l1 (Figure 37). In an early stage of degeneration, only relatively few Aldh1l1 positive cell bodies in the Purkinje cell layer express IKK2-CA. Adjacent to these cell bodies, processes within the molecular layer also display IKK2-CA expression, indicating that indeed these cells are Bergmann glia, whose processes span the molecular layer. Whereas at this stage, the major Aldh1l1 immunoreactivity locates to the Bergmann glia cell bodies in the Purkinje cell layer, this specific staining pattern is lost with progression of degeneration. In late stages of degeneration, prominent Aldh1l and IKK2-CA staining is found in all layers of the cerebellar cortex, most prominently in the molecular layer, suggesting that IKK2-CA expression disrupts the specific structure and localization of Bergmann glia.

To further characterize the structural and functional alterations of Bergmann glia structure and function, GFAP expression was analyzed by immunofluorescence staining and immunoblotting.

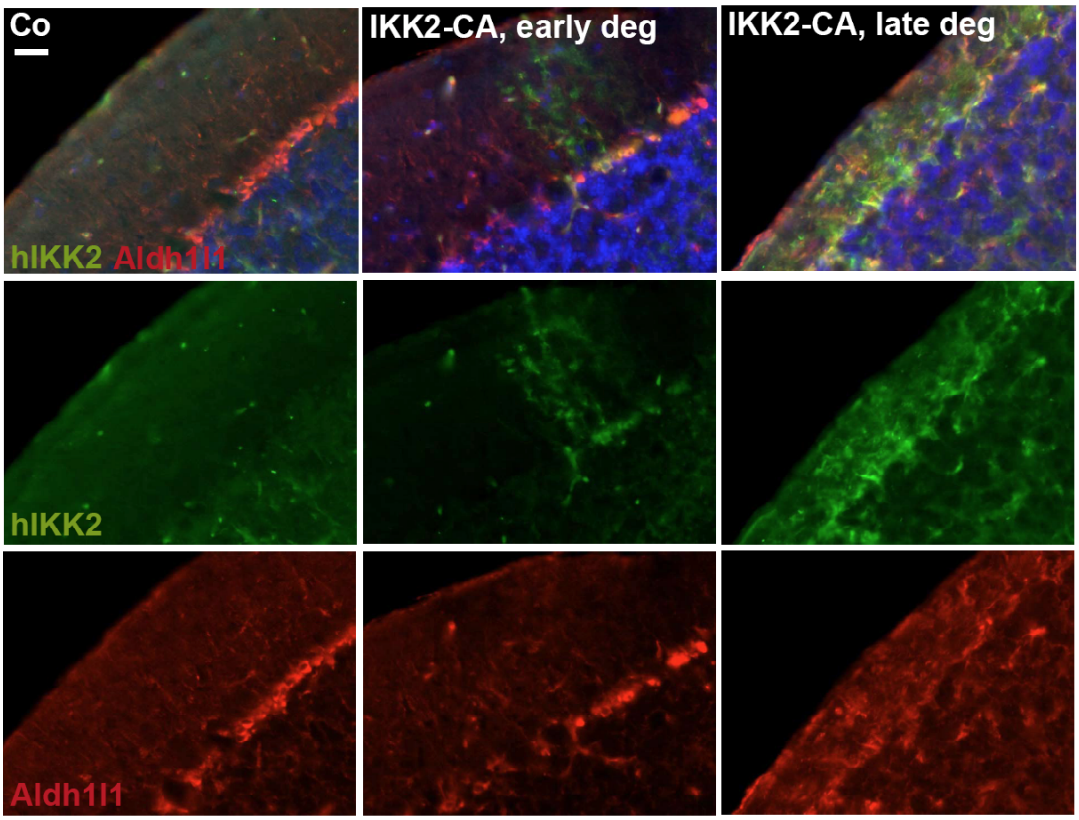

Figure 37: Bergmann glia express the IKK2-CA transgene and have a distorted distribution and morphology in the degenerated cerebellum

Double staining for the transgene (hIKK2) and the astroglia marker Aldh1l1 at 5 months of age shows mosaic transgene expression in Bergmann glia in a cerebellum with mild degeneration, i.e. in an early degeneration phase (middle panels); in this phase the major Aldh1l1 staining localizes to Bergmann glia cell bodies in the Purkinje cell layer, as seen in control animals (left panels); in a heavily degenerated cerebellum/late degeneration phase (right panels), increased Aldh1l1 staining is found, mainly in the molecular layer, indicating astrogliosis, whereas specific location of Aldh1l1 positive Bergmann glia cell bodies in the Purkinje cell layer is lost; correspondingly, transgene staining is also prominent in the molecular layer; merged images (upper panels) show DAPI staining as a nuclear marker; scale bar 20 μm;

Staining for GFAP (Figure 38A) shows the typical radial glial morphology of Bergmann glia in control animals and IKK2-CA expressing animals in an early stage of degeneration, with thin parallel fibers spanning the molecular layer. By contrast, at later stages of degeneration, this structure is severely disorganized. The fibers show prominently increased GFAP immunoreactivity, and they are thicker and completely unoriented, thereby resembling typical characteristics of activated astrocytes.

The increase of GFAP immunoreactivity is also obvious in immunoblotting, where densitometric analysis revealed a 15-fold increased expression at 9 months of age (Figure 38B).

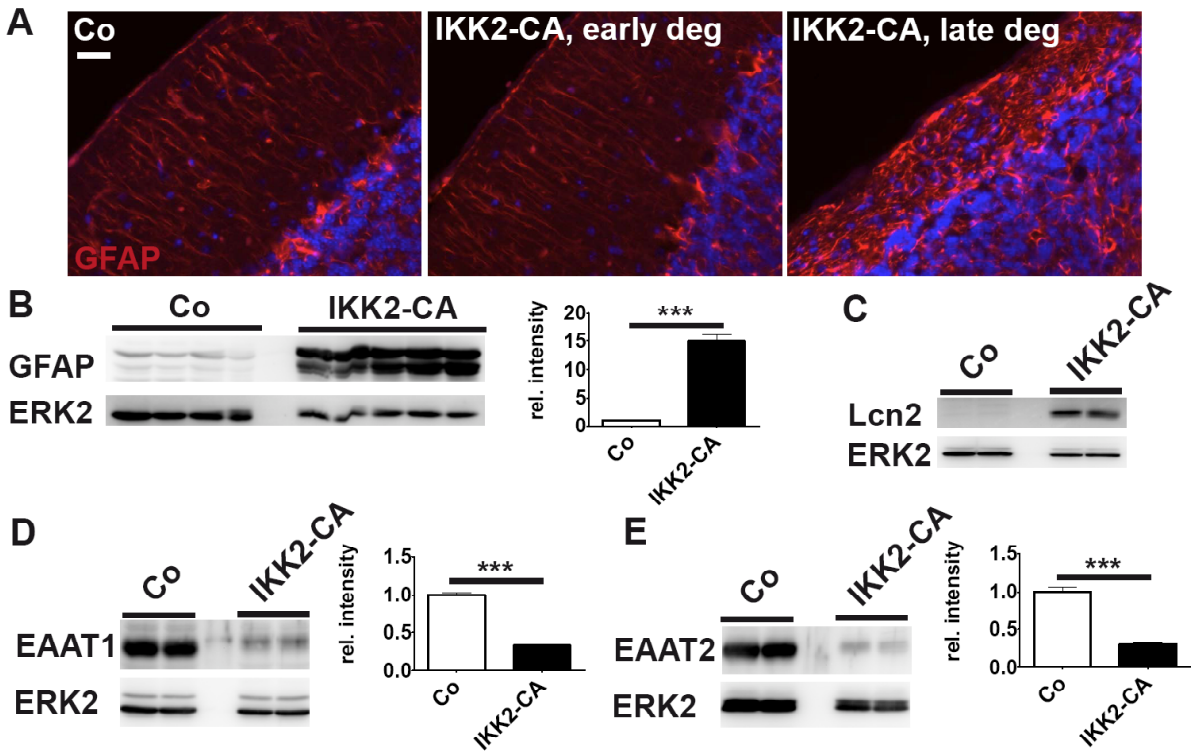

Figure 38: IKK2-CA expression causes astrogliosis, which results in disturbed Bergmann glia structure and glial glutamate transporter downregulation

(A) Staining for the astrocyte intermediate filament GFAP (with nuclear DAPI costaining) at 5 months of age shows disruption of Bergmann glia cytoarchitecture and astrogliosis; in control and mildly degenerated cerebelli the molecular layer is traversed by parallel radial glia processes of Bergmann glia with low GFAP staining, whereas heavily degenerated cerebella show intensely stained, thickened and unorganized processes, indicating a transformation of Bergmann glia from an radial glia-like into an activated astrocyte-like state; scale bar 20 μm

(B) Immunoblotting shows a 15-fold upregulation of GFAP, indicating massive astrocyte/Bergmann glia activation; the two major bands were analyzed by densitometry;

(C) IKK2-CA induces expression of the astrogliosis mediator Lcn2 on protein level

(D) EAAT1 (GLAST) expression is reduced to about 30% in IKK2-CA expressing cerebella

(E) EAAT2 (GLT-1) expression is reduced to about 30% in IKK2-CA expressing cerebella

All immunoblots (B-E) were performed with cerebellar tissue protein extracts of 9 months old animals and show ERK2 immunoreactivity as loading control; values obtained by densitometric quantification were normalized to the loading control ERK2 and then to the mean values of the control samples; statistical analysis: 2-tailed unpaired t-test (n = 4-5), *** $p < 0.001$

Additional evidence for astrogliotic alterations comes from the upregulation of Lcn2, which is a critical regulator and marker of astrogliosis (Lee et al., 2009; Zamanian et al., 2012). As already shown, this factor is highly induced on RNA level by NF-κB in astrocytes (see 3.2.1). On protein level, it is prominently expressed in cerebellar tissue of GFAP/IKK2-CA at 9 months of age, whereas it is virtually absent in control cerebella (Figure 38C).

A key function of astrocytes, which is often disturbed in neuroinflammation, is the reuptake of the excitotoxic neurotransmitter glutamate from the synaptic cleft. The glial glutamate transporters EAAT1/GLAST and EAAT2/GLT-1, which are responsible for this function, are downregulated by various inflammatory cytokines (Tilleux and Hermans, 2007). Such an impairment of glutamate reuptake by Bergmann glia was postulated to be the cause of non-cell-autonomous Purkinje cell degeneration in several mouse models (Cui et al., 2001; Custer et al., 2006; Tao et al., 2011). Indeed in the GFAP/IKK2-CA model, the expression of both EAAT1 and EAAT2 is reduced by about 70% in the cerebellum at 9 months of age (Figure 38D/E), indicating that this mechanism could also be responsible for Purkinje cell degeneration in this model.

3.4.2.4 Early alterations in the cerebellum of GFAP/IKK2-CA mice

To further elucidate the sequence of events and mechanism leading to Purkinje cell degeneration, the early defects in the cerebellum of adult GFAP/IKK2-CA animals around onset of cerebellar atrophy were studied in more detail.

If astrogliosis and Bergmann glia activation and specifically the impairment of glutamate uptake are causative for Purkinje cell degeneration, these astroglial alterations should precede Purkinje cell degeneration. Indeed, astrogliosis mediators as Lcn2 and IL-6 are already upregulated at 2 months of age (see 3.2.1). However at this age, expression of EAAT1 and EAAT2 is not yet altered (Figure 39A/B). This changes dramatically between 2 and 3 months of age: In cerebellar synaptosomal protein extracts of 3 months old GFAP/IKK2-CA animals (Figure 39C/D), both glutamate transporters are downregulated to a similar extent as in 9 month old animals, and GFAP shows a similar induction of about 15-fold. This indicates that there is already strong astroglia activation at 3 months of age, whereas there is not yet prominent Purkinje cell degeneration.

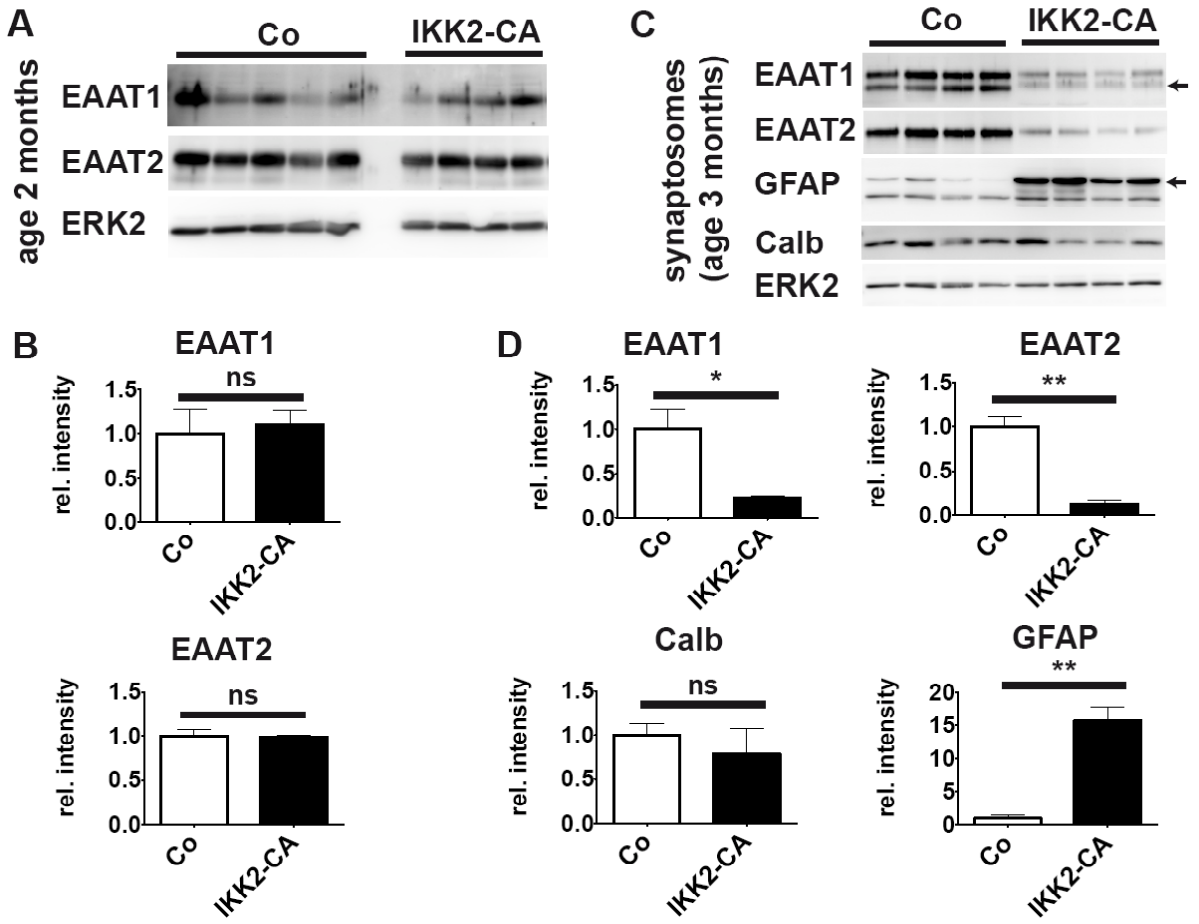

Figure 39: Glutamate transporter downregulation and astroglial activation starts between 2 and 3 months of age

(A) EAAT1 (GLAST) and EAAT2 (GLP-1) expression is not altered at 2 months of age, measured by immunoblot;

(B) Quantification of the immunoblots in (A) shows no alteration in EAAT1/2 protein levels at 2 months of age

(C) Immunoblot of synaptosomal preparations at 3 months of age reveals early astrogliosis preceding Purkinje cell loss, shown by increased GFAP expression, with prominent downregulation of EAAT1 and EAAT2; calbindin immunoreactivity is not altered, indicating no major loss of Purkinje cells; the blot for EAAT1 (arrow) shows also a band for EAAT2, which was detected before on the same blot; likewise the blot for GFAP (arrow) also shows ERK2

(D) Quantification of immunoblots in (C) shows significant downregulation of EAAT1/2 and upregulation of GFAP at 3 months of age, but no significant decrease in calbindin levels;

Immunoblots were analyzed by densitometry, normalized to ERK2 as loading control and then to the mean of the control animals; bar diagrams show mean +/- s.e.m.; statistical analysis: 2-tailed unpaired t-test (n = 4-5); ns not significant ($p>0.05$), * $p<0.05$, ** $p<0.01$;

The absence of major Purkinje cell loss is also confirmed for the same animal cohort by the analysis of calbindin expression, which is similar in control and GFAP/IKK2-CA animals (Figure 39C/D).

Interestingly, although there is not yet a prominent loss of Purkinje cells at that time, the activation of Bergmann glia has already caused irreversible defects (Figure 40).

In particular, an impairment of motor function precedes Purkinje cell loss (Figure 40A), as demonstrated by a shortened beam walking test protocol at 3 months of age (4 consecutive trials with a 12 mm square beam). In this paradigm, the animals show a comparable motor impairment as the animals at 8 months of age (see 3.4.1.1), which suggests that Purkinje cells acquire early defects, which impair their function before they eventually result in their death. Indeed at that time the defects have at least in severe cases already passed a “point of no return”, inevitably leading to the death of the Purkinje cells. This could be shown by the inactivation of transgene expression by doxycycline administration from 3 months of age, which is no more able to rescue Purkinje cell loss (Figure 40B).

Since the prominent downregulation of glial glutamate transporters precedes the onset of Purkinje cells loss and might even more directly coincide with the onset of motor defects and irreversible Purkinje cell damage, it is very likely that indeed excitotoxic stress causes synaptic dysfunction and finally Purkinje cell death.

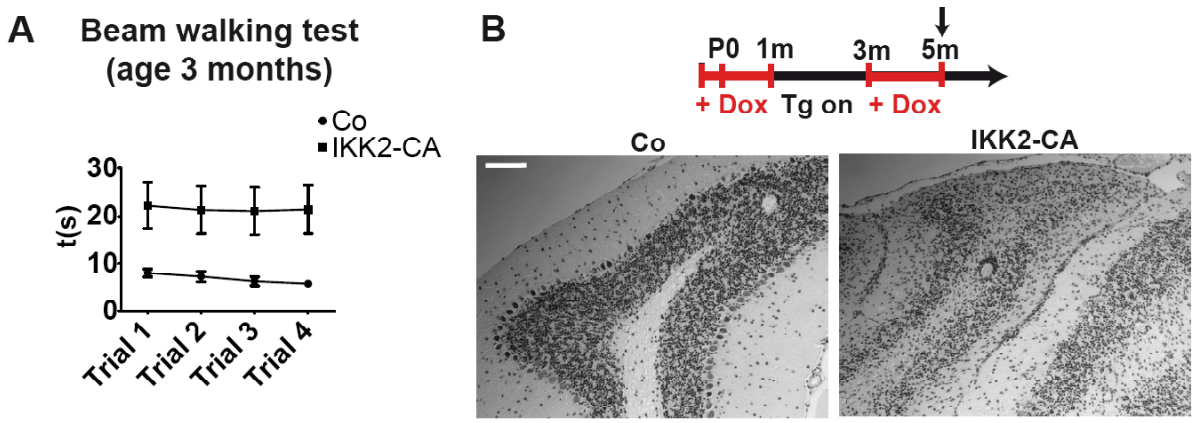

Figure 40: Bergmann glia dysfunction causes early irreversible defects of Purkinje cells

(A) Motor defects are fully developed at the onset of Purkinje cell loss (3 months of age), shown by a simplified 4 trial beam walking test protocol with a 12 mm square beam

(B) Stop of IKK2-CA expression at the onset of Purkinje cell loss by administration of doxycycline from 3 months of age cannot halt Purkinje cell loss; Nissl staining, scale bar 100 μm

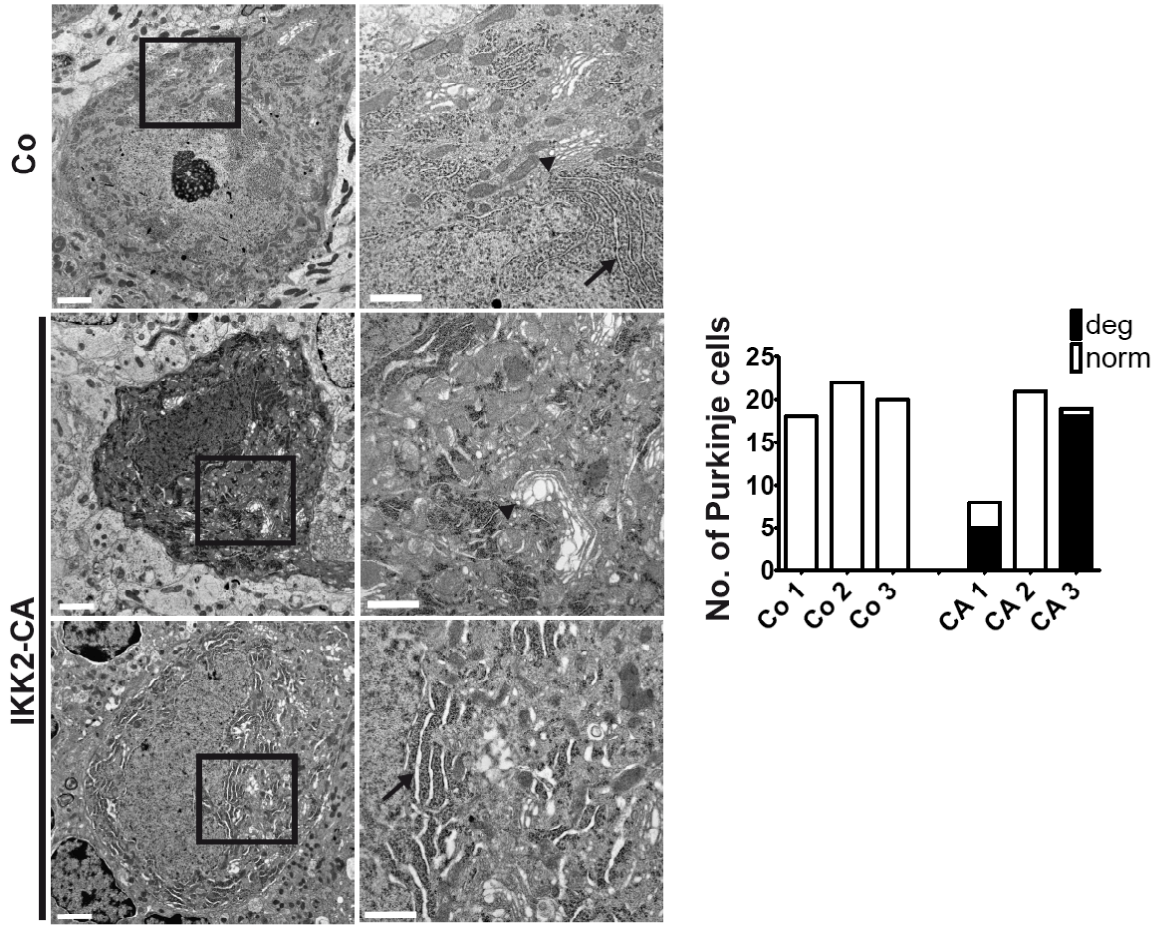

Figure 41: Purkinje cells in IKK2-CA expressing animals show ultrastructural characteristics of excitotoxic dark cell degeneration at 4 months of age

Transmission electron microscopy shows signs of dark cell degeneration of the majority of Purkinje cells in two of three GFAP/IKK2-CA animals at 4 months of age, whereas no degenerating cells are found in controls; note the darkened cytoplasm, irregular morpholgy and dilatation of Golgi cisternae (arrowheads) and endoplasmatic reticulum (arrows); scale bars: 2 μm (left panels), 1 μm (right panels)

This hypothesis is further supported by ultrastructural studies with transmission electron microscopy (Figure 41). In electron microscopic images, the majority of Purkinje cells of 2 out of 3 studied IKK2-CA expressing animals showed a darkened cytoplasm, an irregular cellular and nuclear morphology and a dilatation of the Golgi cisternae and the endoplasmatic reticulum. Such alterations are typical signs of “dark cell degeneration”, which is found in Purkinje cells undergoing excitotoxic degeneration (Perkins et al., 2010). The variable amount of degenerating Purkinje cells might be explained by the variable onset of degeneration (see 3.4.2.2). Of note, in about 60 studied cells of 3 control animals not one single cell was showing

consistent signs of dark cell degeneration.

In summary, these results suggest that IKK2-CA expression induces the early activation of Bergmann glia and leads to the disruption of glutamate uptake by these cells. This causes excitotoxic damage and functional defects of the Purkinje cells, finally resulting in their death by excitotoxic dark cell degeneration.

4 Discussion

Inflammation is an essential response of the organism to pathogens, injury and other threats. It is also a common phenomenon found in various types of CNS disorders, where it is often implicated in disease pathogenesis. Astrocytes are glial cells that have multiple functions in CNS development and homeostasis and are also critically involved in the control of neuroinflammatory processes. The NF-κB signaling pathway is well characterized as a key regulator of inflammation in different cell types in various organs including the CNS.

Therefore understanding the function of NF-κB signaling in astrocytes is of outstanding interest for both basic and applied medical research. The present work characterizes a novel genetic mouse model for the investigation of NF-κB signaling in astrocytes and demonstrates important functions of astroglial NF-κB in neuroinflammation, CNS development and neurodegeneration.

4.1 GFAP promotor driven conditional overexpression of constitutively active IKK2 as a model to study astroglial NF-κB signaling

Mouse models with cell type specific gene inactivation or transgene expression are the tools of choice to investigate gene functions and related signaling pathways in a specific cell type *in vivo*, i.e. in conditions that in the ideal case resemble closely the natural situation also present in the human organism. To study the role of NF-κB signaling specifically in astrocytes *in vivo*, so far only one loss-of-function model (GFAP.IκBα-dn) was studied in detail, which overexpresses a dominant negative, non-degradable IκBα mutant driven by the GFAP promotor (Brambilla et al., 2005). This mutant IκBα lacks the N-terminal activation domain, which prevents its phosphorylation by kinases like IKK2. Thereby its degradation and the nuclear translocation of NF-κB transcription factors is prevented. This model shows no overt phenotype under normal physiological conditions, like similar models (GFAP.IκBα-SS32/36AA) with a mutation of the IKK phosphorylation sites Ser-32 and Ser-36 (Zhang et al., 2005; Crosio et al., 2011). For some pathologic insults, in particular SCI, EAE and retinal ischemic injury, a reduced inflammatory response and an

improved outcome was found in the GFAP.IκBα-dn model (Brambilla et al., 2005; Brambilla et al., 2009b; Dvoriantchikova et al., 2009). This model excludes an important role of basal astroglial NF-κB activity in CNS development and indicates an inflammation promoting function of astroglial NF-κB under pathological conditions, but cannot address the consequences of elevated NF-κB activity under normal physiological conditions.

To investigate this issue, in the present work a new gain-of-function model for astroglial NF-κB signaling, the GFAP/IKK2-CA model, was characterized. This model allows the conditional activation of NF-κB signaling in astrocytes by the GFAP promotor driven expression of a constitutively active IKK2 mutant in a tetracycline-regulated manner.

This binary transgenic system combines some specific features that allow to answer questions that could not be addressed before. It targets NF-κB signaling specifically in GFAP expressing cells as the GFAP.IκBα-dn model, but in contrast to the latter it does activate NF-κB signaling instead of inhibiting it. Differing from other models that activate NF-κB signaling in the brain, as e.g. LPS injection, injury, or similar models, it does not directly activate NF-κB in other cell types and it does not require an additional pathologic stimulus for NF-κB activation, which always also activates additional signaling pathways. Thereby it is suitable to distinguish both cell type and pathway specific effects from more global pathologic stress responses. Finally, in contrast to the constitutively expressed GFAP.IκBα-dn transgene and other conventional transgenic, gene deletion or knock-in approaches, the modulation of the signaling pathway can be reversibly regulated by the administration of doxycycline. This allows the distinction of developmental vs. non-developmental and reversible vs. irreversible consequences of the deregulation of the signaling pathway.

In the specific case of the GFAP/IKK2-CA mouse, it could be shown that NF-κB activation in astrocytes can not only enhance neuroinflammation, as was suggested by the GFAP.IκBα-dn model (Brambilla et al., 2005; Brambilla et al., 2009b; Dvoriantchikova et al., 2009), but is actually sufficient to induce neuroinflammation. Furthermore it could be shown that constitutive IKK2 activation does induce hydrocephalus formation, but only in the early postnatal brain, indicating that this is a developmental defect. Finally, without our conditional gain-of-function-model it would not have been possible to study the mechanism of hydrocephalus formation in detail,

nor to identify the role of astroglial NF-κB activation in adult Purkinje cell homeostasis, because the developmental lethality could not be overcome.

Nevertheless, as with all transgenic systems, the specificity and physiological relevance of this approach has to be considered carefully.
First, the random insertion of a transgenic construct into the genome can disrupt endogenous genes, resulting in transgene function independent phenotypes. In the current study this possibility was excluded by the use of single-transgenic animals of both transgenes as controls, which do not show the main phenotypes, namely neuroinflammation, hydrocephalus formation and cerebellar atrophy. In addition, at least for the developmental phenotype it was shown that a second independent (tetO)7.IKK2-CA founder line could reproduce the phenotype, demonstrating that the location of this transgene at a specific integration site is not responsible for the observed hydrocephalus formation.
The second issue is the cell type specificity of transgene expression, which depends mainly on the promotor of the driver transgene (in this case GFAP.tTA), but also on the transgene integration site. The integration site influences gene expression by the local genomic environment, like close enhancer elements and the local chromatin structure. In the present study it was demonstrated that the GFAP.tTA driven IKK2-CA transgene expression in the brain was mainly found in Aldh1l1 positive astrocytes. Nevertheless, the GFAP promotor, which is used to target transgene expression, is not absolutely specific for astrocytes, but was also described to be expressed at lower levels in astrocyte-related cells like neural stem cells and ependymal cells, but also in peripheral tissues, e.g. in Schwann cells, enteric glia, chondrocytes, fibroblasts and hepatic and pancreatic stellate cells (Brenner et al., 1994; Ding et al., 2009; Middeldorp and Hol, 2011). It is very unlikely that transgene expression by the GFAP promotor activity in peripheral tissues is related to the observed CNS phenotypes, but a possible transgene expression in other astrocyte related CNS glia could not completely be ruled out, and might indeed have an impact on the described phenotypes.
A second point that has to be regarded with caution is how closely the overexpression of the constitutively active IKK2 protein resembles physiological NF-κB activating conditions. The study excludes that the observed phenotypes are due to mere IKK2 protein overexpression, as the even stronger expression of a kinase

inactive IKK2 mutant with the same transgenic approach does not result in any obvious phenotype. Nevertheless, the strong and continuous activity of IKK2-CA might well be more pronounced than IKK2 activity in any physiological condition, thereby resulting in supra-physiological NF-κB dependent and NF-κB independent responses. It was shown in the present study that as expected GFAP/IKK2-CA induces strong RelA nuclear localization and transcription of NF-κB target genes in astrocytes, whereas the action on other putative IKK2 substrates was not investigated.

Despite the potentially supra-physiological NF-κB activation, IKK2-CA overexpression in other organs, such as the exocrine pancreas, the hepatocytes of the liver or the intestinal epithelium, was shown to cause phenotypes that are similar to those found in physiological inflammatory conditions, in these particular studies pancreatitis, inflammatory liver fibrosis and enhanced intestinal tumorigenesis (Baumann et al., 2007; Vlantis et al., 2011; Sunami et al., 2012). For the GFAP/IKK2-CA model, at least on the level of transcriptional responses, the induction of proinflammatory genes in the brain is in a similar range as in other neuroinflammatory disease models. For example, induction of the chemokines CCL2 and CCL5 (100-500 fold) and the cell adhesion molecule ICAM-1 (2-5 fold) is comparable to the situation in an EAE paradigm (Brambilla et al., 2009b). Similarly a several hundred fold induction of C3 and Lcn2 as in the GFAP/IKK2-CA model is found in cerebral streptococcus pneumoniae infections or MCAO respectively (Rupprecht et al., 2007; Zamanian et al., 2012).

This gene expression profile, as well as the relatively mild neurological phenotype in the adult mice (no detected alterations except cerebellar ataxia), indicates that the GFAP/IKK2-CA transgenic system does not cause artificial phenotypes resulting from supra-physiological NF-κB activation, but indeed rather reflects the situation in natural (patho-)physiological neuroinflammatory conditions.

4.2 Role of astroglial NF-κB signaling in astrocyte innate immune functions and the induction of neuroinflammation

The present study strengthens the emerging view of astrocytes as key players of the CNS innate immune system beside microglia.

In the classical view, microglia as the resident brain macrophages are the major innate immune cells in the CNS that initiate neuroinflammation. After detection of a potential threat, they are activated, i.e. they start to produce an array of proinflammatory mediators, which in turn recruit and activate peripheral immune cells and induce inflammation related defense programs of the neural tissue (Tambuyzer et al., 2009; Amor et al., 2010; Glass et al., 2010). In this microglia-centered model, astrocytes enhance the inflammatory response initiated by microglia by the response to microglia-derived cytokines. This enhancement of neuroinflammation is achieved by the additional production of inflammatory mediators and the modulation of the BBB. Indeed it is well established that astrocytes can produce a large number of proinflammatory mediators, including chemokines like MCP-1, RANTES and IP-10, major cytokines like TNF-α, IFNγ, IL-1β and IL-6 and cell adhesion molecules like ICAM-1 that are involved in the recruitment and activation of immune cells (Farina et al., 2007; Wilson et al., 2010).

Nevertheless, the view of microglia as exclusive surveillance cells is questionable, as it is also known that astrocytes themselves can sense and respond to DAMPs/PAMPs, i.e. as microglia they are able to detect potential threats (Farina et al., 2007). As many receptors for these signals (e.g. TLRs) activate NF-κB signaling, which in turn induces the transcription of proinflammatory mediators, this model might also be turned around: In the same manner astrocytes might induce a primary proinflammatory response, which activates a secondary microglial response. Indeed the current study strongly supports this additional alternative model. It shows for the first time that astroglial NF-κB activation is sufficient to induce a neuroinflammatory response, which is characterized by secondary microglia activation, the recruitment of cells of the peripheral adaptive immune system and the production of acute phase proteins by astrocytes. The study also highlights the outstanding importance of NF-κB signaling for inflammatory processes. Many of the important proinflammatory factors that are highly upregulated in the GFAP/IKK2-CA model and are most likely responsible for this inflammatory reaction seem to be direct targets of NF-κB

activation in astrocytes.

In particular, several chemokines (MCP-1, RANTES, IP-10) and cell adhesion molecules (Madcam-1, Icam-1) are highly induced by IKK2-CA in astrocytes, and are well known NF-κB target genes (Gupta et al., 2010). These factors are key mediators of leukocyte infiltration into the brain (Szczucinski and Losy, 2007; Wilson et al., 2010), and are most likely also critical for leukocyte infiltration in the GFAP/IKK2-CA model. Also the acute phase factors Lcn2, C3 and Gbp2 that are induced by IKK2-CA in primary astrocytes are described as NF-κB target genes (Wei et al., 2008; Gupta et al., 2010).

This shows that astrocytes via NF-κB activation are able to recruit peripheral immune cells to the site of inflammation and that they can produce several antimicrobial proteins, i.e. they provide a first line of defense molecules against pathogen invasion, and they also initiate certain steps for the induction of an adaptive immune response.

Beside the recruitment of lymphocytes, the adaptive immune response also requires the presentation of target antigens by professional antigen presenting cells via the MHC class II proteins, and for the elimination of infected or neoplastic cells also antigen presentation on the target cells via MHC class I. Interestingly, while under normal conditions MHC expression in the CNS is very low to absent (Wilson et al., 2010), several MHC proteins of both class I and II are among the most prominently upregulated genes in the brains of the GFAP/IKK2-CA animals. This suggests that astroglial NF-κB activation also activates the antigen presentation capabilities of the CNS, to allow the recruited lymphoid cells to rapidly initiate an effective adaptive immune response. This seems to be achieved mainly indirectly by the activation of antigen presenting cells like microglia. Although the MHC class II invariant chain CD74 was found to be upregulated directly in astrocytes, another MHC class II component (H2-Aa) was induced in brain tissue, but not in primary astrocytes. Therefore the current study does not give a clear indication, whether astrocytes can act as professional APCs *in vivo*, as hypothesized previously (Dong and Benveniste, 2001; Carpentier et al., 2005). Indeed, in an *in vitro* antigen presentation assay astrocytes could stimulate proliferation of PLP-specific T-cells after exposure to a PLP peptide in the presence of TNFα and IFNγ, but only much less by stimulation with TLR agonists (Carpentier et al., 2005). The requirement of such a complex stimulation indicates that NF-κB activation alone is likely not sufficient to induce an

APC function of astrocytes. Nonetheless, the present data and the fact, that TNFα is one factor involved in astroglial APC function, might indicate that NF-κB might boost the APC function of astroglia and thus CNS autoimmunity. To clarify this issue, IKK2-CA expressing astrocytes could be studied in a similar *in vitro* antigen presentation assay as described above.

Altogether, these findings suggest that astroglial NF-κB activation is a key switch that sets the CNS immune system in a preactivated alarm state. As NF-κB activation in this model does not induce the major proinflammatory cytokines TNFα, IL-1β and IL-6 in astrocytes, additional signaling pathways or cell types seem to be required for the production of these cytokines to finally activate a full immune response. Indeed, although NF-κB dependent regulation of these three cytokines is described, other signaling pathways as NFAT-, p38- and JNK-signaling seem to be more important in many circumstances (Simi et al., 2007; Yu et al., 2009; Falvo et al., 2010). In addition, microglia, which are an important and maybe dominant source of these cytokines, might need themselves direct stimulation by PRR activation to induce the production of these cytokines (Tambuyzer et al., 2009; Glass et al., 2010). This would argue for a complementary role of astrocytes and microglia and would be in line with the finding that in contrast to primary astrocytes, the brain tissue of the GFAP/IKK2-CA mice shows a moderate elevation of mRNA levels of these cytokines, suggesting an indirect upregulation in microglia or other cell types.
Although these findings do not call into question the well-established role of microglia in the innate immunity of the brain, they emphasize the view that astrocytes are also key players of likely similar importance in this system, with partially equivalent, but also partially complementary functions (Figure 42).
Beside the demonstration of the important global function of astroglial NF-κB activation for the induction of neuroinflammation, the study also gives insights into the role of NF-κB signaling in astrogliosis, which is a common hallmark of neuropathological conditions and is usually regarded as a secondary consequence of neuroinflammation (Sofroniew and Vinters, 2010). The GFAP/IKK2-CA model suggests that neuroinflammation causes astrogliosis by activating astroglial NF-κB, which in an autocrine loop induces the expression of Lcn2, an important marker and mediator of astrogliosis (Lee et al., 2009; Zamanian et al., 2012).

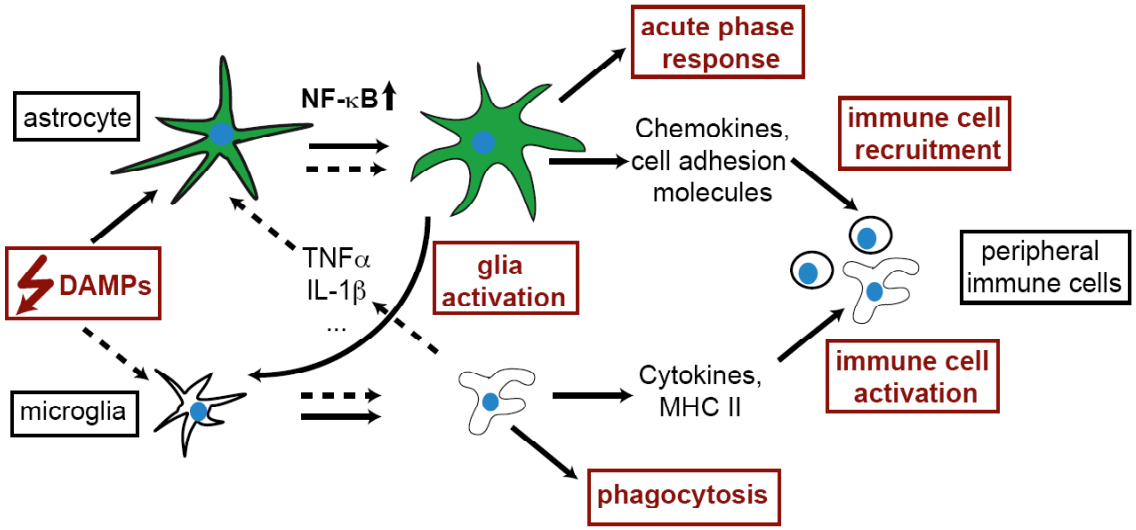

Figure 42: Model of coordinated activation and complementary effector functions of astrocytes and microglia in neuroinflammation

In the classical model of neuroinflammation danger signals (DAMPs) activate microglia, which induce a secondary astrocyte activation by the secretion of inflammatory mediators like TNFα and IL-1β (dashed lines); the GFAP/IKK2-CA model suggests a second additional way of inducing neuroinflammation, in which DAMPs activate astrocytes that in turn induce secondary microglia activation (continuous lines, left side); both cell types seem to have complementary effector functions in the innate immune response (right side): activated astrocytes produce acute phase proteins and factors that recruit peripheral immune cells, whereas activated microglia stimulate the activation of these cells and act as phagocytes.

By contrast, IL-6 and IL-1β, other cytokines that were previously connected to astrogliosis (Correa-Cerro and Mandell, 2007), are only mildly and indirectly upregulated, strengthening the evidence that Lcn2 is in fact a key mediator of astrogliosis. Interestingly, Lcn2 is also implicated in activation and cell death sensitization of microglia, thereby providing one potential mechanism by which astrocyte activation might induce secondary microglia activation (Lee et al., 2007).

The prominent proinflammatory function of astroglial NF-κB signaling found in the GFAP/IKK2-CA model is from a general perspective compatible with the published studies on the GFAP.IκBα-dn mouse model (Brambilla et al., 2005; Brambilla et al., 2009b; Dvoriantchikova et al., 2009). Nevertheless, a direct comparison of the findings in the GFAP/IKK2-CA model with these studies is difficult, as they show a phenotype of blocked NF-κB activation only under pathological conditions. These are required to induce NF-κB signaling in astrocytes, but surely also activate other

signaling pathways and influence the physiology of other cell types. Furthermore, these studies did not distinguish between direct and indirect effects of NF-κB activation, as they only used CNS tissue for their analysis. These different conditions result in marked differences in the proinflammatory gene expression profiles:

In the autoimmune model EAE the major cytokines TNFα and IL-1β are much more prominently induced in the EAE related inflammation than in the GFAP/IKK2-CA model. However the effect of NF-κB inhibition is only moderate, consistently with the finding in the present study, that these cytokines are not directly regulated by NF-κB in astrocytes, but rather by other pathways and/or cells. In the other models, these cytokines show differing induction levels, but again only moderate effects of NF-κB inhibition were observed (less than 3-fold). CCL2/MCP-1 and CXCL10/IP-10 expression is also only partially blocked by the GFAP.IκBα-dn transgene, suggesting that the inhibition of NF-κB is incomplete or other pathways and cell types are important for upregulation of these chemokines in this context. Of note, CCL5/RANTES expression seems to be independent of astroglial NF-κB in the SCI and retinal ischemia model, whereas it is one of the most prominent NF-κB induced genes in the GFAP/IKK2-CA model. The cell adhesion molecule ICAM-1 was consistently moderately upregulated in a partially NF-κB dependent manner in the EAE model and the retinal ischemia model, as also seen in the GFAP/IKK2-CA model. The other highly upregulated genes in the GFAP/IKK2-CA animals were not studied in detail in the GFAP.IκBα-dn studies, although in a microarray screen in the EAE model the complement factors C3 and C1r and several MHC class II genes were deregulated in an astroglial NF-κB dependent manner.

Even more difficult than the comparison of gene expression profiles is the interpretation of the composition of infiltrating cells and other later effects of neuroinflammation, because these are even more dependent on the pathologic stimulus. Of note, in the EAE model, inhibition of NF-κB signaling in astrocytes does not reduce the total amount of infiltrating leukocytes, as would be expected from the GFAP/IKK2-CA model, but is protective rather by shifting the composition of the infiltrating T-cells towards more anti-inflammatory Treg infiltration. In the GFAP/IKK2-CA model a detailed analysis of T-helper-cell subpopulations was not done. Since there are no major Treg populations expected in the control animals, it is unclear, whether there is any corresponding shift to reduced Treg infiltration in the

GFAP/IKK2-CA model, as would be expected from the GFAP.IκBα-dn/EAE model.
In conclusion, the overall increased production of proinflammatory mediators in the GFAP/IKK2-CA model is consistent with the reduced expression in pathological conditions in the GFAP.IκBα-dn model, but the physiological consequences for immune cell infiltration are not comparable, because of a prominent role of NF-κB independent effects in the pathological conditions studied in the GFAP.IκBα-dn model.

4.3 Consequences of astroglial NF-κB activation and neuroinflammation for brain development

Astrocyte related cells serve as NSC and scaffold cells during brain development. These functions are critically dependent on their proper proliferation, migration, differentiation and survival. As inflammatory mediators can interfere with these processes by multiple modes of action, neuroinflammation during brain development often results in severe malformations and functional defects of the CNS. The devastating consequences of developmental neuroinflammation are highlighted in the GFAP/IKK2-CA model. Although transgene expression and the resulting neuroinflammation start around birth, when the major neurogenesis and morphogenesis in most brain regions is already finished, CNS development is clearly impaired in this model, with hydrocephalus formation as the most prominent developmental defect.

4.3.1 Putative mechanisms of hydrocephalus formation in the GFAP/IKK2-CA model

Surprisingly, NF-κB activation in astroglial cells by expression of IKK2-CA results in hydrocephalus formation with high penetrance in early postnatal development, specifically between the onset of transgene expression (around P0 to P2) and P7. Hydrocephalus is a heterogeneous pathology that can be caused by various mechanisms. In the present study, several of these potential mechanisms of hydrocephalus formation in the GFAP/IKK2-CA model were investigated. First, an artifact of overexpression or genomic transgene insertion was excluded as a possible cause of hydrocephalus formation (see 4.1). Second, an obstruction or malformation

of the aqueduct of Sylvius and the subcommissural organ was excluded, which are well established causes of non-communicating hydrocephalus (Rekate, 2008; Huh et al., 2009). In addition, no evidence for fibrotic alterations of the subarachnoid space were found, which are thought to impair CSF resorption in certain types of hydrocephalus, e.g. after cerebral hemorrhagies (Poca and Sahuquillo, 2005).

Another potential cause of hydrocephalus formation are defects of the ependyma, the epithelial-like cell layer that lines the ventricles. These cells possess motile cilia that facilitate the transport of the CSF through the ventricular system, and the absence of these cilia or loss of the complete cells was reported to result in hydrocephalus formation in various mouse models (Huh et al., 2009; Del Bigio, 2010). Indeed in the GFAP/IKK2-CA model a lack of ependymal cilia of the lateral ventricles was found, which is already present at P2, i.e. before the onset of hydrocephalus formation, indicating that this defect is not due damage of the ependyma secondary to hydrocephalus formation, but might have a causative role. It was shown that this lack of ependymal cilia is not caused by the loss of the whole ependymal cells, but that there is probably a defect in cilia formation. Actually, the time of hydrocephalus onset in the first postnatal week corresponds well to the time of ependymal ciliogenesis, which is from P0 to P4 (Spassky et al., 2005). A similar time course of hydrocephalus formation was also found in mice deficient of Celsr2 or Celsr2/3, which have a defect in ependymal ciliogenesis (Tissir et al., 2010). Additional evidence for an impaired cilia formation in the GFAP/IKK2-CA model comes from the observation that hydrocephalus and the lack of cilia can be prevented if the transgene is repressed by doxycycline administration during the critical time period of ependymal ciliogenesis. At later time points of transgene reactivation, these animals do not develop hydrocephalus, although they express the transgene at a similar level and also show neuroinflammation.

While these findings suggest a defect in ependymal ciliogenesis, it cannot be finally excluded that a destruction of the nascent cilia is the reason for hydrocephalus formation. Either there might be a specific sensitivity of the nascent ependymal cilia for destruction by the ventricular macrophages or the lack of ventricular macrophage activation could explain the absence of macrophage mediated destruction of ependymal cilia in animals with delayed transgene activation.

While the present study could show the absence of ependymal cilia as a cellular

mechanism of hydrocephalus formation in the GFAP/IKK2-CA model, most likely because of impaired cilia formation, it could not identify a specific molecular mechanism for this phenotype. A completely cell intrinsic defect of ependymal ciliogenesis might be possible, as ependymal cells express GFAP, dependent on the subpopulation and developmental stage (Jimenez et al., 2001), and consistently subpopulations of ependymal cells were expressing the IKK2-CA transgene. Another possibility would be a non-cell-intrinsic impairment of ependymal ciliogenesis caused by inflammatory mediators that are produced by transgenic astrocytes. Finally, the activated ventricular macrophages could mediate the destruction of the nascent cilia, e.g. via the upregulated complement system, similar to the complement-mediated developmental elimination of synapses by microglia/macrophages (Tahtouh et al., 2009).

Although the molecular mechanism of hydrocephalus formation in the GFAP/IKK2-CA model could not be elucidated and known hydrocephalus related genes were not deregulated in the microarray screen, there are some indirect hints that might link astroglial NF-κB signaling to hydrocephalus formation and ependymal ciliogenesis.
One critical factor for ciliogenesis, whose lack interferes with ependymal ciliogenesis is Foxj1 (Jacquet et al., 2009). In lymphoid cells Foxj1 is able to inhibit NF-κB activation by the induction of IκB proteins (Lin et al., 2004; Lin et al., 2005), thereby its lack might increase NF-κB activation as the IKK2-CA transgene does. Ciliogenesis via Foxj1 can also be disturbed by deregulation of RhoA activation (Pan et al., 2007). In a similar manner, the lack of expression of the myosin familiy protein Myo9a impairs ependymal differentiation mediated by the Rho regulated kinase ROCK (Abouhamed et al., 2009). In the same study it was shown that Myo9a downregulation enhances NF-κB activation in an epithelial cell line, thereby altering the epithelial phenotype. These investigations suggest that cytoskeletal rearrangements via NF-κB and Rho/ROCK signaling are likely critical for ependymal ciliogenesis. Although not experimentally assessed, there are several potential links how astroglial NF-κB activation in the GFAP/IKK2-CA model can disturb the regulation of cytoskeletal dynamics, and in particular the Rho/ROCK pathway. Some of the most prominently deregulated genes in the brain tissue of IKK2-CA expressing mice are Lcn2 and the chemokines CCL5/RANTES, CXCL10/IP-10 and CCL2/MCP-1. These factors were described to signal via the Rho/ROCK-pathway or related Rho-

family proteins, thereby regulating cytoskeletal dynamics and inducing morphological changes and migratory processes (Thelen and Stein, 2008; Lee et al., 2009; Lee et al., 2011a). These cytoskeletal alterations might also interfere with the rearrangements that are required for ependymal ciliogenesis.

Another potential mechanism for hydrocephalus formation beside the impairment of ependymal ciliogenesis programs is the aforementioned macrophage-mediated destruction of ependymal cilia. One putative molecular pathway mediating the destruction of cilia is the complement cascade, that is also implicated in developmental synapse elimination as mentioned above. The ependyma usually protects itself from complement attack by the expression of complement regulators like DAF/CD55, which are upregulated e.g. in case of bacterial meningitis to compensate for the increased complement activation (Canova et al., 2006). However, there might exist conditions with a hyperactivation of the complement system, in which this protective mechanism is overwhelmed, especially if the induction of protective complement regulators is impaired. Indeed, NF-κB activation induces the massive upregulation of C3 and other complement components in the GFAP/IKK2-CA model, which seems not to be accompanied by an prominent upregulation of complement regulators. Second, during neural development, the complement system is prominently activated, as it is involved in developmental synaptic pruning (Tahtouh et al., 2009). This might synergize with the increased complement expression to massively increase the amount of activated complement factors. Evidence that complement activation might contribute to hydrocephalus formation comes from a study which links complement C3 deficiency with decreased intracranial pressure in a mouse model of pneumococcal meningitis, despite an increased bacterial load (Rupprecht et al., 2007). The mechanism of C3 dependent rise of intracranial pressure was not investigated in detail in this study, but others have shown that pneumococcal infections can destroy ependymal cilia (Hirst et al., 2003), suggesting that C3 activation might indeed mediate ependymal cilia distruction.

To finally determine if one of these or even another molecular mechanism is responsible for hydrocephalus formation in the GFAP/IKK2-CA model, further studies are needed, e.g. the combination with genetic models that specifically target the potentially critical pathways.

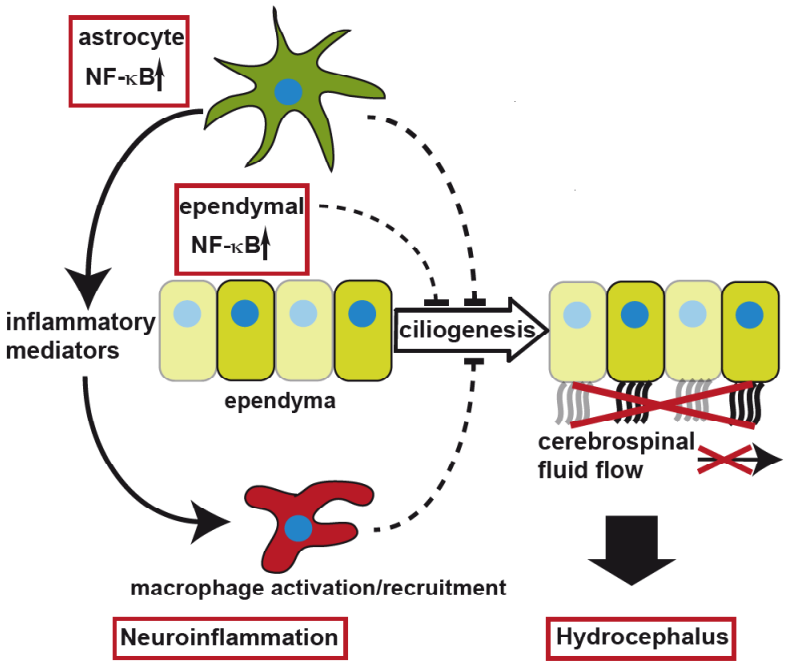

Figure 43: Cellular model of hydrocephalus formation in the GFAP/IKK2-CA mice

NF-κB activation in astrocytes induces inflammatory mediators that cause neuroinflammation, including the recruitment and activation of ventricular macrophages. Activated astrocytes, the secondarily activated macrophages, and/or subpopulations of ependymal cells with constitutive IKK/NF-κB activation interfere with ependymal ciliogenesis by an unknown molecular mechanism. Absence of ependymal cilia impairs CSF flow through the ventricles, resulting in increased CSF pressure and subsequent hydrocephalus formation.

In summary, the characterization of the hydrocephalus formation in the GFAP/IKK2-CA model reveals that NF-κB activation in astrocytes and ependymal cells interferes with ependymal cilia formation, which most likely results in an impaired transport of the CSF through the ventricular system (Figure 43).

4.3.2 The GFAP/IKK2-CA mouse as a model for developmental hydrocephalus formation

Hydrocephalus is very heterogeneous disorder, which can have various causes and pathologic manifestations. Some types of hydrocephalus are congenital genetic disorders, but it is also a frequent complication of neuropathological insults associated with strong inflammatory components such as cerebral infections or hemorrhages. Inflammatory events are associated with NF-κB activation, both as

response and driving force as reported here. Therefore an indirect association of NF-κB signaling with hydrocephalus formation is obvious, although this connection was not studied so far in detail. In fact only one recent study addresses this association, which showed that intraventricular hemorrhages result in nuclear translocation of RelA in ependymal cells in rats, in a paradigm in which also hydrocephalus is induced (Simard et al., 2011). Whereas this work described only a coincidence of NF-κB activation with hydrocephalus formation, our study demonstrates for the first time that NF-κB activation in astrocytes and ependymal cells might have a causative role in certain types of hydrocephalus, in particular hydrocephalus induced by developmental neuroinflammation. Another study indirectly supports a potential causative role of NF-κB signaling in inflammation associated hydrocephalus in a mouse model of pneumococcal menigitis (Koedel et al., 2004), an infectious disease which frequently causes hydrocephalus (Jit, 2010). In this model, intracranial pressure is reduced despite an increased bacterial load when MyD88 is inactivated. MyD88 is a key upstream regulator of the IKK complex in TLR and IL-1 signaling (Hayden and Ghosh, 2008), indicating that impaired NF-κB activation might reduce hydrocephalus formation.

Whether and to what extent astroglial NF-κB activation contributes to hydrocephalus formation in other animal models and in human hydrocephalus remains to be investigated. Various animal models provide several highly differing mechanisms that can cause hydrocephalus, but as these models show marked phenotypic differences and only partially reflect the human pathology, it is not well understood which of these mechanisms might play a role in human hydrocephalus. Non-communicating hydrocephalus can be easily explained by physical blockades of the CSF flow (Rekate, 2008), but in many cases it might be only a secondary event in initially communicating hydrocephalus, as certain animal models like the *hyh* mouse suggest (Wagner et al., 2003).

The mechanisms causing communicating hydrocephalus are much less understood, especially in humans. Inflammation associated fibrosis of the subarachnoid space seems to be important in posthemorrhagic hydrocephalus (Poca and Sahuquillo, 2005). In many human cases, in particular in developmental hydrocephalus, defects of the ependyma, usually a loss of ependymal cells, are frequently observed (Dominguez-Pinos et al., 2005; Sival et al., 2011). These are commonly associated

with the activation of ventricular macrophages (Ulfig et al., 2004; Dominguez-Pinos et al., 2005), as it occurs in the GFAP/IKK2-CA model. In contrast to the GFAP/IKK2-CA model, in the human cases of the aforementioned studies a loss of the entire ependymal cells and not a lack of ependymal cilia was observed. This does not exclude that the cilia themselves are the critical structure responsible for hydrocephalus formation caused by ependymal defects, but rather depicts clear pathological differences between such human cases and the GFAP/IKK2-CA model. However, these studies address only a subset of hydrocephalus types and only a limited number of cases. In addition it was also hypothesized that ependymal cell loss could be a secondary consequence of hydrocephalus formation (Dominguez-Pinos et al., 2005). Overall the relevance of ependymal cilia defects for human hydrocephalus is still under debate. While several animal models clearly show that cilia defects can cause hydrocephalus, ciliopathies in humans are rarely associated with hydrocephalus, and in non-hydrocephalic adult humans frequently a partial loss of ependymal integrity is observed (Huh et al., 2009; Del Bigio, 2010).

These findings highlight that the complex pathogenesis of hydrocephalus most likely cannot be explained by a single mechanism. The results from the GFAP/IKK2-CA model actually suggest that astroglial NF-κB, although probably activated in all cases of inflammation associated hydrocephalus, is only important during brain development as it interferes with cilia formation, but does not induce the destruction of mature cilia. Indeed this might explain in part the increased susceptibility of the developing brain for hydrocephalus formation after inflammatory insults, e.g. in case of congenital toxoplasmosis or lymphocytic choriomeningitis, in which about half of the affected children develop hydrocephalus (Wright et al., 1997; Mack et al., 1999). Ultrastructural alterations similar to the GFAP/IKK2-CA model were indeed found in a model of mumps encephalitis in syrian hamsters, which also displays a highly increased hydrocephalus formation in the early postnatal stage (Takano et al., 1993; Uno et al., 1997). This paradigm further supports the hypothesis that the mechanism elucidated in the present study might be relevant for hydrocephalus formation in CNS infections.

To further clarify the role of astroglial NF-κB signaling and the proposed mechanism in hydrocephalus formation, animals with impaired NF-κB activation in astrocytes should be studied in other inflammation-associated hydrocephalus models. For this purpose, hydrocephalus could be induced in mice with blocked astroglial NF-κB

activation, like the GFAP/IKK2-DN or the GFAP.IκBα-dn transgenic mice, e.g. by intraventricular injection of kaolin, mumps encephalitis or intraventricular bleedings in paradigms that cause hydrocephalus formation (Takano et al., 1993; Uno et al., 1997; Deren et al., 2010; Simard et al., 2011).

4.3.3 Potential mechanisms for hippocampal and cerebellar developmental defects in the GFAP/IKK2-CA model

Beside hydrocephalus formation, there are additional defects in late developing brain regions, in particular the hippocampus and the cerebellum.

Whereas the hippocampus shows a lasting distortion of the dentate gyrus and the CA3 region, which might in part be a consequence of the dilatation of the adjacent lateral ventricles, the development of the cerebellar cortex exhibits a temporary maturation delay. Both the dentate gyrus and the cerebellar cortex at P12 have an appearance that resembles earlier stages of development. In the first case a diffuse structure of the granule layer without organized subgranular zone is observed, in the latter case a thickened external granule layer.

Interestingly, in both regions a prominent postnatal structural reorganization occurs, which depends on the migration of NPCs, a process that among other factors is critically regulated by the CXCL12/CXCR4 chemokine-signaling axis. Defects in this signaling system also cause prominent developmental defects mainly in the cerebellum and the dentate gyrus (Li and Pleasure, 2005; Li and Ransohoff, 2008; Deverman and Patterson, 2009). Although the CXCL12/CXCR4 system does not seem to be affected in the GFAP/IKK2-CA mice, the prominent deregulation of other chemokines might also disturb chemokine-directed migration of NPCs. Indeed CCL2/MCP-1 is known to induce migration and differentiation of SVZ progenitor cells in a stroke model (Liu et al., 2007). Additionally another study detected CCR2, CCR5 and CXCR3 expression on NPCs, which are the main receptors for CCL2/MCP-1, CCL5/RANTES and CXCL10/IP-10 (Tran et al., 2007), the three most prominently upregulated chemokines in the GFAP/IKK2-CA model. Although this study did not address the functions of these receptors on NPCs, it is likely that they are able to induce cell migration of NPCs as known from other cell types.

In summary, the present study suggests that astroglial NF-κB activation seems to influence migration of NPCs, probably by deregulation of chemokine expression,

which was postulated to affect CNS development. This might also be important for adult neurogenesis, which is known to be affected by neuroinflammation (Das and Basu, 2008).

4.4 Consequences of astroglial NF-κB activation and neuroinflammation for CNS homeostasis

The binary tetracycline-regulated transgene expression system allows to study the effects of GFAP/IKK2-CA on the adult organism even though expression of the transgene during development is lethal, because the expression of the transgene can be reversibly blocked by doxycycline. The block of transgene expression up to the age of 4 weeks avoids hydrocephalus formation and other developmental defects and thus allows to investigate the outcome of IKK2-CA induced chronic neuroinflammation at later stages. Of note, despite a prominent inflammatory phenotype, the animals survive for a long time without any severe disease symptoms. The only defect that becomes apparent after around 2 months of transgene reactivation are deficits in motor function. This defect is associated with an atrophy of the cerebellum, a phenotype that resembles the pathology of cerebellar ataxias, a group of rare neurodegenerative disorders.

4.4.1 Influence of GFAP/IKK2-CA induced neuroinflammation on brain function and behavior

Many inflammatory cytokines have a dual role in the immune system and the nervous system. Therefore, neuroinflammation influences brain function as well as behavior, and it is also supposed to be involved in the pathogenesis of psychiatric diseases such as depression. Furthermore, inflammation is known to influence CNS homeostasis and neuronal survival, thereby contributing to neurodegenerative disorders that are characterized by a progressive impairment of brain functions such as motor coordination and memory.

The prominent neuroinflammatory phenotype in the GFAP/IKK2-CA model implicates alterations in brain function and behavior of these mice. Surprisingly, these animals usually do not show obvious behavioral alterations except distinct movement abnormalities.

When motor function was investigated in detail, older GFAP/IKK2-CA mice showed marked impairments of motor coordination and balance, as was shown by the rotarod test at 9 months of age and the beam walking test at 3 and 9 months of age. These impairments did not progress beyond a certain point, i.e. complete limb paralysis or similar symptoms were not observed. Such defects in motor coordination without a complete loss of muscle control are called ataxias and can be caused by an impaired cerebellar function, which is the crucial brain region for coordination and fine-tuning of movements (Apps and Garwicz, 2005; Manto and Marmolino, 2009). Indeed the motor defects in the GFAP/IKK2-CA model are associated with a prominent atrophy of the cerebellum and a corresponding loss of a large fraction of Purkinje cells, which is a typical presentation of many cerebellar ataxias. The motor defects precede macroscopic cerebellar atrophy, which might well be explained by functional impairments of Purkinje cell function before the actual loss of these cells. However, it cannot be excluded that other brain parts might play a role in the manifestation of these early defects.

In contrast to these severe deficits in motor coordination, other brain functions assessed in the present work are not prominently affected in the GFAP/IKK2-CA model. It is well described that neuroinflammation can cause sickness behavior with depression-like symptoms, e.g. decreased activity, reduced interest in the environment and increased fear behavior (Dantzer et al., 2008). Surprisingly, except for a mild reduction in overall activity, neither in the open field test nor in the elevated plus maze test differences in the fear and exploratory behavior of the GFAP/IKK2-CA mice could be detected, arguing for the absence of sickness behavior or a depression-like state. Although the brain regions that control these behaviors were not studied in detail, a lack on inflammation in these regions is rather unlikely, as they are located in the brain stem (Dantzer et al., 2008), which is generally quite strongly affected by the IKK2-CA induced neuroinflammation. More likely, the relatively low induction of IL-1β and TNFα might the absence of classical sickness behavior, since these cytokines seem to be the major mediators of such behavioral alterations (Dantzer et al., 2008).

Another component of inflammation induced sickness behavior is an impairment of learning and memory, which was shown in both animal models and human studies (Yirmiya and Goshen, 2011). In the GFAP/IKK2-CA model learning and memory was investigated in the Morris water maze test. In this assay, no clear differences

between IKK2-CA expressing animals and controls were observed, although the interpretation of these results is complicated by the impaired swimming performance of the GFAP/IKK2-CA expressing animals, which might hide subtle differences. In addition to several other results obtained in the present study, this suggests that neuroinflammation induced by NF-κB activation in astrocytes is relatively mild, despite a prominent inflammatory infiltration, and resembles rather a preactivation of the immune defense systems than a full pathologic neuroinflammatory state. This might be due to the lack of a specific stimulus for the induction of several important proinflammatory cytokines like TNFα, IL-1β and IL-6, which mediate several typical pathological consequences of inflammation. TNFα and IL-1β might also be critical for the learning and memory deficits observed in other models of inflammation, as several studies suggest that directly manipulate these cytokines. Additionally, as moderate physiological levels of these cytokines seem to be required for an optimal learning and memory performance, low grade inflammation might not have any adverse influence on these functions or might even improve them (Yirmiya and Goshen, 2011).

In summary, the performed experiments imply that astroglial NF-κB activation and the resulting neuroinflammation in adult animals have rather mild consequences compared to other models of neuroinflammation. This might be due to the incomplete activation of the inflammatory response because of the minor induction of certain key cytokines like TNFα and IL-1β. Although most brain regions are affected by the GFAP/IKK2-CA induced neuroinflammation, the only remarkable functional impairment is the ataxia phenotype that is a consequence of the Purkinje cell degeneration and cerebellar atrophy, which is discussed in detail in the next sections.

4.4.2 Potential mechanisms of Purkinje cell degeneration caused by NF-κB activation in astrocytes

A wide range of studies link inflammatory processes to neurodegenerative diseases, but in most cases the contribution of neuroinflammation to the pathogenesis and the underlying mechanisms are not well understood. A few paradigms so far directly demonstrated that inflammatory stimuli can indeed induce neurodegeneration *in vivo*, e.g. a PD model in which systemic LPS administration was shown to induce the degeneration of dopaminergic neurons in the substantia nigra (Qin et al., 2007). This

study hypothesized that the high density and strong activation of microglia in the substantia nigra renders these neurons especially sensitive for inflammation-induced degeneration.

The GFAP/IKK2-CA model also shows such a region/cell type specific sensitivity for inflammation-induced neurodegeneration. In this case, inflammatory activation of astroglial cells, including the Bergmann glia, seems to be responsible for the selective non-cell-autonomous degeneration of the Purkinje neurons. Bergmann glia are highly specialized cells, which are critical for the homeostasis of Purkinje cells, as their loss or dysfunction is indeed sufficient to cause Purkinje cell degeneration in several models that specifically target astroglial cells.

One study demonstrated that the ablation of certain subpopulations of GFAP expressing cells including Bergmann glia results in the degeneration of Purkinje cells (Cui et al., 2001). Later, some models with more subtle manipulations of Bergmann glia confirmed this requirement of proper Bergmann glia function for Purkinje cell survival (Custer et al., 2006; Tao et al., 2011; Wang et al., 2011). These and other studies showed a downregulation of the glial glutamate transporters EAAT1/GLAST and/or EAAT2/GLT-1 as in the GFAP/IKK2-CA model, and suggested this as actual cause of the observed Purkinje cell degeneration (Cui et al., 2001; Custer et al., 2006; Perkins et al., 2010; Shiwaku et al., 2010; Tao et al., 2011). In addition, some of these studies directly showed an impaired glutamate uptake, increased excitability and impaired survival of cerebellar Purkinje cells and/or granule cells in co-cultures with Bergmann glia *ex vivo* (Custer et al., 2006; Perkins et al., 2010; Shiwaku et al., 2010; Tao et al., 2011). Consistent with the present study, others also found dark cell degeneration of Purkinje cells in ultrastructural studies as *in vivo* evidence for excitotoxicity (Custer et al., 2006; Perkins et al., 2010; Tao et al., 2011).

These results strongly indicate that the reuptake of glutamate by Bergmann glia is necessary to prevent excitotoxic damage of the Purkinje neurons, which have large a dendrite tree with a lot of glutamatergic synaptic input by the cerebellar granule neurons and the climbing fibers from the inferior olive (Apps and Garwicz, 2005). According to this model, any disruption of the specific Bergmann glia differentiation, structure and function interferes with glutamate uptake from Purkinje cell synapses and can thereby result in their degeneration. The underlying mechanism could be the downregulation of glutamate transporters, the retraction of Bergmann glia processes from the synapses or the loss of the complete cells.

Indeed the wide range of different disorders associated with Purkinje cell degeneration implies multiple mechanisms converge in one final phenotype, which is cerebellar ataxia due to Purkinje cell degeneration. In the case of the GFAP/IKK2-CA model, constitutive NF-κB activation in Bergmann glia and other astroglia results in the transformation from the specific radial glia-like phenotype to an activated astrocyte-like phenotype. The underlying mechanism is probably the massive induction of para- and autocrine mediators of astrogliosis, like in particular Lcn2, but also IL-6 and IL-1β (Correa-Cerro and Mandell, 2007; Lee et al., 2009).

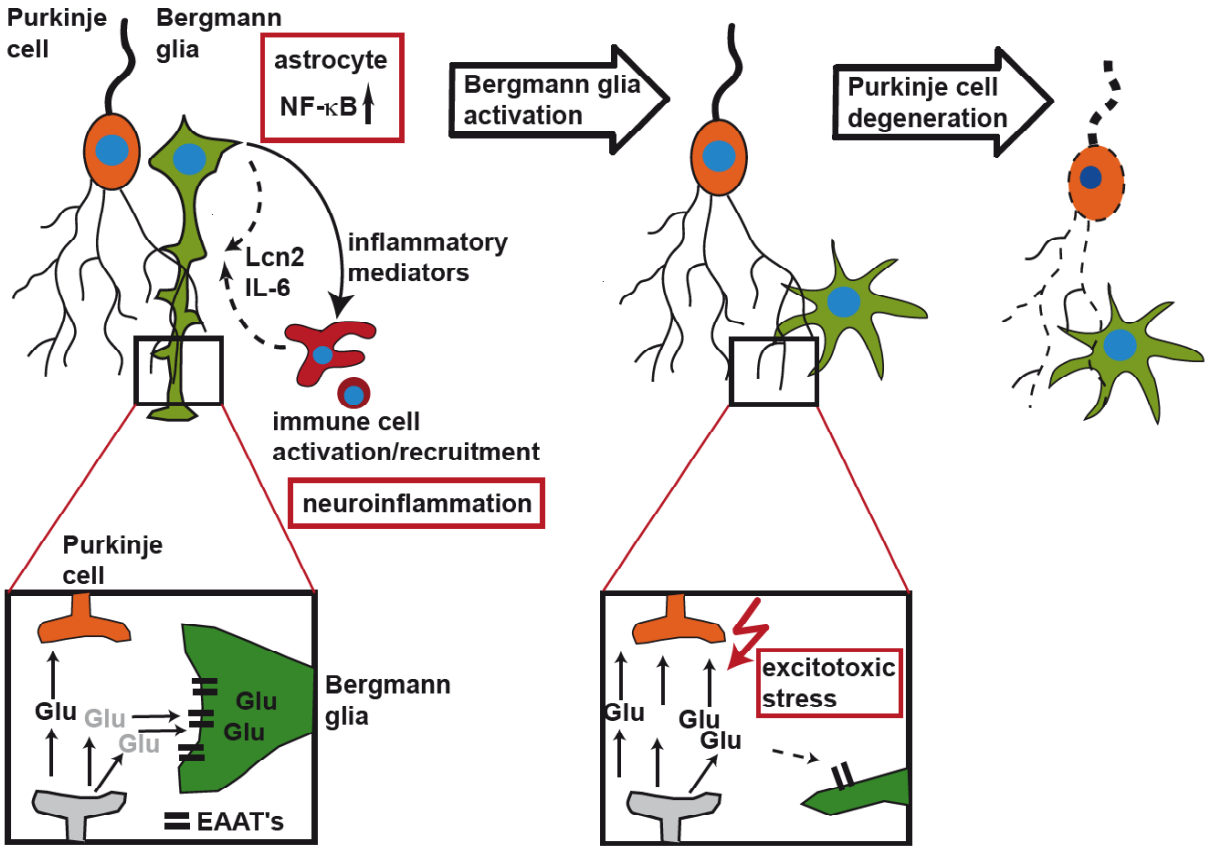

Figure 44: Model of inflammatory excitotoxic Purkinje cell degeneration caused by NF-κB induced Bergmann glia activation in the GFAP/IKK2-CA mice

Astroglial NF-κB signaling induces the expression of proinflammatory factors that cause neuroinflammation including the recruitment and activation of microglia and peripheral immune cells. Activated astrocytes and immune cells produce mediators of astrogliosis like Lcn2 and IL-6 (left side). These mediators induce activation of Bergmann glia, causing morphological changes and a downregulation of glial glutamate transporters (EAAT1/2), which disrupts glutamate uptake by Bergmann glia and results in excitotoxic stress for Purkinje neurons (middle). This finally leads to excitotoxic Purkinje cell degeneration (right side).

This massive morphological change most likely results in the retraction of processes from the Purkinje cell synapses and is accompanied by a downregulation of the glial glutamate transporters EAAT1/GLAST and EAAT2/GLT-1. Although these transporters are described as NF-κB target genes (Kim et al., 2003; Kim et al., 2011) and would therefore be expected to be upregulated, they were shown to be downregulated in various neuroinflammatory conditions and by multiple cytokines, including the NF-κB inducers IL-1β and TNFα (Tilleux and Hermans, 2007). Surprisingly, it was shown that blocking of NF-κB activation prevented the downregulation of EAAT1 and EAAT2 in a model of hypoxia, indicating that NF-κB activation can actually repress glutamate transporter expression under certain conditions (Dallas et al., 2007). For EAAT2, the interaction of NF-κB with N-Myc was described to be responsible for the stimulus dependent downregulation in response to TNFα, but not to EGF stimulation (Sitcheran et al., 2005).

The findings from the current study and the discussed earlier publications suggest a general model how neuroinflammatory insults in the cerebellum could induce Purkinje cell degeneration (Figure 44). Briefly, neuroinflammatory stimuli induce NF-κB activation in astrocytes and Bergmann glia, which results in the recruitment of immune cells and the induction of astrogliosis mediators. These induce the activation of Bergmann glia, resulting in the disruption of their specific structure and the downregulation of their glutamate transporters, leading to excitoxic stress and finally the degeneration of the Purkinje neurons.

4.4.3 The GFAP/IKK2-CA mouse as a model of autoimmune/ autoinflammatory cerebellar degeneration

The GFAP/IKK2-CA model with its strong permanent activation of NF-κB signaling in astrocytes might represent a supraphysiological condition, but a number of findings in both human cases and animal models suggest that the proposed mechanism of inflammation mediated Purkinje cell degeneration might be involved at least in some types of cerebellar ataxias. While for most genetic cerebellar ataxias only little is known about the general mechanisms and the contribution of neuroinflammation and astrogliosis to these diseases, many acquired forms are clearly associated with inflammatory and autoimmune conditions, although the specific role of NF-κB

signaling in these diseases remains to be investigated.

Only a few studies addressed a potential involvement of inflammation in hereditary forms of ataxia. One study showed an upregulation of inflammatory markers in SCA3, while another one demonstrated early astrogliosis with altered distribution of EAAT1 in SCA1 (Evert et al., 2001; Giovannoni et al., 2007). More detailed functional/mechanistic studies are not published to date. Even less is known about the contribution of NF-κB signaling to these diseases, only one study on SCA7 reports an impairment of NF-κB activation in neurons as found in other polyglutamine disorders, which results in a reduced neuroprotection *ex vivo* (Wang et al., 2007).

By contrast, among the acquired ataxias many are associated with inflammatory and autoimmune reactions. Acquired cerebellar ataxias can have diverse inflammation associated causes, such as injury, stroke, infections like neurosyphilis and neuroborreliosis, and superficial siderosis, which is a deposition of iron in pial and subpial regions of the brain, probably caused by repeated subarachnoid bleedings (Manto and Marmolino, 2009; Klockgether, 2010, 2011). Other causes of acquired ataxias such as chronic alcohol abuse or other toxic insults also can have inflammatory components or otherwise influence astroglia. Indeed ethanol can activate NF-κB signaling in cultured astrocytes at concentrations in a range of human blood alcohol levels after alcohol consumption (Blanco et al., 2004). Several autoimmune disorders can also cause cerebellar ataxias with a prominent loss of Purkinje cells, most prominently paraneoplastic diseases. These disorders are frequently associated with antibodies against cerebellar antigens, but can also cause cerebellar atrophy in cases without evident autoantibodies (Dalmau and Rosenfeld, 2008; Klockgether, 2010). Remarkably, experiments with antibodies or T-cells directed against such cerebellar antigens were not able to induce a similar cerebellar ataxia phenotype in animal models (Dalmau and Rosenfeld, 2008; Kazarian and Laird-Offringa, 2011), indicating that the specific adaptive immune response is not required or at least not sufficient to cause Purkinje cell degeneration. This might indicate that the general inflammatory component of the autoimmune response might be more important for the loss of Purkinje cells than the specific antigen recognition by the adaptive immune cells. This hypothesis is supported by the fact that other autoimmune disorders that are not primarily related to the cerebellum can also cause cerebellar ataxia, e.g. celiac disease, systemic lupus erythematosus, Sjögren syndrome or autoimmune thyroiditis (Manto and Marmolino, 2009).

The cerebellar atrophy in the GFAP/IKK2-CA mice argues for a view that NF-κB mediated inflammation and activation of Bergmann glia might be the decisive driving force in autoimmune/inflammatory cerebellar degeneration, but not a specific attack of the adaptive immune system against Purkinje cells. Indeed, in contrast to T-cell or antibody transfer experiments, the cerebellar pathology of the GFAP/IKK2-CA model is quite similar to the human pathology, as far as this can be judged from the few human studies. One study on anti-Yo autoantibody associated Purkinje cell degeneration describes a nearly complete loss of Purkinje cells, a moderate loss of granule cells accompanied by gliotic activation of Bergmann glia and other astrocytes, together with prominent microgliosis and moderate infiltration of T-cells (Giometto et al., 1997), which is remarkably similar to the phenotype of the GFAP/IKK2-CA mice.

Although the phenotypical similarity between the GFAP/IKK2-CA model and human autoimmune/inflammatory cerebellar degeneration has to be clarified further, the present study indicates that the GFAP/IKK2-CA model might be a useful tool to investigate pathogenesis of these disorders, as currently no good animal model for such diseases is available. It also suggests that anti-inflammatory and anti-excitotoxic approaches rather than specific T- or B-cell targeting medications might be promising for the therapy of such diseases. In particular, the specific inhibition of astrogliosis and Bergmann glia activation might be a promising approach for the treatment of these diseases.

However, our study also clearly shows that most likely only a very early intervention might be beneficial, because the repression of IKK2-CA at 3 months of age, i.e. at the onset of Purkinje cell loss, cannot stop the further loss of Purkinje cells. Beside individual differences between specific patients, this might also in part explain the variable effectiveness of the current treatment approaches with anti-inflammatory drugs such as corticosteroids, and plasmapheresis or Rituximab for the elimination of autoantibodies or B-cells respectively (Marmolino and Manto, 2010; Pelosof and Gerber, 2010). New experimental treatment strategies also include anti-excitotoxic drugs such as the glutamate release inhibitor riluzole (Marmolino and Manto, 2010). Such anti-excitotoxic strategies have also been successfully used in animal models of Purkinje cell degeneration after other inflammation associated insults such as hypoxia or cardiac arrest (Kanthasamy et al., 1999; Barenberg et al., 2001), indicating that this might be a broadly applicable approach to treat cerebellar ataxias.

4.5 The GFAP/IKK2-CA mouse as a model for the investigation of the role of neuroinflammation in neurological disorders

When IKK2-CA expression is activated after completed brain development, the GFAP/IKK2-CA mice develop a chronic neuroinflammatory phenotype without obvious disease symptoms except cerebellar ataxia. Similar moderate neuroinflammation without the prominent sickness behavior of strong acute inflammation is found in many CNS disorders, including neurodegenerative diseases and psychiatric conditions (Stolp and Dziegielewska, 2009; Glass et al., 2010; Dean, 2011). In many cases, the connection of the individual disorder with inflammation is purely associative. If there is evidence for a pathogenic or protective role of inflammation, it is often derived from relatively artificial models such as cell culture or from the acute administration of strong inflammatory stimuli such as LPS. The genetic inhibition of inflammatory pathways in models of neurological disorders is another approach to functionally address this issue.

However, so far there are only few approaches to experimentally enhance neuroinflammation, especially to mimic the chronic moderate neuroinflammation that is usually found in CNS disorders. Such models, combined with models of neurological diseases could give valuable insights in the contribution of inflammation to the pathogenesis of these diseases. This might be the intrinsic disease-associated inflammation, but it could also help to clarify how and to what extent inflammation caused by non-symptomatic infections or chronic inflammatory and autoimmune disorders increase disease susceptibility or progression. For example, systemic administration of LPS results in a long term induction of TNFα in the brain and is sufficient to induce a marked loss of dopaminergic neurons in the substantia nigra, suggesting that peripheral inflammatory insults might increase the susceptibility for PD (Qin et al., 2007). Another approach used the GFAP promotor driven expression of CCL2 to induce macrophage recruitment to the brain in an AD model. This leads to an increased Aβ deposition, suggesting that inflammatory events might enhance AD progression (Yamamoto et al., 2005).

For future studies to investigate the link between neuroinflammation and neurological disorders *in vivo*, the GFAP/IKK2-CA model is a valuable additional tool with several advantages compared to the aforementioned models: It induces a moderate chronic

neuroinflammation, which is in many points similar to that in various neurological disorders. In addition, it can be conditionally regulated in a reversible manner, thereby allowing to bypass developmental effects of neuroinflammation. In contrast to the chronic LPS model, it does not affect the whole organism, and it does not require external interventions with possible pathological consequences like several other models that use intracranial application of inflammatory stimuli. Therefore the GFAP/IKK2-CA model of neuroinflammation can be combined with models of neurological disorders without introducing any additional alterations that might complicate the interpretation of the results.

A first study using such an approach was performed and investigated the GFAP/IKK2-CA mice in an MPTP induced model of PD, which showed marked differences to the LPS induced model described above (Oeckl et al., 2012). In contrast to the LPS model and a model which pharmacologically inhibited NF-κB signaling in the MPTP paradigm (Ghosh et al., 2007), no influence of neuroinflammation and NF-κB signaling on the loss of dopaminergic neurons was found in the GFAP/IKK2-CA mice after MPTP intoxication. This indicates that possibly microglia are the more important players in the inflammatory events promoting the degeneration of dopaminergic neurons in the substantia nigra. Another possible explanation is that astrocyte-mediated inflammatory processes are actually important in PD, but the acute toxic type of neurodegeneration in the MPTP does not reflect very closely the cell death mechanisms in PD, where the neurons are slowly degenerating under conditions of chronic stress that might be enhanced by inflammatory conditions. To further clarify this issue, other models of PD should to be investigated in the presence of the GFAP/IKK2-CA transgene system.

In the same manner, the GFAP/IKK2-CA model could be used to investigate the role of neuroinflammation and astroglial NF-κB signaling in other neurodegenerative diseases such as AD or ALS. Although inhibition of astroglial NF-κB in the GFAP.IκBα-SS32/36AA mouse did not show an effect on disease progression (Crosio et al., 2011), it is not clear whether neuroinflammation from ALS independent origin might modify disease pathogenesis, as some studies suggest. Indeed there is evidence from genetic approaches that show that both microglia and astrocytes are involved in ALS pathogenesis (Glass et al., 2010).

In addition, potential depression related symptoms of the GFAP/IKK2-CA mice should be investigated in more detail, as there is mild reduction of overall activity in

these mice, and a study shows that Ido1, one of the key enzymes responsible for cytokine induced depression, can be induced in astrocytes by TLR3 ligands in a NF-κB and IRF3 dependent manner (Suh et al., 2007).

4.6 Conclusions

The present study provides new insights in the function of NF-κB signaling in astrocytes, the role of astrocytes in neuroinflammation and the consequences of neuroinflammation and astroglial NF-κB signaling for CNS development and homeostasis.

It demonstrates for the first time that astrocytes via NF-κB activation do not only enhance neuroinflammation, but are actually able to induce a global neuroinflammatory response that sets the CNS immune defense in a preactivated alarm state. Thereby the study stresses the emerging view that astrocytes are key players in the innate immunity of the CNS that seem to be equally important as microglia, which are traditionally regarded as the major innate immune cells of the CNS.

The study also reveals a critical impact of neuroinflammation on brain development. By demonstrating that astroglial NF-κB activation interferes with ependymal cilia formation, the study provides a potential link between developmental neuroinflammation and hydrocephalus formation. For the future, this could open up new perspectives for the understanding and treatment of hydrocephalus, a frequent complication of disorders with strong neuroinflammatory components, such as brain infections or subarachnoid bleedings, especially during early childhood.

Finally, the present work also gives new insights in the role of inflammation in neurodegenerative diseases. It highlights the special susceptibility of the cerebellar Purkinje neurons for non-cell-autonomous degeneration because of their critical dependence on the support functions of Bergmann glia. It suggests a putative mechanism for the loss of Purkinje cells in autoimmune/inflammatory cerebellar ataxias, a group of rare but devastating neurodegenerative disorders of the cerebellum. In this model, the autoimmune/inflammatory processes in the cerebellum induce an activation of NF-κB signaling in Bergmann glia, leading to their astrogliosis-like activation. This results in an impaired glutamate uptake by Bergmann glia that finally results in excitotoxic Purkinje cell degeneration. As this principle of

inflammation induced astroglial dysfunction is supposed to be a general principle in many neurodegenerative diseases, the GFAP/IKK2-CA mouse model that was characterized in this study might provide a new valuable tool for the investigation of neurodegenerative disorders. This is in particular true for autoimmune/inflammatory cerebellar ataxias, whose pathology is fairly well reflected by this transgenic mouse and for which so far no good animal model is available.

5 Abbreviations

3m	age 3 months
Aβ	Amyloid beta
Ac.-α-Tub	acetylated alpha-tubulin
AD	Alzheimer's disease
Aldh1l1	aldehyde dehydrogenase 1-like 1
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APC	(professional) antigen presenting cell
APC	adenomatous polyposis coli (gene)
Aqp4	aquaporin 4
ATP	adenosine-5'-triphosphate
BBB	blood brain barrier
Bcl-2/-3/…	B-cell lymphoma 2/3/...
BDNF	brain derived neurotrophic factor
C1s/3/4/…	complement factor 1s/3/4/…
CA	constitutive active
Calb	calbindin
CCL	chemokine C-C-motif ligand
CCR	chemokine C-C-motif receptor
CD11b/45/…	cluster of differentiation
cDNA	complementary DNA
CNS	central nervous system
CNTF	ciliary neurotrophic factor
Co	control
COX-2	cyclooxygenase 2
Cp	crossing point
Cp	Ceruloplasmin
CSF	cerebrospinal fluid
CXCL	chemokine C-X-C-motif ligand
CXCR	chemokine C-X-C-motif receptor
DAMP	danger associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole

DEPC	diethylpyrocarbonate
DG	dentate gyrus
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DN/dn	dominant negative
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-triphosphate
Dox	doxycycline
DTT	Dithiothreitol
E18.5	embryonic stage/day 18.5
EAAT1/2	excitatory amino acid transporter 1/2 (GLAST/GLT-1)
EAE	experimental autoimmune encephalitis
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGL	(cerebellar) external granule layer
ER	endoplasmatic reticulum
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum
Gbp	guanylate-binding protein
GFAP	glial fibrillary acidic protein
GFAP/IKK2-CA	GFAP.tTA x (tetO)7.IKK2-CA transgenic mice
GLAST	glutamate aspartate transporter (EAAT1)
GLT-1	glial glutamate transporter 1 (EAAT2)
Glu	glutamate
HBSS	Hank's balanced salt solution
HD	Huntington's disease
hIKK2	human IKK2
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IDO	indoleamine-2,3-dioxygenase
IκB	inhibitor of NF-κB
IKK	inhibitor of NF-κB kinase

IKK2-CA	constitutively active IKK2
iNOS	inducible nitric oxide synthetase
IL-1/2/…	interleukin 1/2/…
IFNα/β/γ	interferon alpha/beta/gamma
IP-10	interferone gamma-induced protein 10 (CXCL10)
Lcn2	Lipocalin 2
LPS	Lipopolysaccharide
LTD	long-term depression
LTP	long-term potentiation
LV	lateral ventricles
Madcam	mucosal vascular addressin cell adhesion molecule
MAPK	mitogen activated protein kinase
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemotactic protein 1 (CCL2)
MHC	major histocompatibility complex
min	minute
MnSOD	manganese superoxide dismutase
MPTP	1-methyl-4-phenyl-1,2,3,6-terathydropyridine
mRNA	messenger-RNA
MS	multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NEMO	NF-κB essential modifier
NeuN	neuronal nuclei
NF-κB	nuclear factor κB
NGF	nerve growth factor
NO	nitric oxide
NPC	neural progenitor cell
NSC	neural stem cell
P0/2/7/...	postnatal day 0/2/7/...
PAGE	polyacrlyamide gel eletrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PC	Purkinje cell
PCR	polymerase chain reaction

PD	Parkinson’s disease
PE	Phycoerythrin
PFA	paraformaldehyde
PLP	proteolipid protein
PMSF	phenylmethanesulfonylfluoride
PRR	pattern recognition receptor
qPCR	quantitative realtime-PCR
RANTES	regulated upon activation normal T-cell expressed and secreted (CCL5)
RIPA	radioimmunoprecipitation assay
RLU	relative lightunits
RNA	ribonucleic acid
rpm	rounds per minute
ROS	reactive oxygen species
RT	room temperature
SOD1	superoxide dismutase 1
SCA	spinocerebellar ataxia
SCI	spinal cord injury
SCO	subcommissural organ
SD	standard deviation
SDF-1	stromal cell-derived factor 1 (CXCL12)
SDS	sodiumdodecylsulfate
SEM	scanning electron microscopy
s.e.m.	standard error of the mean
TBI	traumatic brain injury
TBS	tris buffered saline
TEM	transmission electron microscopy
tetO	tet operator/tetracycline responsive element
Tg	transgene
TGF-β	transforming growth factor beta
Th/T_H	helper-T-cell
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor alpha receptor
Treg	regulatory T-cell

tTA	tetracycline controlled-transactivator
UBD	ubiquitin binding domain
UPL	Universal Probe Library (Roche)
UV	ultraviolet irradiation
VCAM	vascular cell adhesion molecule
wks	weeks

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Erklärung

Ich versichere hiermit, dass ich die Arbeit selbstständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich und inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

Ulm, den .....

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Acknowledgements

I want to thank all who supported me to let me reach this point where I am now.

I thank Prof. Dr. Thomas Wirth for giving me the opportunity to work on this thesis at the Institute of Physiological Chemistry, for the supervision of this work and for his great support and suggestions.
Further, I also want to thank Prof. Dr. Jan Tuckermann for agreeing to revise/evaluate this thesis.
I am deeply grateful to Dr. Bernd Baumann for giving me the opportunity to work on this interesting topic, for always letting me work freely to realize my own ideas, and for his great support whenever I needed help.

I want to thank all the other people who were involved in this project, gave suggestions and and helped me with a lot of technical work, especially:

- Prof. Dr. Paul Walther and the technical staff of the Central Facility for Electron Microscopy for the help with the electron microscopy experiments
- Dr. Karlheinz Holzmann and the staff of the Microarray Facility for the help with the gene expression microarray experiment
- Ute Leschik, Bianca Ries, Melanie Gerstlauer for the excellent technical assistance
- Alexander Magnutzki, who performed some initial studies on the adult phenotype of the GFAP/IKK2-CA mice for his master thesis

I thank all my lab colleagues and the former and present members of the Institute of Physiological Chemistry for all the help and the good atmosphere.

I want to thank my brother for checking again the final version of the thesis.

Finally I want to thank my mother, my brother and all the friends who were at my side during the last years, and those who had an earlier part in bringing me to this point, my former teacher Dr. Helmut Moßner and my father, who unfortunately could not see where this way led me now.

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Publications

Lattke M, Magnutzki A, Walther P, Wirth T, Baumann B (2012)
NF-κB activation impairs ependymal ciliogenesis and links neuroinflammation to hydrocephalus formation.
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J Neurosci 32:5688-5703.

Poster Presentations and Talks

Lattke M, Magnutzki A, Walther P, Wirth T, Baumann B
IKK2 activation in astrocytes initiates neuroinflammation resulting in impaired brain development and homeostasis
Poster presentation
Keystone Meeting “NF-kappaB Signaling and Biology: From Bench to Bedside” (2012)
Whistler, Canada

Lattke M, Magnutzki A, Walther P, Wirth T, Baumann B
IKK2 activation in astrocytes initiates neuroinflammation resulting in impaired brain development and homeostasis
Poster presentation
6. Nachwuchswissenschaftler-Meeting der GBM Studiengruppen “Biochemische Pharmakologie und Toxikologie” und “Rezeptoren und Signaltransduktion” (2012)
Günzburg, Germany

Lattke M, Magnutzki A, Wirth T, Baumann B
Astrocyte-specific IKK2 activation induces neuroinflammation and results in hydrocephalus development
Poster presentation
10th International Congress of Neuroimmunology (2010)
Sitges, Spain

Lattke M, Wirth T, Baumann B
Astrocyte-specific IKK2 activation is sufficient to induce neuroinflammation and results in impaired brain development
Short talk and poster presentation
Keystone Meeting “NF-kappaB in Inflammation and Disease” (2010)
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Functional analysis of the IKK2/NF-κB-system in astrocytes

Poster presentation

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Poster presentation

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