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GENE EXPRESSION PROFILING AFTER EXPERIMENTAL PERINATAL ASPHYXIA AND THE EFFECTS OF COMPLEMENT DERIVED ANAPHYLATOXIN C3a

PROFILOVÁNÍ GENOVÉ EXPRESE PO EXPERIMENTÁLNÍ PERINATÁLNÍ ASFYXII A ÚČINKY ANAFYLATOXINU C3a ODVOZENÉHO Z KOMPLEMENTU

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Hradec Králové 2009 **Hana Šourková**

„Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.“

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ABSTRACT

Background: The complement system is involved in neuroprotection and brain repair after brain damage. To understand the molecular mechanisms of these processes, we performed gene expression profiling using quantitative real-time polymerase chain reaction (qPCR), which is the most accurate modern strategy for gene expression analysis.

Project: Our project was directly aimed at expression profiling of selected genes potentially involved in loss and rescue of neural tissue during three weeks after hypoxic-ischemic brain injury, an experimental model of perinatal asphyxia. Recent experiments have shown that over-expression of C3a under the control of the GFAP promoter (C3a/GFAP) reduced hippocampal injury after left common carotid artery ligation in neonatal mice by 50%, compared to wild type mice. Here, we assessed how the local expression of C3a/GFAP transgene affects gene expression profiles. Gene expression was measured on samples from hippocampus ipsilateral and contralateral to the injury and ipsilateral part of cortex, taken at the time of injury, 6 and 24 hours; 3, 7 and 21 days after the injury.

Results: Our data showed that the regulation of gene expression after hypoxic-ischemic injury differs in timing and intensity and may also be region dependent. The analysed genes belong to families related to neuromediator release and deactivation (SYN II, SYN III, GS), proliferation (NES, SOX2, DMN), apoptosis (Bax, Bad, BclXL, 14-3-3 eta), neuronal maturation (ENO2, TubB3), microglia activation (Aif 1, CD68 antigen); to families of inflammatory cytokines (Il1β, Il6, TNFα), complement anaphylatoxin receptors (C3aR, C5aR, C5L2), growth factors (GAP43, NGF) and genes related to the transgene function (C3a/GFAP, GFAP, C3).

Conclusion: Our study showed selected processes of the brain response to the hypoxic-ischemic injury within three weeks after the insult, which might help to uncover potential therapeutic targets for reducing brain damage.

We showed slight differences in trends in expression of the complement receptors and inflammatory cytokines. However, our study did not find any significant differences in gene expression profiles of C3a/GFAP over-expressing mice and wild type mice and as such, we still do not understand the mechanisms involved in C3a neuroprotection.

ABSTRAKT

Úvod: Komplement se účastní ochrany a reparace mozku po jeho poškození. Pro porozumění molekulárním mechanismům těchto procesů jsme provedli profilování genové exprese s využitím kvantitativní polymerázové řetězové reakce v reálném čase (qPCR), která je jednou z nejpřesnějších moderních strategií pro analýzu genové exprese.

Projekt: Projekt byl zaměřen na profilování exprese vybraných genů potenciálně ovlivňujících ztrátu a ochranu neuronální tkáně, a to během tří týdnů po hypoxicko-ischemickém poškození mozku, modelujícím perinatální asphyxii. Nedávné experimenty ukázaly, že exprese C3a pod kontrolou GFAP promotoru (C3a/GFAP) zmenšila poškození hipokampu po ligaci levé karotidy u novorozených myší o 50% ve srovnání s kontrolními myšmi. Projekt rovněž posuzuje jak místní exprese C3a/GFAP transgenu ovlivňuje expresi vybraných genů. Exprese genů byla analyzována na vzorcích z ipsilaterální a kontralaterální části hipokampu a ipsilaterální části mozkové kůry, které byly získány v čase poranění, 6 a 24 hodin; 3, 7 a 21 dní po něm.

Výsledky: Výsledky ukázaly, že se regulace genové exprese po hypoxicko-ischemické příhodě liší v čase a intenzitě a může být také odlišná v příslušných oblastech mozku. Zkoumané geny lze zařadit do rodin se vztahem k uvolnění a deaktivaci neurotransmiteru (SYN II, SYN III, GS), proliferaci (NES, SOX2, DMN), apoptóze (Bax, Bad, BclXL, 14-3-3 eta), maturaci neuronů (ENO2, TubB3), aktivaci mikroglií (Aif 1, CD68 antigen); do rodin prozánětlivých cytokinů (Il1β, Il6, TNFα), receptorů pro anafylatoxiny komplementu (C3aR, C5aR, C5L2), růstových faktorů (GAP43, NGF) a genů ve vztahu k funkci transgenu (C3a/GFAP, GFAP, C3).

Závěr: Naše analýza poodhalila vybrané procesy v odpovědi mozku na hypoxicko-ischemickou příhodu během tří týdnů po zásahu, čímž by mohla pomoci poodkrýt potenciální terapeutické cíle omezující poškození mozku. Naše data naznačila rozdílné tendece v expresi receptorů pro anafylatoxiny komplementu a prozánětlivé cytokiny, nicméně nenalezla žádné významné rozdíly v profilech normálních myší a myší exprimujících C3a/GFAP transgen, a tudíž neuroprotektivní mechanismus C3a anafylatoxinu neobjasnila.

ABBREVIATIONS

14-3-3	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein
Aif 1	allograft inflammatory factor 1
Bad	BCL2-associated agonist of cell death
Bax	BCL2-associated X protein
Bcl2	B-cell leukemia/lymphoma 2
BclXL	BCL2-like 1
C3	complement component 3
C3a	complement derived anaphylatoxin C3a
C3a/GFAP	transgene expressing C3a under the control of GFAP promoter
C3a/GFAP	mice expressing C3a/GFAP transgene
C3aR	complement component 3a receptor 1
C5aR	complement component 5a receptor 1
C5L2	receptor for C5a, C3a complement derived anaphylatoxins
CD68	CD68 antigen
cDNA	complementary DNA
Ct	cycle of threshold
Cl Hip	contralateral hippocampus
DMN	synemin, desmuslin
Eno2	neuronal enolase
GAP43	growth associated protein 43
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
HI	hypoxia-ischemia
IFs	intermediate filament proteins
Il1β	interleukin 1β
Il6	interleukin 6
Ip Cx	ipsilateral part of brain cortex
Ip Hip	ipsilateral hippocampus
MCAO	middle cerebral artery occlusion
mRNA	messenger RNA
NES	nestin

NGF	nerve growth factor
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RT	reverse transcription
Sox2	SRY (sex determining region Y)-box 2
SYN III	synapsine III
SYN II	synapsine II
Tm	melting temperature
TNFα	tumor necrosis factor α
TubB3	tubulin-β3
Wt	wild type mice

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1. INTRODUCTION

Neurological disorders affect millions of people worldwide, but current treatment is still inadequate. Perinatal asphyxia is a hypoxic-ischemic complication that occurs during child birth. The resulting brain damage is a major cause of acute mortality and chronic disability in infants and children [1]. Statistics suggest an incidence of systemic asphyxia in 2-4 out of 1000 full-term infants [2]. For these children, novel strategies for brain repair that target regeneration, plasticity and rehabilitation are needed.

Brain injury, is associated with a number of processes, ranging from days to weeks [1, 3-5] that include oxidative stress, excitotoxicity, inflammation, cell death and lastly, regeneration and repair. The outcome after injury depends on the severity of the insult, on the region, which is affected, and also on the age at which it occurs [1]. In order to develop safe and effective treatments it is necessary to fully understand these processes and their temporal patterns.

Complement is a component of the humoral immune system. Activation of the complement cascade leads to the formation of C3-convertase, an enzymatic complex that cleaves the central molecule of the cascade, the third complement component (C3). This cleavage generates C3a, a fragment with anaphylatoxic properties. In the CNS, astrocytes, microglia and neurons are able to produce most of the complement proteins [6]. In the adult mammalian CNS complement has been shown to promote both basal and ischemia-induced neurogenesis [7]. Recently, a 50% reduction in neuronal loss after experimental perinatal asphyxia was shown (unpublished) in mice expressing C3a/GFAP compared to wild type mice, suggesting that C3a might be an important therapeutic target in perinatal asphyxia and that further understanding of its role in brain repair may facilitate the development of a novel treatment paradigm.

Gene expression profiling is a technique that uses quantitative real-time polymerase chain reaction (qPCR, one of the most sensitive high throughput applied methods) to quantify mRNA levels. In general, mRNA levels may correlate to protein levels and as such can be used to monitor cell functions. Thus, gene expression profiling can be used to understand changes in cellular processes following an insult and might help to understand the role of genes and their functions.

2. BACKGROUND

2.1 HYPOXIC – ISCHEMIC NEONATAL BRAIN INJURY

2.1.1 HYPOXIC-ISCHEMIC EXPERIMENTAL MODEL OF PERINATAL ASPHYXIA

Various models of ischemic brain injury are used for research experiments. The model of perinatal asphyxia is often performed by unilateral common carotid artery ligation followed by systemic hypoxia caused by inhalation of low levels of oxygen usually in an oxygen-nitrogen mixture [2]. In rodents it is performed around postnatal day 7, which represents histologically similar stage of brain development to that of a 32-34 week gestation human fetus or newborn infant [2].

The damage is largely restricted to the cerebral hemisphere ipsilateral to the common carotid artery occlusion and it is most apparent in cerebral cortex especially in the distribution regions of the middle cerebral artery (Fig. 1), in hippocampus, striatum and thalamus [2, 8].

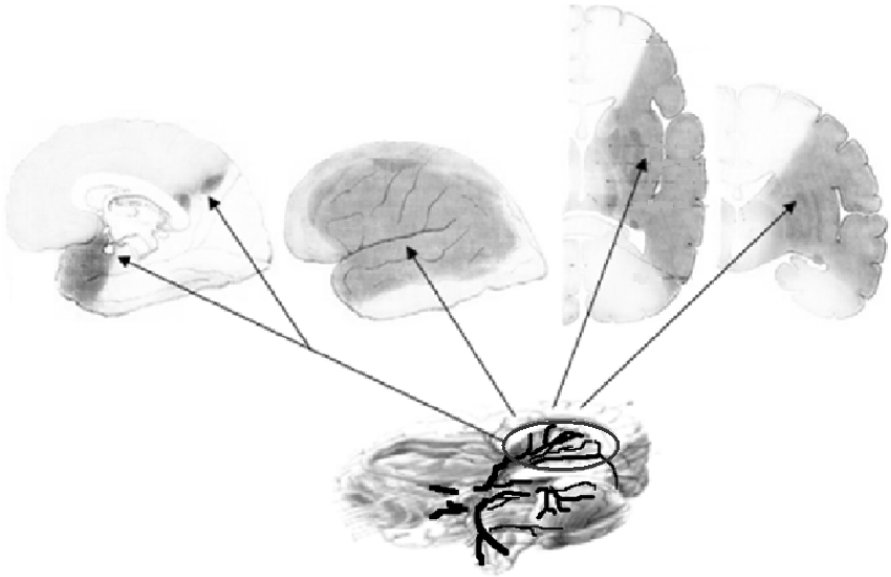

Fig. 1 Regions with blood supply by middle cerebral artery [9] (lateral view from inside of hemisphere, outside cortex and cranial deep inside structure)

2.1.2 MECHANISMS OF BRAIN RESPONSE TO PERINATAL ASPHYXIA

The response to brain injury consists of a number of processes, including cell death, inflammation, excitotoxicity, oxidative stress and brain repair. The response can last up to weeks following the original insult (Fig. 2).

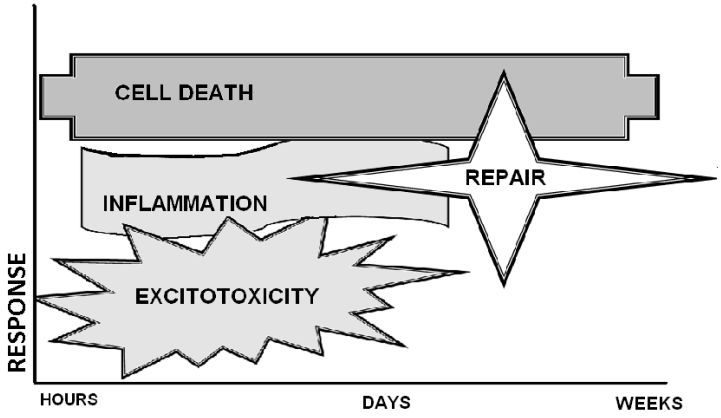

Fig. 2 Time line of the response mechanism after brain injury: excitotoxicity, inflammation, cell death and repair [1]

2.1.2.1 EXCITOTOXICITY

Excitotoxicity is caused by over-stimulation of neurons by excitatory neurotransmitters which may lead to neuronal death [1, 10]. Glutamate is the major excitatory neurotransmitter in the CNS important for development of brain damage [11-13].

After a hypoxic-ischemic insult, cerebral metabolism becomes anaerobic, which is less efficient and causes a rapid depletion of energy reserves [12]. Due to the lack of energy, transcellular ion-pumps are not able to maintain ionic gradients. The reduction of ionic gradients leads to loss of membrane potential and spontaneous depolarization followed by neurotransmitter release [12] (Fig. 3).

The release of glutamate allows a large increase in cytosolic Ca^{2+} [14] and causes mitochondrial calcium overload following by cessation of energy production.

Thus the excessive release of glutamate amplifies the lack of energy (Fig. 3) [12] and triggers activation of many deleterious enzymes by intracelullar Ca^{2+} increase.

Experimental data indicates that the excitotoxicity (the excessive activation of glutamatergic neurotransmission) leads to cell death [1] and the blockade of glutamatergic receptors has been shown to be neuroprotective in hypoxic-ischemic injury [11].

The initial excitotoxic trigger may be reduced by astrocyte up-take of glutamate [1] and its deactivation by glutamine synthetase (GS) (Fig. 3). Glutamine synthetase is an aminotransferase that converts ammonia and glutamate into glutamine [15].

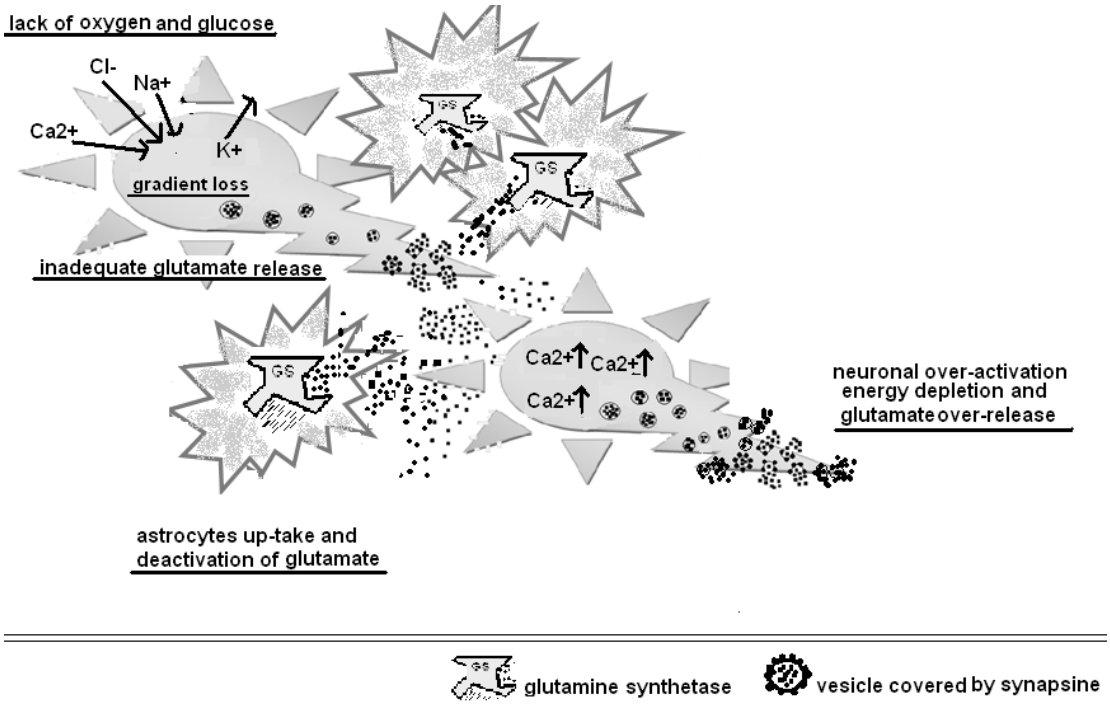

Fig. 3 The lack of oxygen and glucose followed by depolarization causes an increase of glutamate release, which amplifies the deficit of energy. Glutamate is released from synapsine covered vesicles via fusion of the vesicles with the presynaptic membrane [16] and may be up-taken by astrocytes.

2.1.2.2 INFLAMMATORY CYTOKINES

Cytokines are small soluble glycoproteins produced by one cell that alter the behavior or properties of another cell [17].

Inflammatory cytokines Il1β, Il6 and TNFα and their receptors are present in the CNS [18]. Cytokines have been implicated in the pathophysiology of focal cerebral ischemia and the levels of Il6, Il1β, TNFα have been shown to increase after brain ischemia [18-21].

Inflammatory cytokines seem to play both harmful and beneficial roles [12].They have been shown to contribute to infarct progression in the post ischemic period [19, 22, 23], while other studies show neuroprotection. TNFα protects neurons against metabolic and excitotoxic insults in vitro [24], and TNFα receptor knockout mice were shown to have increased neuronal cell degeneration following ischemia and excitotoxic injury, suggesting a critical role for endogenous TNFα in regulating the cellular response to brain injury [12, 25]. Administration of exogenous IL-6 following ischemia produced by middle cerebral artery occlusion results also in marked neuroprotection [12, 26].

2.1.2.3 CELL DEATH

The mechanism of neuronal cell death following hypoxia–ischemia includes neuronal necrosis and apoptosis [12, 22]. Cell death depends on the affected region of the brain and the severity of the insult [1, 3]. Neurons may also die acutely in ischemic core by necrosis but in a more delayed apoptotic fashion in the surrounding penumbra [8, 22]. There is a suggestion that apoptosis plays a prominent role in the evolution of hypoxic-ischemic injury in the neonatal brain and may be more important than necrosis after injury [1, 5].

Cell dying by apoptosis degrades its own DNA according to an internal program of self-destruction. Apoptosis is an energy dependent process, distinguishable from necrosis, by the presence of cell shrinkage, chromatin condensation and genomic fragmentation, while the membrane is relatively preserved with the absence of an inflammatory response [12, 22].

Apoptosis or programmed cell death is regulated by proteins of the Bcl2 family [22, 27]. This family includes pro-apoptotic and anti-apoptotic members. The pro-apoptotic members such as Bad and Bax promote apoptosis by opening of the permeability transition pore, decreasing in the mitochondrial membrane potential and the release of cytochrome c into the cytoplasm followed by activation of caspase 9 and directing to apoptotic cell death [14, 27-30]. An increase of anti-apoptotic proteins such as BclXL, Bcl2 and 14-3-3 can reduce the degree of hypoxia-ischemia induced cell death by sequestration of pro-apoptotic members [4, 27, 31, 32].

Thus the relation of Bax and Bad and their anti-apoptotic homologues Bcl2,14-3-3 and BclXL seems to be a critical determinant of relative resistance of cells to apoptotic cell death [14, 22, 27].

2.1.2.3 CELLULAR RESPONSE TO THE BRAIN INJURY

Various cell types are needed for a brain repair after an insult. Their response to the injury may be performed by activation, inhibition or changes in the differentiation process.

STEM CELLS

Neural stem cells (Fig. 4) can be defined as self-renewing and multipotent cells, which make them capable for replication and giving rise to the three main cell types found in the CNS: neurons, astrocytes and oligodendrocytes [33, 34]. In adult brain there are two areas of maintaining neural stem cells: dentate gyrus of hippocampus and subventricular zone [33, 35-37]. They provide a possibility to contribute to post-injury brain regeneration and repair.

The stem cell stage can be characterized by expression of SOX2, nestin and GFAP. It is followed by a more differentiated progenitor stage (Fig. 4). Progenitor cells have still a possibility to undergo several cycles of proliferations but their ability to differentiate is limited. The development of functional cells from progenitors also involves several stages of differentiation, including expression of tubulin-β3 and neuronal enolase in neurons and GFAP and glutamine synthetase in astrocytes [33, 37-40].

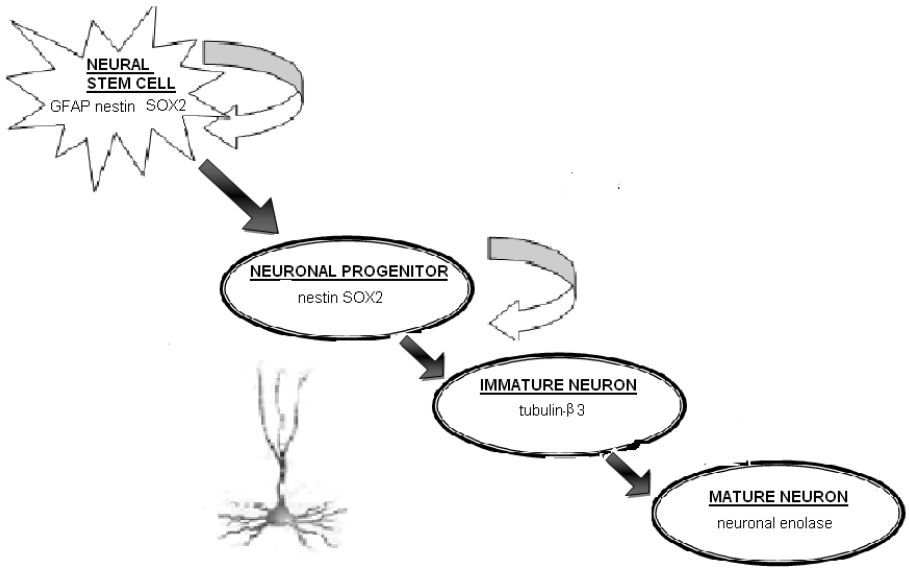

Fig. 4 Process of development of neuron from stem cell includes several cell stages, which may be characterized by activation of specific genes.

ASTROCYTES

Astrocytes are the most common cell type in the CNS [33]. Their essential role in the adult brain is regulating the chemical content of the extracellular space [41]. Astrocytes also produce a large number of growth factors and other molecules, which can affect other cell types in their morphology, proliferation and differentiation [33, 42].

Astrocytes become reactive after an injury during a process known as reactive astrogliosis. They become hypertrophic and up-regulate intermediate filament proteins, GFAP, vimentin and nestin. Reactive astrocytes differentially express hundreds of genes compared to resting astrocytes [33]. Apart from intermediate filaments, these include glutamine synthetase, inflammatory cytokines and growth factors [33, 43], which are all involved in the response to injury.

Reactive astrocytes play a beneficial role at an early stage after neurotrauma, when they help to mediate excess glutamate levels, but at a later stage, astrocytes and other glial cells accumulate in the area of injury and give rise to a glial scar, which is a major impediment of axonal regeneration [22, 33, 35].

The stages of development and reactivity of astrocytes might be also distinguished by intermediate filament proteins (IFs) presence [33]. Nestin and synemin form IFs in astrocyte precursors and in immature astrocytes [44]. In maturing astrocytes, the expression of GFAP increases while nestin disappears [33, 45]. Reactive astrocytes up-regulate GFAP and re-express nestin and synemin [33, 45-47]. The glutamine synthetase also increases during maturation [48] and activation [49].

MICROGLIA

Microglia are considered the immune cells in the brain. Microglia are activated within hours after an injury and within days can transform into macrophages [22]. After an injury they remove debris from dying neurons and glia [22, 41]. Microglia have also been shown to play a positive role in neurotrophin production in response to complement derived anaphylatoxin C3a induction after an insult [50]. It has been shown in a model of spinal cord injury, that activated microglia express Aif 1, while further activation leads to the expression of CD68 antigen, which can cause axonal retraction [51].

2.1.3 COMPLEMENT

The complement system is a component of the humoral immune system involved in inflammation, opsonization, and cytolysis [7, 35]. Complement is made up of a large number of distinct plasma proteins [17] functioning as enzymes, enzyme inhibitors, or enzyme cofactors.

The complement activation involves a cascade of proteolytic steps in which an inactive precursors protein is cleaved to yield active molecules. There are three pathways by which the effector functions of complement can be activated (Fig. 5). The classical pathway is triggered by antibody binding to an antigen. The lectin pathway is initiated by binding of a serum lectin to mannose containing proteins or to carbohydrates in bacteria or viruses. The alternative pathway is initiated when a spontaneously activated third complement component (C3) binds to the surface of a pathogen. The alternative pathway also provides an amplification loop for the classical pathway (Fig. 5) [17].

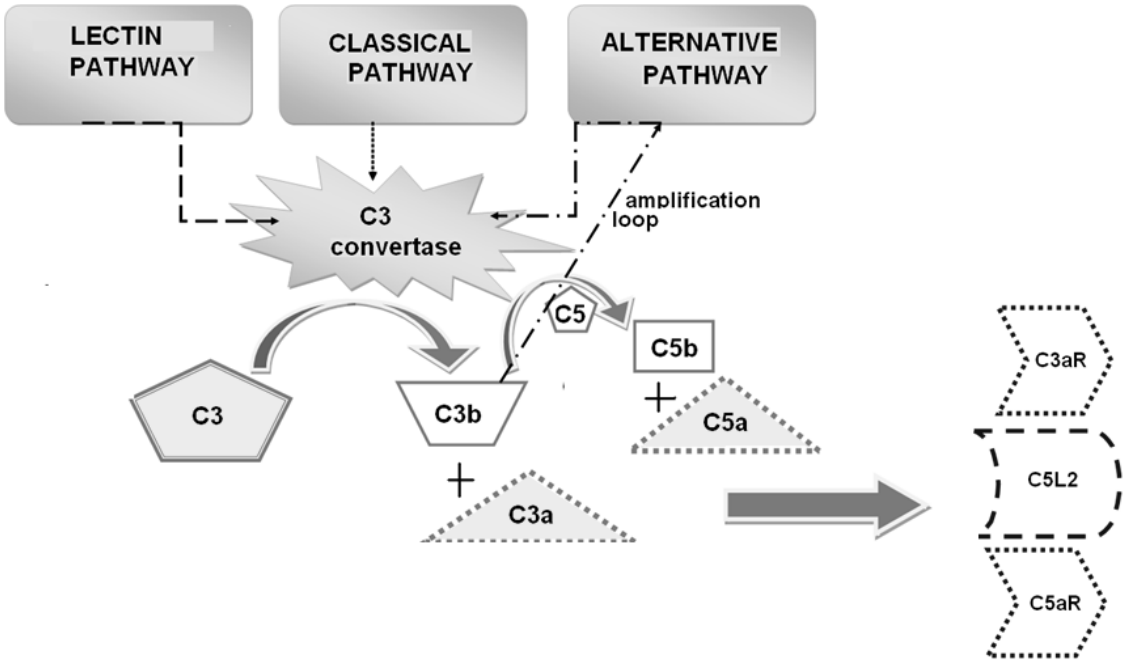

Fig. 5 Pathways of complement activation leading to the formation of complement anaphylatoxins C3a and C5a, which are ligands for the receptors C3aR, C5L2 and C5aR.

Complement activation results in the formation of C3-convertase, an enzymatic complex that activates the central molecule of the cascade, C3 (Fig. 5*).* The proteolytic cleavage of C3 generates C3a, a small fragment with anaphylatoxic properties (see 2.1.3.1) and C3b. C3b can bind to the surface of the microbial cell or to the antibody to act as a component of the C5 convertase that can trigger the terminal part of the cascade. This in turn generates C5a anaphylatoxin through the proteolytic activation of C5 (Fig. 5) and culminates in the assembly of the cytolytic membrane attack complex on the target surface [35].

2.1.3.1 C3a PEPTIDE

The proteolytic release of C3a is one of the major biological consequences of complement activation (Fig. 6).

Fig. 6 The C3a sequence of C3 encoding gene [52]

C3a, C4a and C5a are all complement-derived anaphylatoxins. Their systemic activity is described as a potent pro-inflammatory mediator, that causes smooth muscle contraction, histamine release from mast cells and increased vascular permeability. Overproduction contributes to autoimmune diseases such as for example rheumatoid arthritis, to septic shock and myocardial ischemic injury among other conditions [17].

C3a and C5a are both chemoattractant molecules. Whereas C5a has broad pro-inflammatory effects, the effects of C3a appear to be more selective and somewhat anti-inflamatory. C3a affects the expression levels of inflammatory cytokines Il1β, TNFα, Il6 [53, 54].

In mice, C3a and C5a are also critical for liver regeneration by promoting hepatocyte proliferation [35, 55].

2.1.3.2. COMPLEMENT AND C3a IN THE BRAIN

Production of complement proteins in the CNS is now well described in microglia, astrocytes and neurons [6, 35, 56]. C3a and C5a receptors C3aR and C5aR are expressed in the normal uninjured CNS.

It has been shown that neural stem cells in vitro and neuroblasts in vivo also express both C3aR and C5aR [7] and that the expression of these receptors increases after focal cerebral ischemia [57]. A novel receptor C5L2 can also bind these anaphylatoxins with predominant C5a affinity [58]. It may serve to modulate C5a biological functions [59] due to more equal affinity to active and inactive anaphylatoxins; a removal of C-terminus arginine abrogates the binding of anaphylatoxins to C3aR, C5aR but not to C5L2 [60].

Complement inhibitors are also present in the CNS, but mainly in glia and not in neurons, which makes them susceptible to complement-mediated cell lysis [6, 61].

Local production of complement by resident cells is increased following brain ischemia [35, 57, 62, 63].

After injury, complement activation has been suggested to exacerbate the inflammatory response therefore contributing to secondary tissue damage as shown by systemic complement depletion [64], using C3 deficient mice or C3aR-antagonist treatment [65]. All of these studies showed a reduction in the size of infarct after cerebral ischemia. There is also evidence that the negative role of C3a depends on type of hypoxic-ischemic injury, because treatment with C3aR antagonist showed reduced infarct only after transient but not permanent cerebral artery occlusion [66].

Interestingly, C3a and C5a are also neuroprotective [67-69] (Fig. 7). Exposure to C3a induces de novo expression of nerve growth factor in microglial cells in vitro [50] and protects against neuronal excitotoxicity [69].

It has been shown, that C3a reduces hippocampal brain injury and increases hippocampal neurogenesis after hypoxia-ischemia in neonatal mice [67].

Studies using transgenic animals lacking C3 or C3aR blockade also show that complement positively regulates basal neurogenesis in adult mice, conceivably through C3aR signaling. Also, mice lacking C3 had reduced ischemia-induced neurogenesis in SVZ and at the infarct border, despite larger infarcts [7]. Interestingly, mice

expressing C3a and C5a under the control of a GFAP promoter, present in astrocytes, and neural stem cells, did not show any alterations in basal neurogenesis [70].

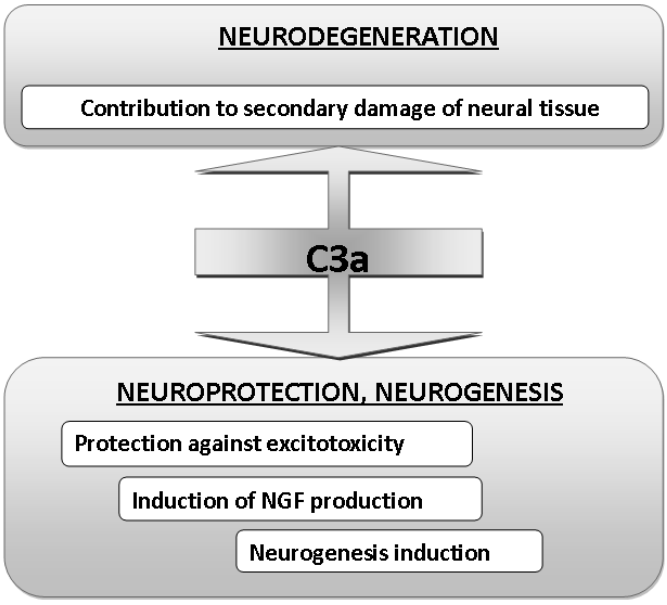

Fig. 7 Positive and negative roles of C3a in neural tissue damage

2.2. TRANSGENE MODELING STUDY

Transgenic models are a commonly used approach to elucidate the role of certain protein or peptide in an organism. The knocked-out gene model can be used, where the gene function is disabled, and then determine the missing function. It is also possible to form a transgenic model with the expression of gene, which is commonly not expressed in the particular part of the organism, and study the effects there [71].

The transgene consists of the genetic information for the required protein and the regulatory region which determines the cell type and time point for the transgene expression [71].

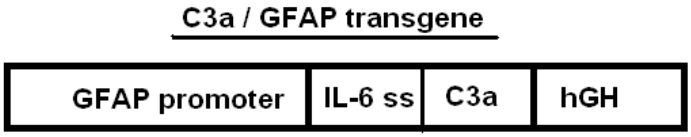

Fig. 8 Model of transgene in which C3a expression is driven by GFAP promoter. This model allows the expression of the C3a peptide in brain [72] by cells expressing GFAP (see 2.1.2.3.).
(GFAP promoter - regulatory region,
IL-6 ss - signal sequence to ensure proper C3a secretion,
C3a – C3a encoding region,
hGH - human growth hormone polyadenylation sequence)

2.3 QUANTITATIVE REAL-TIME PCR mRNA ANALYSIS

mRNA quantification using quantitative real-time PCR includes several experimental steps (Fig. 9).

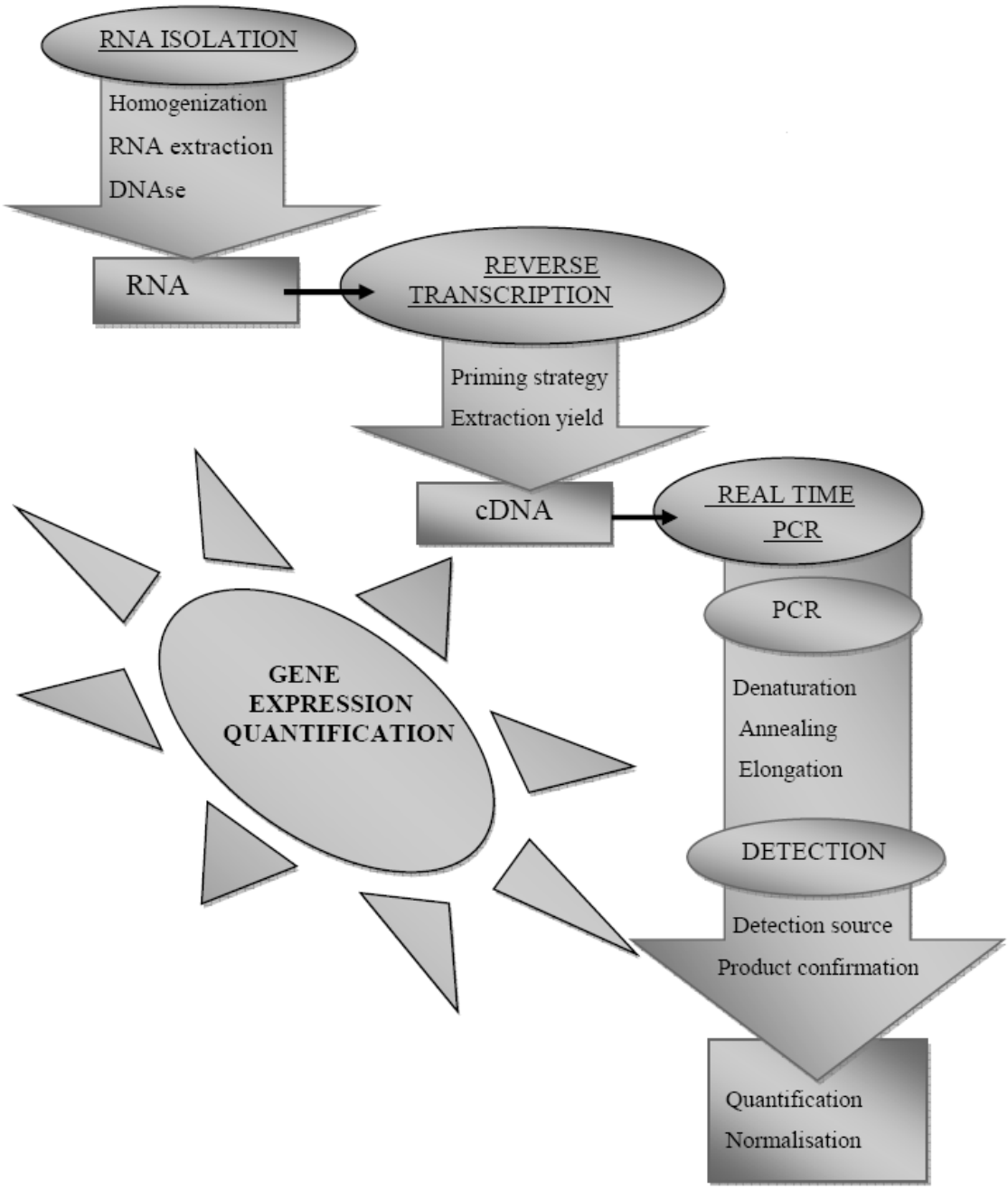

Fig. 9 Systematic overview of all steps in gene expression quantification using real-time PCR

2.3.1 RNA ISOLATION

SAMPLE HOMOGENIZATION

To allow an effective RNA isolation tissue samples need to be disrupted and homogenized. The nucleic acids need to be stabilized and protected against degradation. Homogenization usually includes both mechanical and chemical treatment.

The mechanical processing can be performed by fast-shaking and efficiently completed by crushing with steal beads present in the tube with the tissue sample, e.g. Tissue Lyser (Qiagen).

The chemical treatment facilitates the decomposition of cell structure and inactivates functional proteins such as RNAses, which may degrade the RNA template [73].

RNA EXTRACTION

RNA isolation (Fig. 10) is usually performed by solid phase separation in a column. Fixation of RNA to the column enables repetitive washing and concentration of nucleic acids in the sample.

If the sample comes from tissue containing high amount of lipids, a liquid extraction can precede solid phase separation [74].

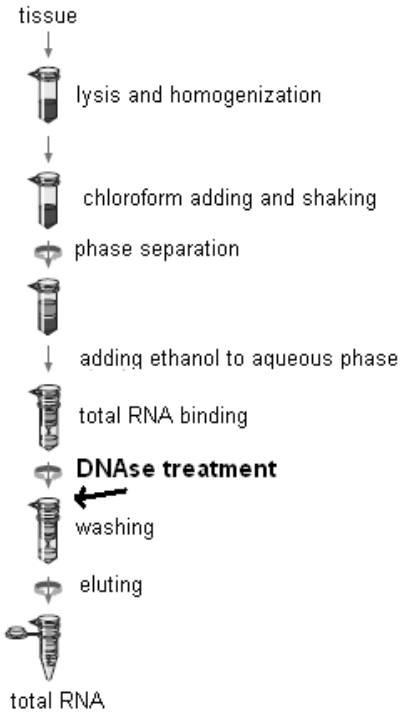

Fig. 10: RNA isolation including liquid extraction, solid phase separation and DNAse treatment

DNAse TREATMENT

Genomic DNA in the sample can affect RNA analysis. First, false measurement of spectroscopic concentration may occur, because the total amount of nucleic acid found by spectroscopy is used to equalise the amount mRNA in the sample (see in 5.2.2). Second, because all cells have one or more copies of a gene in the genomic DNA, they may be amplified and contribute to inaccurate quantification. It is appropriate to remove DNA from the samples by DNAse treatment. DNA is cleaved by DNA-specific nuclease and washed out of the column (Fig. 10) [74, 75].

2.3.2 REVERSE TRANSCRIPTION

Reverse transcription (RT) is an enzymatic reaction where complementary DNA (cDNA) is generated from mRNA. RT is used to generate DNA for qPCR, because RNA is less stable than DNA. This is the most vulnerable step, in terms of reproducibility, in the entire analysis [75]. The variability in RT is due to reverse transcriptase and its inhibitors, the priming strategy and the secondary and tertiary structure of mRNA template [76].

Reverse transcriptase is an enzyme used by retroviruses to incorporate its own coding RNA information into host DNA. Several commercial reverse transcriptases are available today. They are in most cases engineered from Moloney murine leukemea virus or the Avian myeloblastosis virus and modified to improve thermostability or to decrease endonuclease activity (Tab. 1). The RT efficiency between enzymes may vary up to 100-fold [77].

Tab. 1: Example of reverse transcriptases [78] offered by Invitrogen®

Enzyme name	Enzyme source	RNase Activity
SuperScript® III Reverse Transcriptase	MMLV Mutant	Reduced
SuperScript® II Reverse Transcriptase	MMLV Mutant	Reduced
ThermoScript™ Reverse Transcriptase	AMV Mutant	Reduced
MMLV Reverse Transcriptase	MMLV	Yes
Cloned AMV Reverse Transcriptase	AMV	Yes

For transcription initiation the reverse transcriptase needs a double stranded oligonucleotide-starting sequence. This is obtained by using primers. RT primers are single-stranded synthetic oligonucleotides, which can be designed using three different priming strategies: random hexamers, gene specific primers and oligo(dT)primers (Fig. 11) [79].

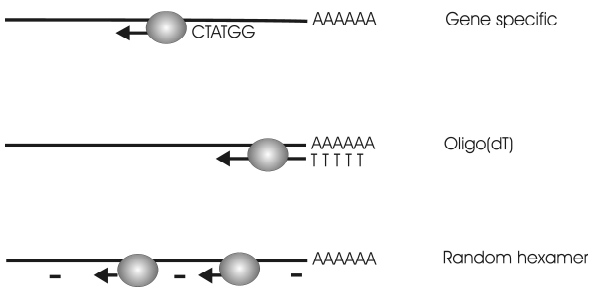

Fig. 11 Principles of RT priming strategy

Random hexamers are randomly ordered six-base long primers. Random hexamers can bind to many places of the single-strand RNA and thus increase the possibility of reverse-transcription initiation, but high concentrations may cause the generation of short cDNA fragments, which are not able to cover the PCR needed sequence [79].

Oligo(dT)- primer is an oligomer consisting of only thymidine. It binds to the specific mRNA poly-(dA)-tail sequence that is present at the 3´end of most mRNA molecules in eukaryotes. However, only one priming position for each mRNA may cause a lower yield of cDNA [79].

Gene specific oligonucleotides bind only to the specific sequence present in the mRNA of interest, mainly transcribing this RNA. They should provide transcription of the targeted mRNA but may also cause less cDNA yield for the same reason as oligo(dT) primers.

All mRNAs are unique in sequence. They differ in secondary and tertiary structures and as such, no universal optimal priming method exists. Therefore, combination of priming strategies can be used to reach a higher RT yield. The most common is to use a mixture of random hexamers and oligo(dT) [79].

RNA PURITY, EXTRACTION YIELD

Reproducibility of RT also may be severely affected by RNA concentration, consequently the same concentration of RNA should be used in all samples that are compared [79]. Extraction yield allows the use of approximately an equal amount of template in the RT reaction.

The absorbance measurement can be used to determine the nucleic acid concentration. Using the absorbance obtained at its characteristic wavelength (260nm) the concentration can be calculated from equation (Eq.1).

$$A = \varepsilon.c.l \qquad (Eq.1)$$

Where ε is specific absorbance constant, c is molar concentration, l is distance that light ray passes through the sample, A is the absorbance at 260nm.

It is also possible to assess RNA purity by spectroscopic analysis using the unique absorbance properties of each molecule. The maximal absorbance of nucleic acids is at the wavelength 260 nm [75], proteins at 280 nm and contaminating salts absorb mainly at 230 nm. The absorbance curve can be used as a purity assessment, where an absorbance ratio between 260/280 nm represents protein contamination and a 260/230 nm ratio demonstrates the salts present in the sample. The nucleic acid absorbance curve should have one peak at 260 nm, protein contamination ratio in the range of 1.9 – 2.1 and a salt ratio between 1.6- 2.1 (Fig. 12).

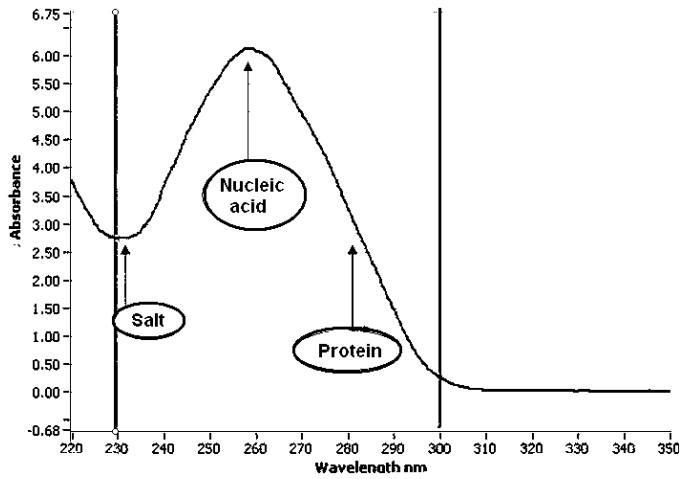

Fig. 12 Absorbance profile of nucleic acid

2.3.3 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

Polymerase chain reaction (PCR) is a cyclic enzymatic reaction for exponential amplification of a specific DNA fragment. This amplificated amount of specific DNA allows its detection, which is traditionally performed by agarose gel electrophoresis [71].

Quantitative real-time PCR (qPCR) is based on conventional PCR amplification. In contrast to classic PCR detection and quantification by electrophoresis, qPCR includes a detection process during the PCR cycling, which makes it possible to measure the amount of product after each cycle of the reaction and enables much more accurate quantification [76].

PCR consists of three parts: denaturation, annealing and elongation (Fig. 13).

These steps are generally repeated between 30 to 50 times [71].

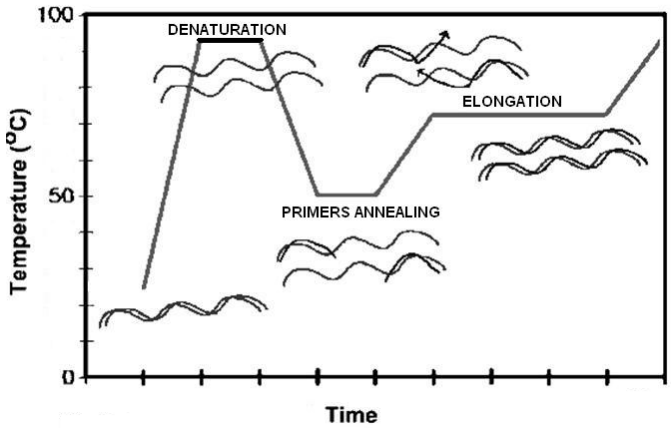

Fig. 13 Main principles of PCR cyclic unit

DENATURATION

Double-stranded DNA consists of two complementary strands. During denaturation double-stranded DNA is separated into single-stranded DNA by heating usually at 95˚C (Fig. 14) [71].

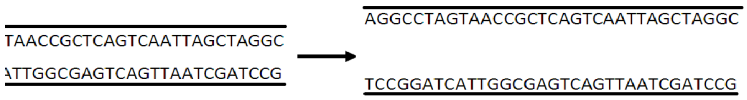

Fig. 14 Denaturation

PRIMERS ANNEALING

PCR primers are sequence specific oligonucleotides used in pairs [71]. Each pair contains a forward and reverse primer, which can be designed by commercial software such as Premierbiosoft-primer design or Primer3.

Primers bind to the single-stranded DNA template (Fig. 15).

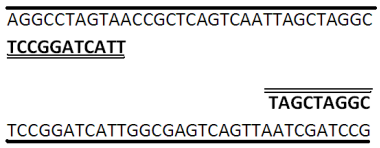

Fig. 15 Annealing

They can also bind to each other due to intermolecular or intramolecular interactions between primers and form secondary structures that greatly reduce their availability to annealing and as such, amplification [71].

Primers must be specific for the sequence of interest, so as not to target other genes in the mixture. To improve the specificity it is necessary to avoid regions of homology. Commonly, primers are designed and then blasted (compared to the genomic database) to test the specificity.

To avoid genomic DNA amplification, which may affect the mRNA (cDNA) analysis, primers may be also designed to span an intron [80].

The length of primers is usually 17-30 nucleotides.

Annealing temperature should correspond to the melting temperature of the primer. The melting temperature is the temperature at which half of double-stranded nucleic acid sequence is dissociated to single-strands. Annealing temperature is the temperature used for primers to bind to a template and it also should differ as little as possible between the reverse and forward primers.

ELONGATION

In the elongation step, DNA polymerase generates a new DNA strand using the old one as a template (Fig. 16). The DNA polymerase used in PCR was obtained from bacteria in thermal waters because of its ability to retain enzymatic capability at temperatures up to 95˚C, used in PCR for denaturation of DNA strands [71].

DNA polymerase uses magnesium cations-Mg^{2+} as cofactors for a nucleotide binding and matches free nucleotides, according to their complementarity, to the template strand to elongate the nascent strand in its 5´-3´direction. This is done at the optimum temperature for the function of DNA polymerase [71].

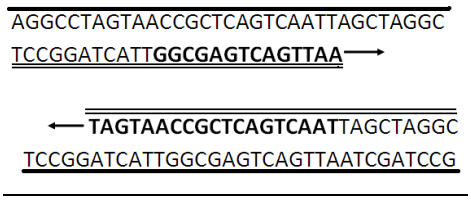

Fig. 16 Elongation

DETECTION

qPCR detection of the product is mediated by an optical unit that illuminates the sample and records the emitted fluorescence. The signal source can be divided into two different reporter categories according to specificity; nonspecific dyes and probes [76].

Non-specific dyes (Fig. 17) are not sequence dependent. The most commonly used dye in qPCR is SYBR Green I. It binds to the groove in double stranded DNA and as such, gives a low signal from single stranded DNA [76].

Fig. 17: Non-specific dye intercalation and light emission [81]

Probes (Fig.18) are sequence specific oligonucleotides or their analogs coupled to one or two dyes. In order to be used a specific sequence for the region that should be detected must be found [76].

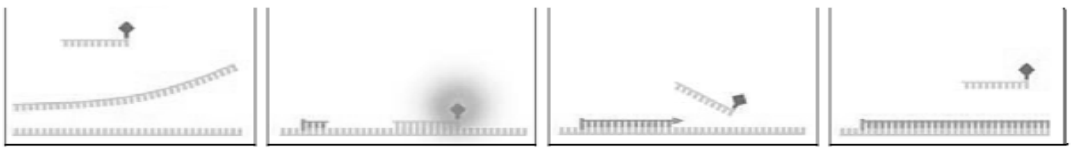

Fig. 18: Probe binding and signal emission [81]

Probes are able to distinguish specific products so they are suitable for the detection of several sequences amplified at the same time in the same tube.

Nonspecific dyes are universal for double stranded sequence detection. To distinguish different products, a melting curve analysis should be used [76].

The melting curve analysis is performed by signal detection during the continuous increase of temperature at the end of amplification (Fig. 19). The melting curve analysis enables also detection of primer-dimer formation or some other undesirable double-stranded products [76].

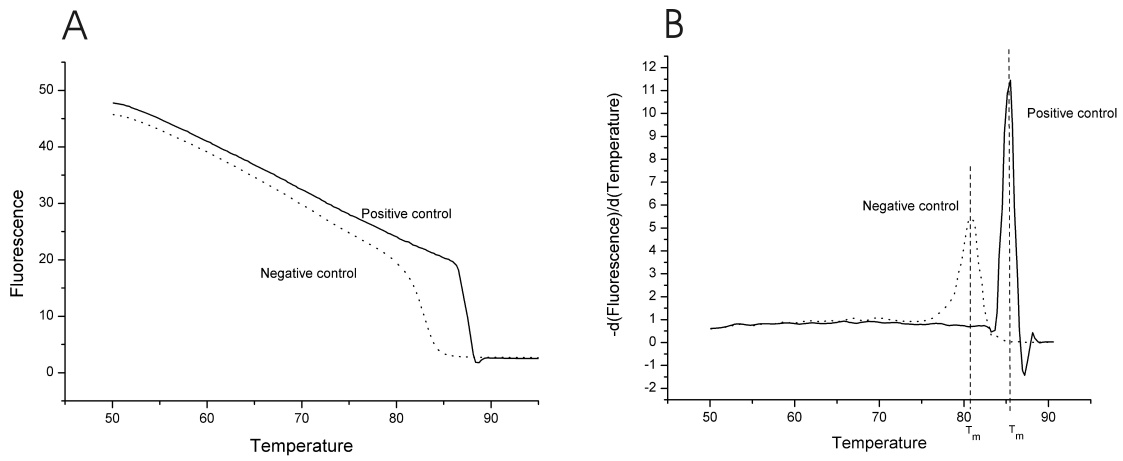

Fig. 19 Melting curve and second derivation of melting curve [76]

The gradual decrease of the signal with an increase in temperature (shown by the melting curve) correlates to the separation of double-stranded DNA (to which the dye can bind) into single-strands (to which the dye cannot bind) (Fig. 19).

The melting temperature (Tm) is defined as the temperature where 50% of double-stranded DNA has been separated and relates to the product sequence according to the length and sequence. Tm is obtained from the negative derivation of the melting curve (Fig. 19) [76].

Alternatively, electrophoretic analysis can be used to confirm the specificity and the purity of the amplified product by finding just one product of the expected length.

2.3.4 QUANTIFICATION

At a certain point during cycling when enough PCR product has accumulated, the fluorescence signal increases significantly above the noise level (Fig. 20). This point is referred to the cycle of threshold (Ct) or crossing point (Cp). It is defined by a threshold line or second derivate maximum of the amplification curve. For accurate quantification the Ct must be determined in the exponential part of the amplification curve [76].

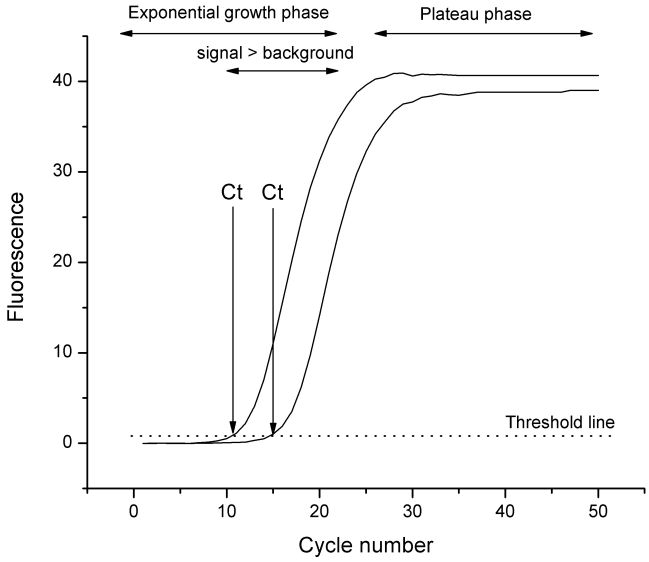

Fig. 20 Set-up of the threshold line crossing the amplification curve to determine the Ct value [76]

BASIC MATHEMATIC MODEL

The fluorescence signal *I*, in the exponential part of the amplification curve is proportional to the amount of target DNA, *N*, in the sample:

$$I = k \cdot N \qquad \textit{(Eq.2)}$$

where *k* is an assay instrument and detection chemistry dependent constant (fluorescence units per target DNA molecule, varies e.g. with different probes, dyes) [76].

The basic equation describing the real-time PCR cDNA amplification is:

$$N_{ct} = \eta\, N_0 \,.\, (1+E)^{Ct-1} \qquad \textit{(Eq.3)}$$

N_{ct} is the number of target DNA molecules present at the cycle of threshold or second derivate maximum, N_0 is the number of initial DNA molecules, η is the reverse transcription efficiency, E is the PCR efficiency (E=1 corresponds to 100% efficiency), (ct -1) is the threshold cycle number reduced by one because the first cycle is used to make double stranded DNA from single stranded cDNA generated in the reverse transcription [76].

The PCR efficiency is assumed to be independent of N_0 over the studied concentration range. The PCR efficiency can be estimated from calculating the slope of the curve of dilution series of genomic DNA or purified PCR product cDNA (Fig. 21) [76].

$$E = 10^{-1/slope} \text{--} 1 \qquad \textit{(Eq.4)}$$

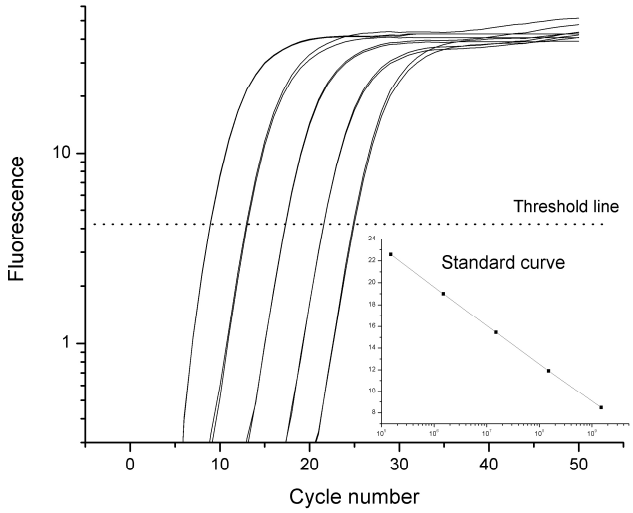

Fig. 21 Amplification curves of dilution series and standard curve of their Ct values [76]

NORMALIZATION

Usually the relative quantification is used for gene expression analysis. The gene of interest may change in expression levels in the studied situation, while other reference genes should remain constant. Ideally, reference gene expression is not affected by changes in the environment but all genes are regulated to a certain degree in certain situations. It is suitable to screen genes involved in different cell functions to find the least affected genes. In order to decrease a possible regulatory effect, combinations of genes may be used [75, 79].

By using (Eq.2) and (Eq.3) and assuming the same experimental setup and the same reverse transcription efficiency, the normalization ratio *Z* (Eq.5) of target and reference genes can be found [76].

$$Z = \left[\frac{N_{0_{target}}}{N_{0_{reference}}}\right] = \left[\frac{(1+E_{reference})^{Ct_{reference}-1}}{(1+E_{target})^{Ct_{target}-1}}\right] \qquad \textit{(Eq.5)}$$

3. THE AIM OF THE STUDY

To understand the changes in gene expression after experimental perinatal asphyxia in different brain regions in wild type mice and in a transgenic mouse model of C3a expression under the control of the GFAP promoter (C3a/GFAP).

1. To select genes involved in processes potentially important for reduction of neural tissue loss and to design experimental set-up for analysis of their expression profiles.
2. To analyse temporal gene expression profiles of the selected genes.
3. To compare the gene expression profiles among the ipsilateral, contralateral hippocampus and the ipsilateral cortex.
4. To do expression analysis of the C3a/GFAP transgene.
5. To compare the effects of C3a/GFAP expression to the expression profile of the selected genes.

4. MATERIALS AND METHODS

4.1 MICE AND HYPOXIA-ISCHEMIA

Tissue was obtained from the ipsilateral and contralateral hippocampus and ipsilateral cortex of wild type mice and transgenic mice, over-expressing C3a under the control of a glial fibrillary acidic protein (GFAP) promoter (*C3a/GFAP)* [67, 72], after unilateral hypoxia-ischemia.

Hypoxia-ischemia model was performed on postnatal day 9 by left common carotid artery ligation followed by treatment in 10% oxygen in nitrogen humidified gas mixture for 30 min [67]. Samples were collected at 0h, 6h, 24h, 3d, 7d and 21d after injury and from uninjured 9 days old controls.

4.2 RNA PURIFICATION

Tissue was frozen at -80˚C and homogenizated for RNA extraction with a Tissue Lyser (Qiagen) in QIAzol (Qiagen) according to the manufacturer's instructions (Lipid Tissue RNAeasy Mini Kit). RNA extraction, including DNAse treatment, was performed with Lipid Tissue RNAease Mini Kit also as described by the manufacturer's instructions (Qiagen). The RNA purity and concentration was measured by a NanoDrop-spectroscopy.

4.3 REVERSE TRANSCRIPTION

Reverse transcription (RT) was performed in two steps.

First, random hexamers (5uM), oligo(dT) (5uM) and dNTP (500uM) were mixed with 1ug of total RNA in a total volume of 6.5ul. Final concentrations are shown in parentheses. Samples were preincubated for 5 min at 65˚C and then put on ice for 2 min.

Second, SuperScript III Reverse Transcriptase (10U/ul) (Invitrogen), First-Strand Buffer (1x) (Invitrogen), DTT detergent (5mM) and RNAseOut (2U/ul) (Invitrogen) were added to a final volume of 10ul. Samples were incubated at 50˚C for 60 min, then

for 15 min at 55˚C (SuperScriptIII optimum) and lastly for 15 min at 70˚C to deactivate the reverse transcriptase. The final cDNA product was diluted to 200ul.

RT negative control samples, where the reverse transcriptase was exchanged for water, were included into the RT-run for control of genomic contamination.

4.4. QUANTITATIVE REAL-TIME PCR

Primers were designed by using Primer3 [82] and Netprimer [83]. The specificity of designed primers was confirmed using the BLAST algorithm [84]. Functionality of primers was checked by qPCR, melting curve analysis and all PCR products were verified by agarose gel electrophoresis (1,5% w/v). (For primers sequence and amplicon length see 7. Supplements).

Real-time PCR experiments were performed with mastermix containing iQ SYBRGreen Supermix (1x) (Bio-Rad), primer mix (0,4uM/each) and cDNA in a total volume of 20 ul. PCR negative control samples (with no cDNA template) and RT negative control samples were included to exclude possible contamination.

Real-time PCR was performed in a LightCycler 480 (Roche Diagnostics) starting with 3 min at 95˚C for predenaturation of the Taq-Antibody followed by 40-45 amplification cycles, consisting of denaturation at 95˚C for 20 s, 20 s annealing of primers at 60˚C and 20 s elongation at 72˚C. The cycling ended with a temperature gradient for melting curve analysis.

For the transgene C3a/GFAP assay the following protocol was used: denaturation (95˚C for 20s), annealing (56˚C for 20s) and elongation (72˚C for 30s) [72].

The Ct value was determined using the maximum second derivate function in LightCycler software (Roche Diagnostics).

4.5 DATA ANALYSIS

Variations between real-time PCR runs were compensated with identical control samples used in all measurements. To normalize gene expression data, the expression of two least variable expressed genes PKG1 and B2M were used. These were evaluated by reference gene tests that tested 12 potential reference genes [85].

For relative quantification, the following equation (Eq.6) was used assuming 90% PCR efficiency for all assays.

$$Z = \frac{N_{0\ target}}{N_{0\ reference}} = \frac{(1+0{,}9)^{\left(\frac{ct\ ref_1 + ct\ ref_2}{2}\right)}}{(1+0{,}9)^{ct\ target}} = 1{,}9^{ct\ ref_{1,2}, mean - ct\ target} \qquad (Eq.6)$$

N_0 is the number of initial DNA molecules, *ct target* is Ct value of target gene, *ct* $ref_{1,2}$ *mean* is the mean of Ct values of reference genes.

The Z-values for the wild type mice control samples were arbitrarily set to one to simplify the comparison between groups.

The arithmetical mean was calculated together with the standard error using equation (Eq.7).

$$SE = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n(n-1)}} \qquad (Eq.7)$$

Where SE is standard error of the mean, x_i is the gene expression of each mouse, $\bar{x}$ represents the mean of all mice in the group and n is the number of animals in the group. Here n= 2-7.

4.5. RESULTS LEGEND

Expression levels were measured at the time of injury (*0h*), 6 hours (*6h*), 24 hours (*24h*); 3 days (*3d*), 7 days (*7d*) and 21 days (*21d*) after hypoxic-ischemic injury. 9 day old uninjured mice were used as uninjured controls for both wild type (*Wt*) and C3a/GFAP over-expressing transgenic mice (*C3a/GFAP*). Measurements were done in the contralateral hippocampus (*Cl Hip*), ipsilateral hipocampus (*Ip Hip*) and ipsilateral cortex (*Ip Cx*).

All values are relative to wild type uninjured control mice *(c)*, which were taken as 1.

5. RESULTS AND DISCUSSION

5.1. TRANSGENE FUNCTION RELATED PROFILES:

C3a/GFAP, GFAP, C3

C3a/GFAP transgene expression was up-regulated during the first six hours about 15-fold in *Ip Hip* and *Ip Cx*. In *Ip Hip* the 8-fold up-regulation was maintained during the first week (Fig. 22).

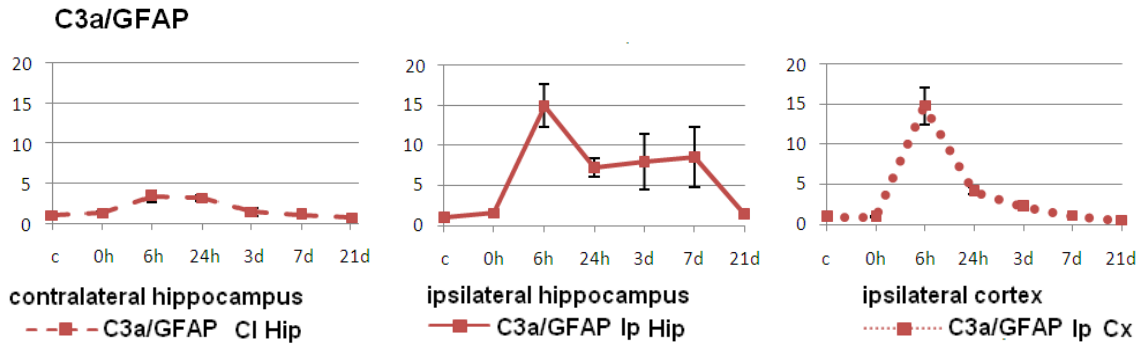

Fig. 22 Expression of genes connected to transgene function: C3a/GFAP

The maximum up-regulation of endogenous C3 (35-fold) was after *7d* in *Ip Hip*, while there was no change in the expression of C3 in the *Ip Cx*, and *Cl Hip* (Fig. 23).

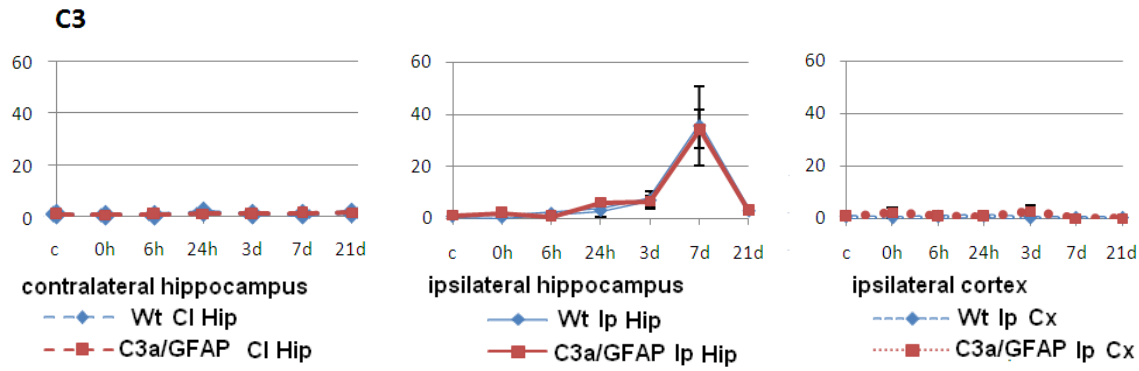

Fig. 23 Expression of genes connected to transgene model function: C3

GFAP expression was up-regulated after *6h* and reached its maximum (a 5-fold increase) expression after *24h* (Fig. 24).

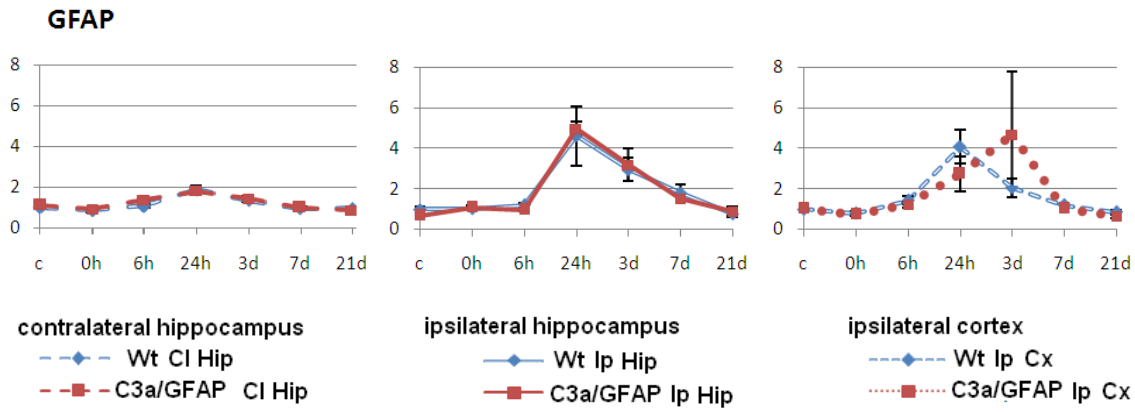

Fig. 24 Expression of genes connected to transgene model function: GFAP

➤ The up-regulation of C3a/GFAP transgene occured before the up-regulation of C3 even before maximal up-regulation of GFAP.

The time lag between normal C3 and transgene C3a/GFAP up-regulation could allow the C3a/GFAP transgenic mice to differentially affect the response to hypoxia-ischemia.

One reason for up-regulation of C3a/GFAP earlier than GFAP could be explained by that the transgenic promoter may not include all regulatory regions of the true GFAP promoter or the location of the genomic insert may affect the transgenic C3a/GFAP promoter. The gap between *6h* and the next detection point (*24h*) could also have masked a detection in the up-regulation in GFAP shortly after *6h*.

➤ Significant regional differences were also seen in the expression profiles of C3.

5.2. NEURONAL MATURITY PROFILES: ENO2, TUBB3

The expression profile of tubulin-β3 (TubB3) was most affected by injury during the first 6 hours in the *Ip Hip*; there was a 3-fold decrease in *C3a/GFAP* and slightly smaller decrease in *Wt* animals. After *24h*, all of the expression profiles showed a decrease. There was a 7-fold lower expression level in *Cl Hip*, a 10-fold lower level in *Ip Hip Wt* and a 5-fold decrease in *C3a/GFAP* compared to the expression levels from *21d* with uninjured controls *(c)* (Fig. 25).

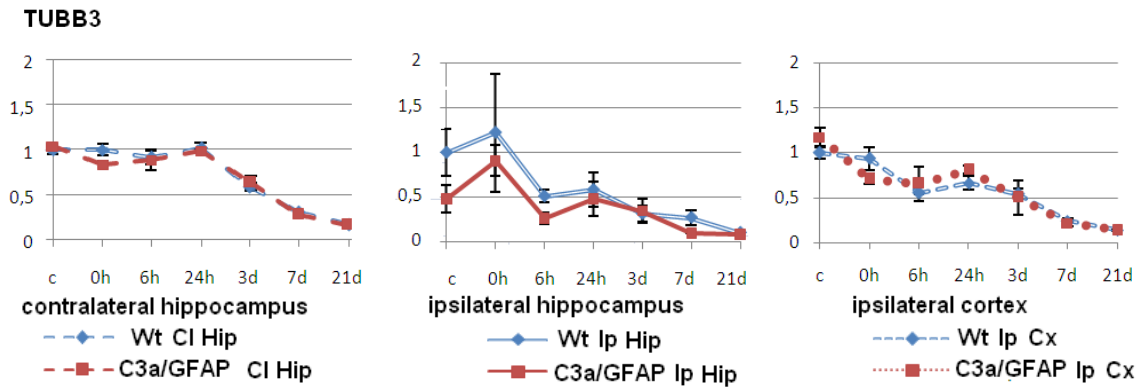

Fig. 25 Expression profiles of genes connected to neuronal maturity: TubB3

The expression profile of neuronal enolase increased gradually in *Cl Hip* and *Ip Cx*, but after *24h* showed no changes in the *Ip Hip* (Fig. 26).

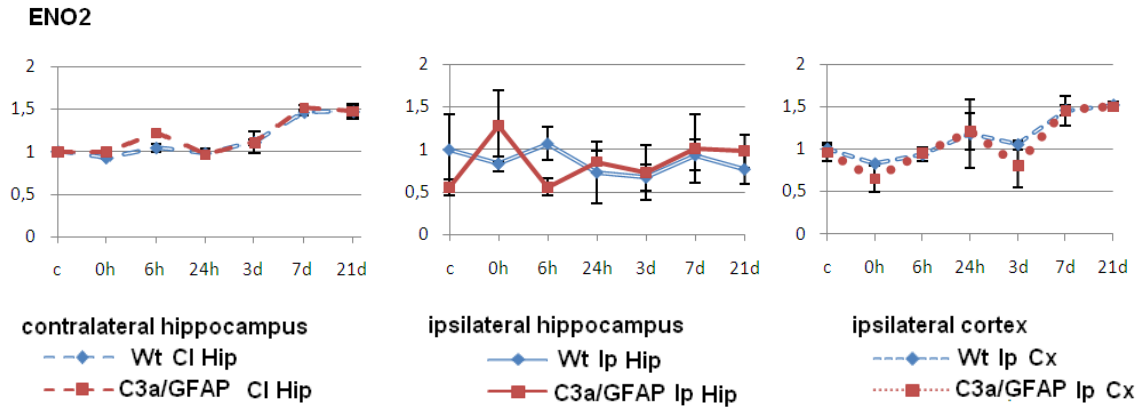

Fig. 26 Expression profiles of genes connected to neuronal maturity: ENO2

➤ TubB3 showed a decrease in expression over time while the neuronal enolase was slightly up-regulated. Although the acute response of TubB3 seems to be more affected by hypoxia-ischemia (*Ip Hip*) than neuronal enolase, the final levels (*21d*) of TubB3 expression were more similar in all regions compared with neuronal enolase. Differences in profiles of TubB3 and ENO2 may correspond to the stage of neuronal differentiation, when certain genes are preferably expressed. The expression of TubB3 may have been affected earlier as TubB3 is present in more immature neurons while ENO2 is a marker of mature neurons.

5.3. NEUROMEDIATOR RELEASE AND DEACTIVATION PROFILES: SYN II, SYN III, GS

Synapsin II expression showed a 3-fold decrease in expression during the first 24 hours after injury in *Ip Hip*. After *24h*, the expression profile seemed to be stable in all regions. At *21d* in *Ip Hip*, expression was about 2-fold lower than uninjured controls *(c)* and other regions (*Cl Hip, Ip Cx)* (Fig. 27).

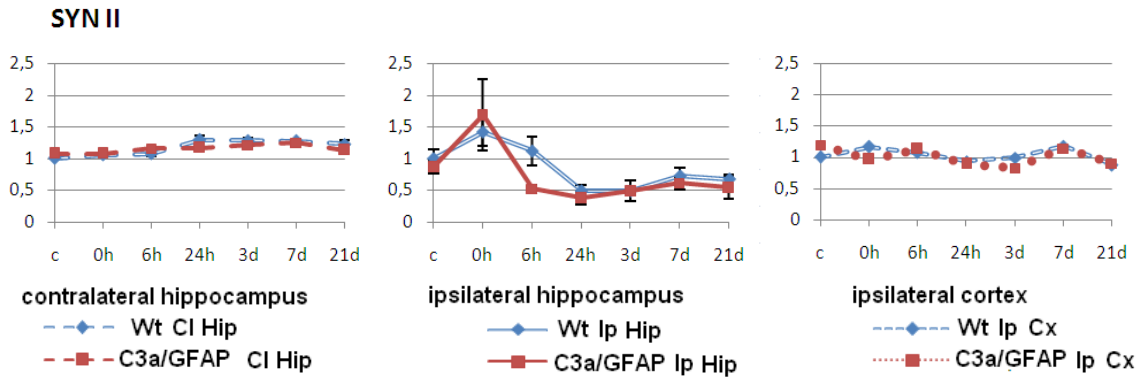

Fig. 27 Gene expression profile related to neurotransmission: SYN II

Synapsin III expression showed a quick down-regulation during the first 6 hours in *Ip Hip*. Decrease in *Ip Cx* most was prominent between *24h* and *3d*. An overall down-regulation of Synapsine III was visible in all regions (Fig. 28).

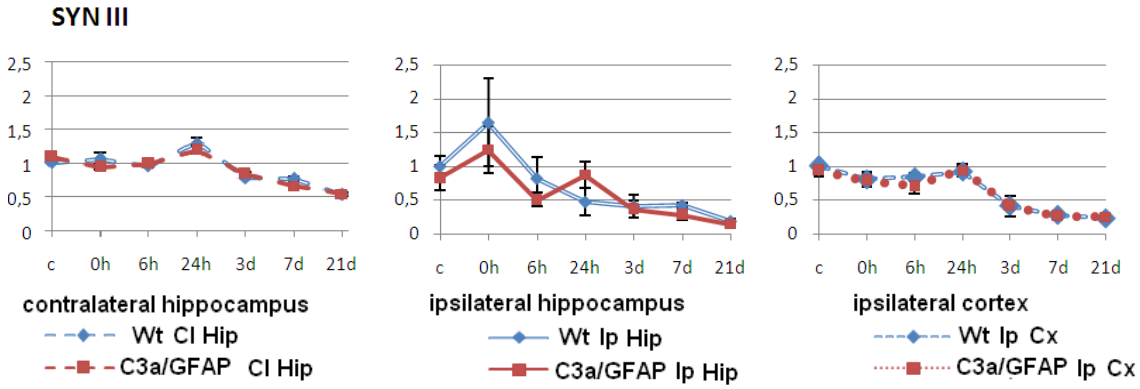

Fig. 28 Gene expression profile related to neurotransmission: SYN III

Glutamine synthetase expression showed a trend towards increased expression in all regions after the *3d*. The most prominent peak was seen in *Ip Hip* at the *7d*. The final levels in all regions were quite similar with around a 2-fold up-regulation when compared to uninjured controls *(c)*. *C3a/GFAP* mice in *Ip Hip* also showed a trend towards decrease in *Cl Hip* during the first 3 days (Fig. 29).

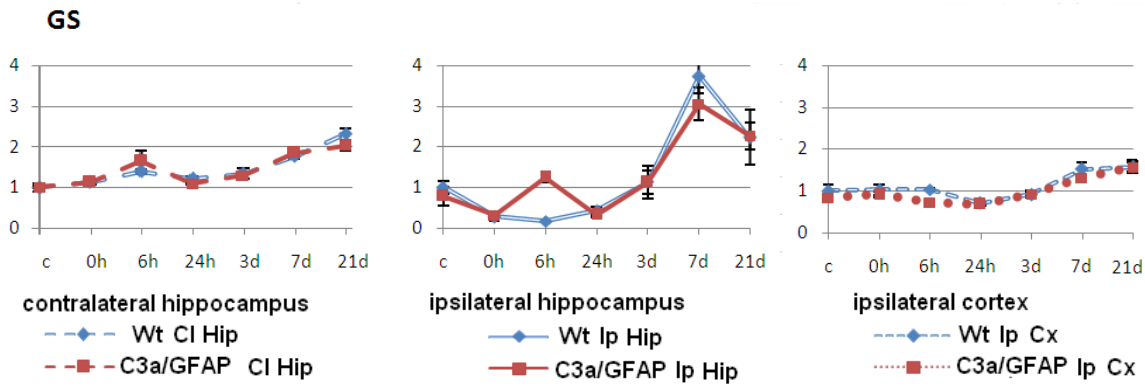

Fig. 29 Gene expression profile related to neurotransmission: GS

➤ The different expression profiles for SYN II and SYN III are not fully understood.

➤ GS is highly expressed by reactive astrocytes but can also increase during the maturation of astrocytes. Thus the increase in all brain regions could be caused by the maturation of astrocytes in combination with an additional increase in expression by reactivity of astrocytes, especially in *Ip Hip*.

➤ Our expression profiles of genes potentially related to glutamate release and deactivation show that the neuroprotective effect of C3a after excitotoxicity, which has been previously described [69] is not achieved by differential expression of these genes.

5.4. INFLAMMATORY CYTOKINES PROFILES: IL1β, IL6, TNFα

Il1β expression was up-regulated 12-fold directly upon injury (*0h*) in *Ip Hip*. In contrast, in the *Cl Hip* only *C3a/GFAP* was up-regulated *0h*. After the *24h* no regulation was seen when compared to uninjured control *(c)*. Compared to other regions, no response was detected in *Ip Cx* profile (Fig. 30).

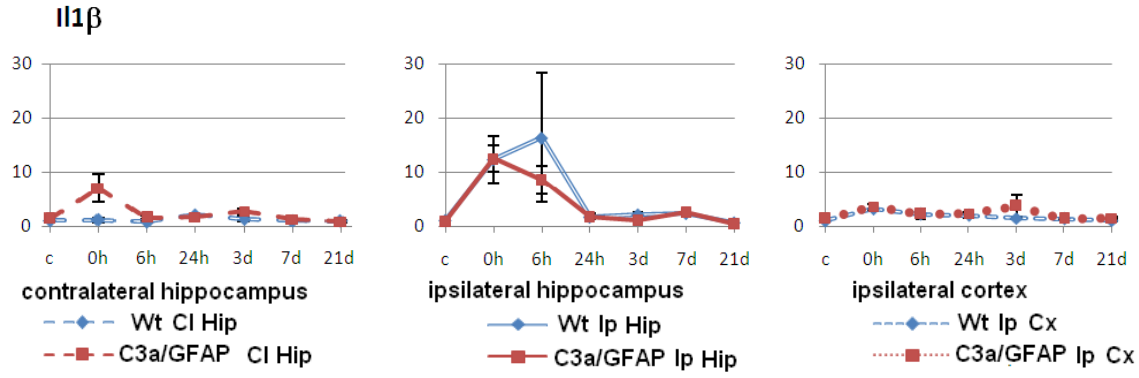

Fig. 30 Gene expression profiles of inflammatory cytokines: Il1β

Expression of Il6 showed a similar profile in the *Ip Cx* and *Ip Hip*, with the maximal up-regulation during first 6 hours. In *Ip Hip* the regulation level in *Wt* at *6h* was approximately 3-fold higher than in *C3a/GFAP* and 8-fold higher when compared to uninjured controls *(c)* (Fig. 31).

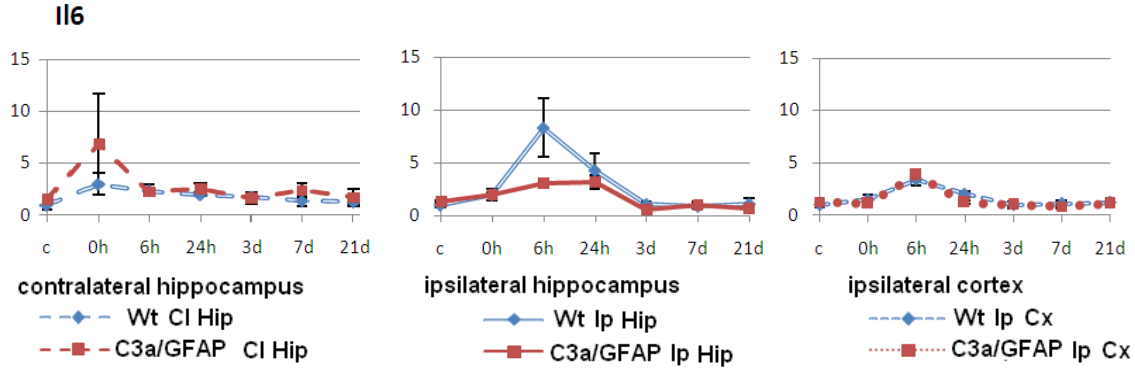

Fig. 31 Gene expression profiles of inflammatory cytokines: Il6

In *Ip Hip*, TNFα expression increased directly upon injury, maximum expression was seen at *6h* with 10-fold up-regulation compared to the uninjured controls *(c)*. The down-regulation from *6h* till *7d* was much greater in *Wt*. In the *Ip Cx*, there was a significant decrease that ended at *21d* with a 20-fold lower level of expression, compared to uninjured controls *(c)*. Other regions displayed no apparent regulation (Fig. 32).

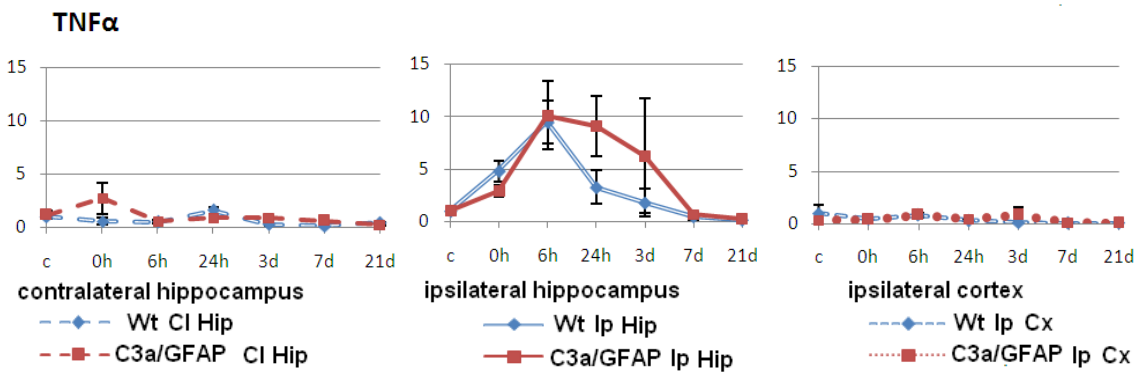

Fig. 32 Gene expression profiles of inflammatory cytokines: TNFα

➤ All genes showed the most regulation in the Ip Hip by 6 hours, reflecting their role in acute inflammation. In *Cl Hip* and *Ip Hip*, *Wt* and *C3a/GFAP* mice showed slight differences in expression, which might indicate the involvement of C3a in the regulation of Il1β, Il6 and TNFα expression. Three weeks after injury Il1β and Il6 expression levels in *Wt* and *C3a/GFAP* mice were more or less comparable to uninjured controls *(c)*, while TNF*α* expression reached a 5-fold lower level in *Ip Hip* and a 20-fold lower level in *Ip Cx* compared to uninjured controls *(c)*.

➤ Inflammatory cytokine expression also showed regional differences.

Differences in Il6, Il1β, and TNFα expression have been also shown in different rodents, injury models, brain regions and age of animals [19, 20].

5.5. MICROGLIA ACTIVATION PROFILES: AIF 1, CD68 ANTIGEN

Aif 1 was down-regulated in all regions after *24h* until the last time point (*21d*). Slight recovery of expression levels was seen in the *Cl Hip* (Fig. 33).

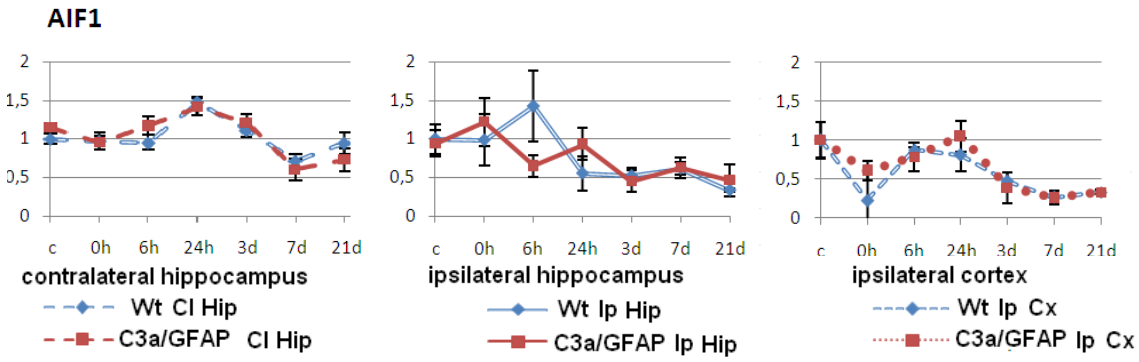

Fig. 33 Expression profiles of genes connected to microglia activation: Aif 1

In the *Ip Hip,* CD68 antigen expression increased until the *3d* when it reached its maximum (3 and 4.5-fold for *C3a/GFAP* and *Wt*, respectively). In the *Ip Cx* the expression profiles were similar but there was less pronounced regulation and an earlier peak (*24h*) in *Wt* (Fig. 34).

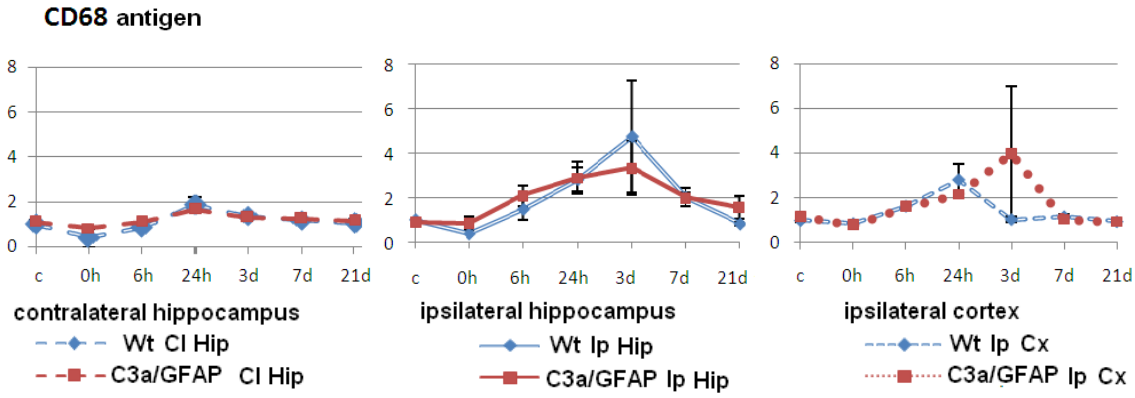

Fig. 34 Expression profiles of genes connected to microglia activation: CD68 antigen

➤ Expression of AIF 1 and CD68 correspond to microglia activation after injury. It has been shown [51] that activated microglia first express Aif1, but upon increased activation, microglia express the CD68 antigen. Thus the increase of CD68 antigen expression in first week, together with the decrease of Aif1 expression occurring after *6h* seems to reflect the progressive activation of microglia in developing inflammation.

5.6. COMPLEMENT ANAPHYLATOXIN RECEPTORS PROFILES: C3aR, C5aR, C5L2

The gene expression profiles of C3a and C5a receptors showed similar patterns in all regions. In the *Ip Cx* and *Ip Hip* up-regulation was seen during the first week. Maximum expression was seen at *24h* in *Wt* (*3d* in *C3a/GFAP*). The up-regulation was most pronounced in *Ip Hip* for both receptors. *Cl Hip* showed no marked regulation (Fig. 35).

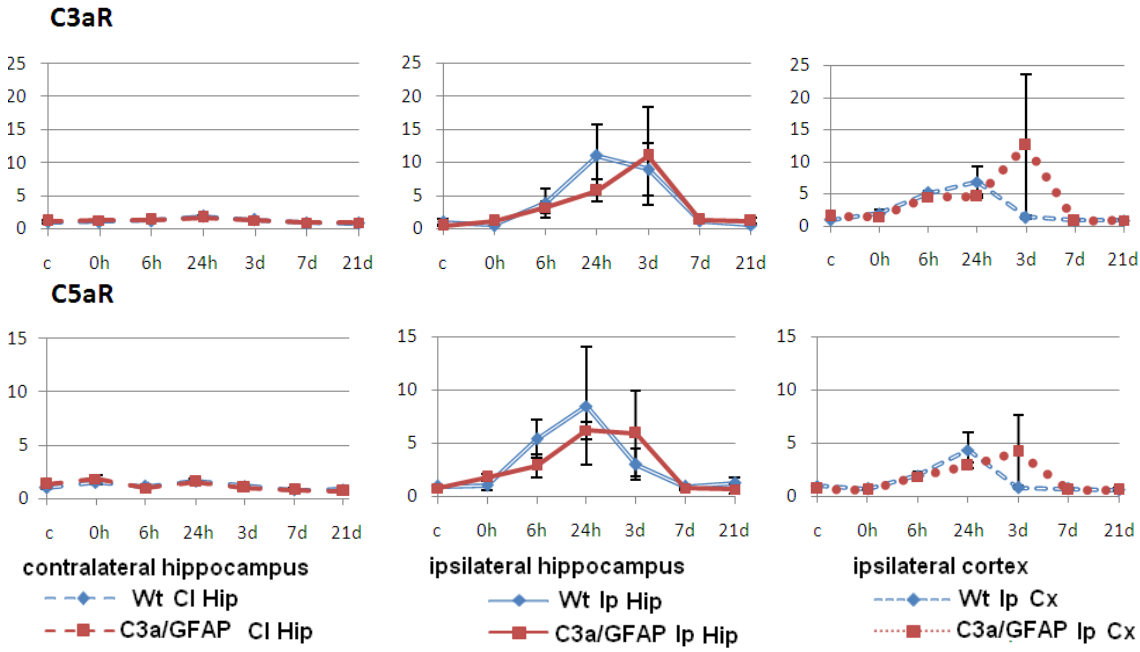

Fig. 35 Gene expression profiles of complement anaphylatoxin receptors: C3aR, C5aR

C5L2 expression was slightly up-regulated and maximum expression was seen at *3d* in the *Ip Cx* and *Cl Hip*. In the *Ip Hip*, expression decreased during the first day, while an up-regulation was seen until *7d*. The levels at *21d* in the *Ip Hip* were 4-fold lower than uninjured controls *(c)* (Fig. 36).

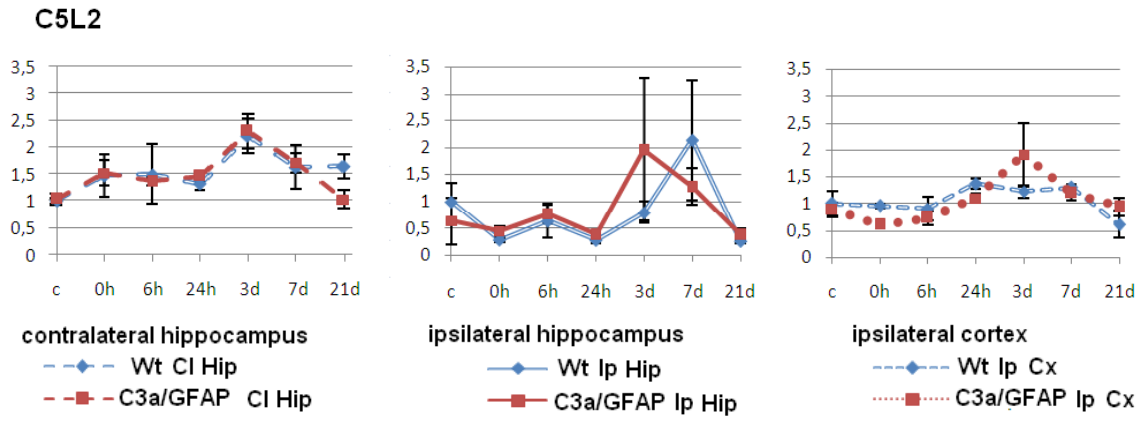

Fig. 36 Gene expression profiles of complement anaphylatoxin receptors: C5L2

➤ All receptor expression profiles showed a similar shift in expression between wild type and C3a/GFAP mice especially in the *Ip Hip*, suggesting a regulation of expression of complement receptors by their ligands. Notably, the expression levels of all receptors increased earlier than C3 expression (See Fig. 23).

Our results are similar but not identical to other studies, where middle cerebral artery occlusion was used in mice and rats [57, 62].

5.7. SELF-RENEWAL CAPACITY RELATED PROFILES: NES, SOX2, DMN

Expression levels were all down-regulated over time regardless of genotype and brain region. The down-regulation seen was about 2-fold lower at *21d* except for Nes, which had 5-fold lower expression at *21d* compared to uninjured controls *(c)* (Fig. 37).

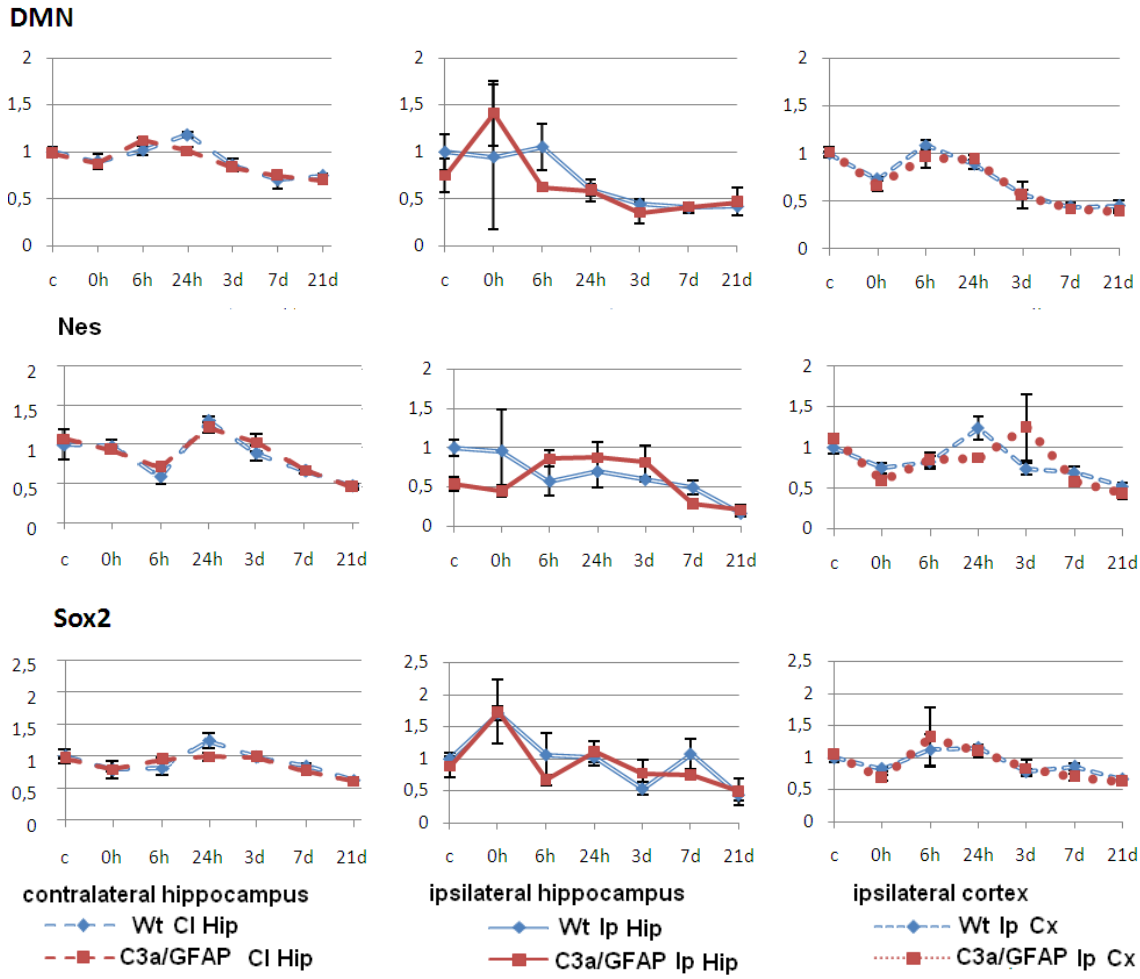

Fig. 37 Expression profiles of genes related to cells with self-renewal capacity: Sox2, Nes, Dmn

➤ The general decrease in expression of these genes could reflect the end of brain development and changes in the activity of certain cell types. The turning points in expression profiles may be caused by a combination of astrocyte activation, ischemia-induced neurogenesis, functional tissue loss by injury and a decrease in proliferative capacity, which is linked to this stage of postnatal development.

5.8. GROWTH FACTORS PROFILES: GAP43, NGF

GAP43 expression was slightly up-regulated in *Ip Hip*, but then after *24h* down-regulation was seen with minimum expression (6-fold) at *21d* in all regions (Fig. 38).

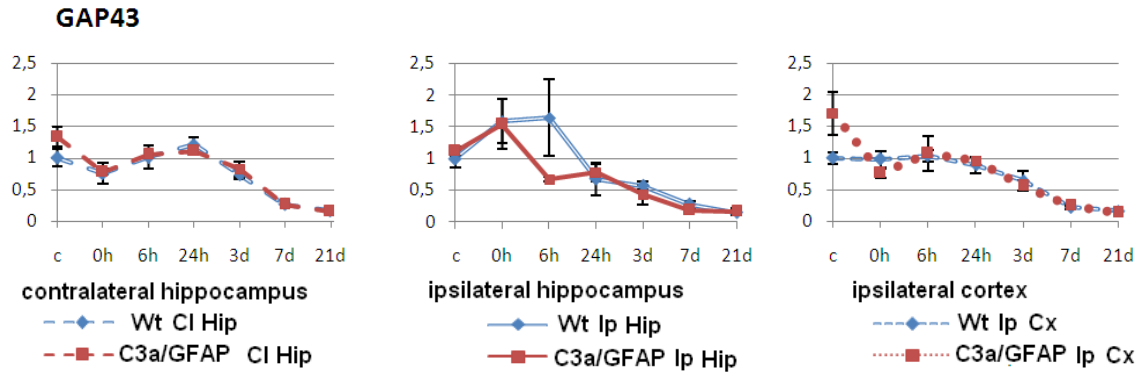

Fig. 38 Expression profiles of growth factors: GAP43

NGF expression was slightly up-regulated in the *Ip Cx* and *Cl Hip*, while in the *Ip Hip* the expression remained unaltered or slightly down-regulated (Fig. 39).

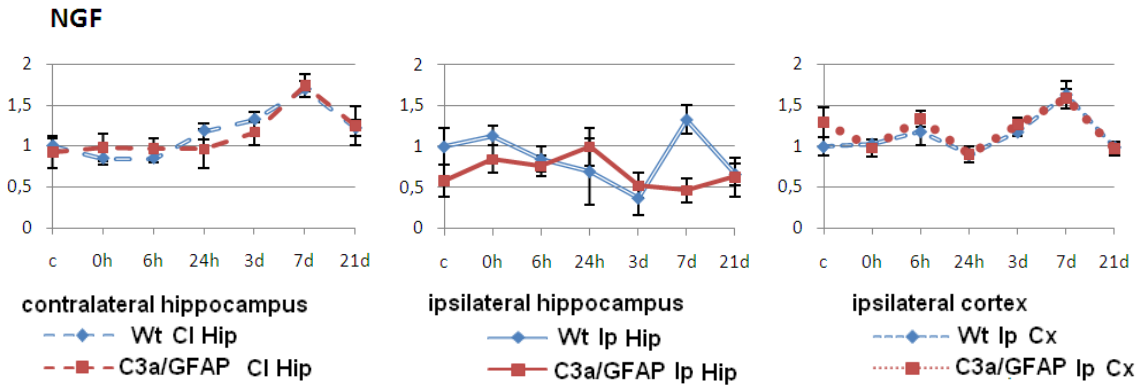

Fig. 39 Expression profiles of growth factors: NGF

➤ The NGF expression profiles in wild type mice seem to correlate with other reports [86]. However, the NGF expression profile does not confirm induction of NGF expression by C3a anaphylatoxin as described in human brain [50].

5.9. APOPTOSIS RELATED PROFILES: BAX, BAD, BCL XL, 14-3-3 ETA

The highest response was detected in the *Ip Hip* for all genes. 14-3-3 eta and Bad expression increased around 2-fold from *3d* to *7d*. In the *Ip Cx*, Bad and Bax expression showed a decreasing trend. Bax expression showed the highest (4-fold) down-regulation in the *Ip Hip* between *24h* and *3d*. All other profiles did not show any clear regulation (Fig. 40).

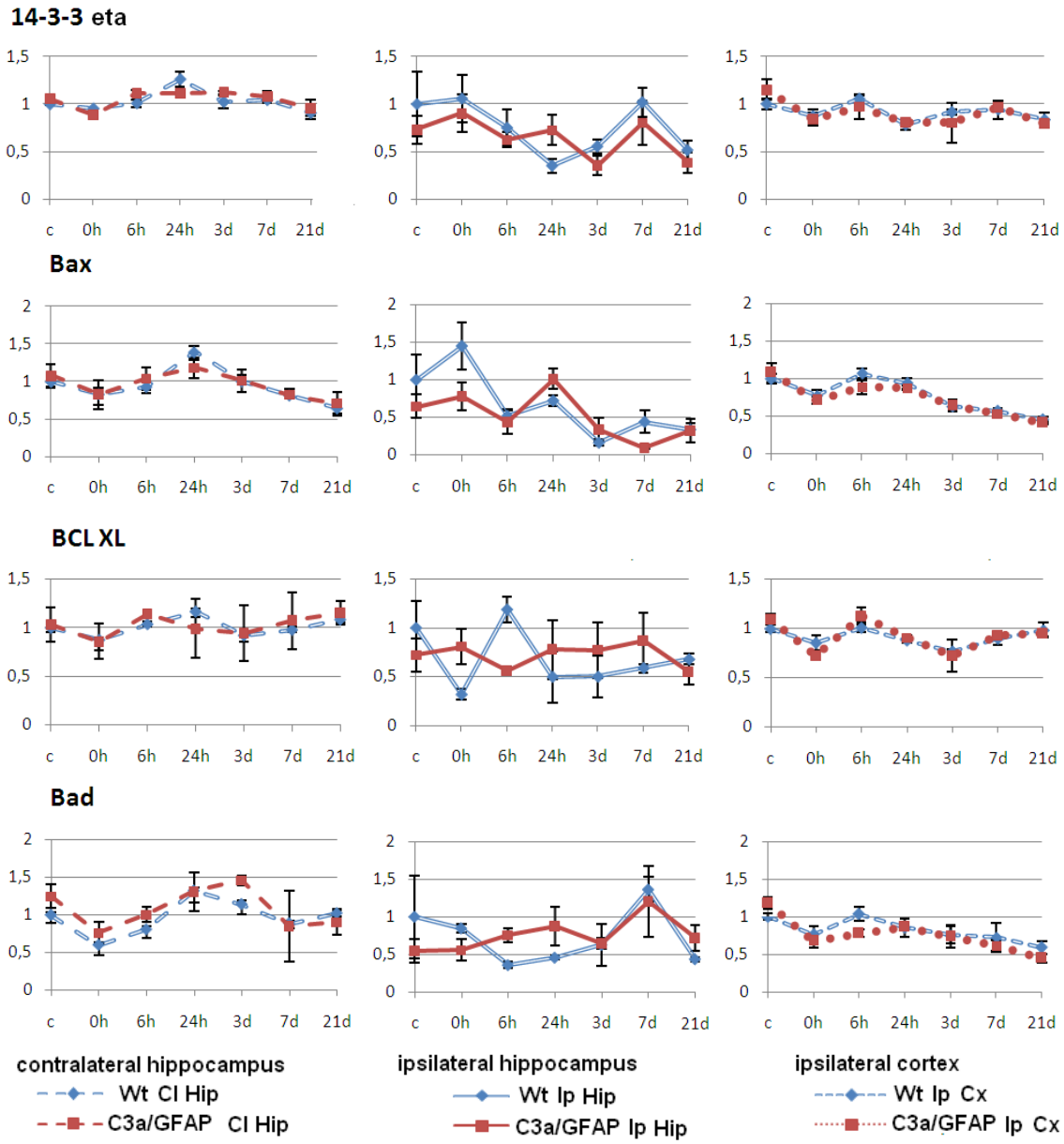

Fig. 40 Expression profiles for genes involved in apoptotic cell death: 14-3-3 eta, Bax, Bad, BclXL

➢ Expression profiles of genes involved in the apoptotic processes showed a decrease in expression (around 2-fold lower levels) compared to uninjured controls *(c)*, irrespective of their pro-apoptotic or anti-apoptotic role. The apoptosis related gene expression (apart from Bad) profiles were similar between the *Ip Cx* and the *Cl Hip.*

5.10. RESULTS OVERVIEW

The most significant findings obtained by gene expression profiling during and after hypoxic-ischemic brain injury are summarized here.

Timing The most prominent changes in gene expression in the ipsilateral hippocampus were seen for C3a/GFAP, Il1β, Il6, SOX2, TubB3 by 6 hours; Gap43, Syn II, Syn III, GFAP, C3aR, C5aR, 14-3-3 eta by 1day; and C3, GS, CD68, C5L2 by 7 days.

Intensity C3, TNFα, Il1β, C3a/GFAP and C3aR were genes with the most prominent changes in expression.

Regions The gene expression profiles of most genes showed similar trends, with higher regulation in the ipsilateral hippocampus, when compared profiles of regions. However, the expression pattern of several genes C3, GS and TNFα seemed to be more region specific.

The results confirmed complement system involvement in processes following HI and the direct up-regulation of the expression of inflammatory cytokines during acute inflammation response. The activation of microglia increases with time and peaks after 24 hours after HI.

The results confirmed the functionality of the C3a/GFAP transgene and slight differences in expression trends of the complement receptors and inflammatory cytokines between C3a/GFAP mice and wild type mice.

6. CONCLUSION

The understanding of mechanisms following hypoxic-ischemic injury may allow us to develop treatments that can rescue neural tissue. Our study was an attempt to understand the processes that occur after experimental perinatal-asphyxia and the role of complement derived anaphylatoxin C3a using gene expression profiling.

For this purpose we selected genes and designed experiments for their expression profiles analysis. Selected genes were connected to neuromediator release and deactivation (SYN II, SYN III, GS), inflammatory cytokines (Il1β, Il6, TNFα), complement anaphylatoxin receptors (C3aR, C5aR, C5L2), growth factors (GAP43, NGF), proliferation/stem cell markers (NES, SOX2, DMN), apoptosis (Bax, Bad, BclXL, 14-3-3 eta), neuronal maturation (ENO2, TubB3) microglia activation (Aif 1, CD68 antigen) and genes related to the transgenic model used (C3a/GFAP, GFAP, C3).

Our data show that in the hippocamus, and to a lesser extent cerebral cortex, ipsilateral to the injury, the expression of number of genes differs in timing and intensity. These data help to map selected processes following hypoxic-ischemic injury in the neonatal brain. To be able to distinguish basal developmental processes from injury-induced plasticity responses in the contralateral hemisphere, it would be necessary to perform expression profiling in additional uninjured control mice at different ages.

Our data confirmed the expression of C3a/GFAP in the C3a/GFAP transgenic model in response to hypoxia-ischemia, which shows a beneficial increase in C3a before C3 expression up-regulation. However to determine its dose-effect, it would be necessary to determine the amount of C3a peptide derived from this transgene and to compare it to the C3a peptide derived from natural C3 during this time.

Slight trends towards a difference in complement receptors and inflammatory cytokine expression were seen, but we did not find any significant difference between C3a/GFAP over-expressing mice and wild type mice in the profiles of the analysed genes. The reason may be that because neuroprotective mechanism of C3a may not involve the regulation of selected genes or the effect may be only detectable at protein level. Alternatively the expression of C3a/GFAP may have only local effects, which are masked by gene expression measurments of all cells in the respective region.

7. SUPPLEMENTS

Primers and amplicon length for tested genes and a summary of the importance of the genes chosen for analysis.

G	Gene name		A	Official gene abbreviation
R	Relation to analysis			
L	Amplicon length (bp)	R	Reference	
P	Forward primer sequence		Reverse primer sequence	

G	Allograft inflammatory factor 1		A	Aif 1
R	Moderate activated microglia marker			
L	157	R	[51]	
P	ACTTCATCCTCTCTCTTCCATCC		TCTCACACTTCCCCTTTCCA	
G	BCL2-associated agonist of cell death		A	Bad
R	Apoptosis - proapoptotic			
L	102	R	[27, 30]	
P	GCAGCCACCAACAGTCATC		CTCCTCCATCCCTTCATCCT	
G	BCL2-associated X protein		A	Bax
R	Apoptosis - proapoptotic			
L	168	R	[27, 28] [29]	
P	GCTGGACACTGGACTTCCTC		CTCAGCCCATCTTCTTCCA	
G	BCL2-like 1		A	BclXL/BclX
R	Apoptosis - antiapoptotic			
L	152	R	[27, 30, 32]	
P	AGAGAGGCAGGCGATGAGTT		ACGATGCGACCCCAGTTT	
G	Beta-2 microglobulin		A	B2M
R	Reference gene			
L	/	R	tested	
P	TATAA panel of reference genes			
G	CD68 antigen		A	CD68 antigen
R	Microglia stage connected to deleterious effect to neuronal regeneration			
L	104	R	[51]	
P	GTCCCTCTTGCTGCCTCTC		GCTGGTAGGTTGATTGTCGTC	
G	Complement component 3a receptor1		A	C3aR1
R	Classical receptor for C3a anaphylatoxin			
L	141	R	[57, 62, 66, 69]	
P	TGTTGGTGGCTCGCAGAT		GCAATGTCTTGGGGTTGAAA	

G	**Complement component 5a receptor1**		A	**C5aR1**
R	Classical receptor for C5a anaphylatoxin			
L	126	R	[57, 62]	
P	TACCACAGAACCCAGGAGGA		CGCTTCGGGAGGTGAATG	
G	**Complement central component 3**		A	**C3**
R	Central component of complement activation; generating C3a			
L	130	R	[7, 64, 65]	
P	GCCTCTCCTCTGACCTCTGG		AGTTCTTCGCACTGTTTCTGG	
G	**G protein-coupled receptor 77**		A	**Gpr77 (C5L2)**
R	Novel receptor for complement derived anaphylatoxins modulating inflammatory response of complement			
L	113	R	[58-60]	
P	CCGACTTGCTTTGTTGTGTG		CAGAATGGTGATGGAGGACA	
G	**Glial fibrillary acidic protein**		A	**GFAP**
R	Expressed mainly by astrocytes; reflect maturation and activity of astrocytes			
L	125	R	[45]	
P	AACCGCATCACCATTCCT		CGCATCTCCACAGTCTTTACC	
G	**Growth associated protein 43**		A	**GAP43**
R	Neuronal regeneration			
L	245	R		
P	CACTGATAACTCCCCGTCCT		GGTCTTCTTTACCCTCATCCTG	
G	**Glutamate-ammonia ligase (glutamine synthetase)**		A	**Glul (GS)**
R	Deactivation of glutamate; maturity and activity of astrocytes			
L	142	R	[49]	
P	CGCAAAGACCCCAACAAG		ATTCCTGCTCCATTCCAAAC	
G	**Interleukin 1β**		A	**Il1b**
R	Proinflammatory cytokine; increase after HI			
L	175	R	[19, 20, 42]	
P	AGTTGACGGACCCCAAAAG		CCACGGGAAAGACACAGG	
G	**Interleukin 6**		A	**Il6**
R	Proinflammatory cytokine; increase after HI			
L	277	R	[19, 26, 42]	
P	TTCCATCCAGTTGCCTTCTT		GGTAGCATCCATCATTTCTTT	

G	**Mutant gene**		A	**(C3a/GFAP)**
R	Expression of C3a after activating GFAP promoter			
L	/	R	[72]	
P	TCAGTACAGTTGATG		TGGGCATGGAGTGGCAACTT	
G	**Nerve growth factor**		A	**NGF**
R	Neuron regeneration			
L	129	R	[86, 87]	
P	ACCACAGCCACAGACATCAA		GCACCCACTCTCAACAGGA	
G	**Nestin**		A	**Nes**
R	Intermediate filament in self-renewing cells			
L	233	R	[37, 46]	
P	AGCAACTGGCACACCTCAA		GGTATTAGGCAAGGGGGAAG	
G	**Neuronal enolase**		A	**Eno2**
R	Maturation of neurons			
L	234	R	[38]	
P	AGGCTGGCTACACGGAAA		CGTTGGCTGTGAACTTGG	
G	**Protein kinase,cGMP-dependent,typeI**		A	**PKG1**
R	Reference gene			
L	/	R	tested	
P	TATAA panel of reference genes			
G	**Synemin (desmuslin)**		A	**Dmn**
R	Intermediate filament in reactive astrocytes			
L	132	R	[44, 47]	
P	CTACCCCGACAGACCTCCTT		TTAGATACCCCTGCCACCAC	
G	**Synapsine II**		A	**SYN II**
R	Fusion of glutamate vesicle with presynaptic membrane, regulation of the reserve pool of synaptic vesicles			
L	153	R	[16]	
P	ATGGCAAAGATGGCAAAGA		GTGGAGACAGGGCAGGAG	
G	**Synapsine III**		A	**SYN III**
R	Fusion of glutamate vesicle with presynaptic membrane, regulation of the reserve pool of synaptic vesicles			
L	202	R	[16]	
P	TGCCTCCTTCTCATCTCCA		GTCGCCATTCACTTTCTTCC	
G	**SRY (sex determining region Y)-box 2**		A	**Sox2**
R	Expressed in neural stem cells, progenitor cells; important in neurogenesis			
L	93	R	[39]	
P	AAAAACCACCAATCCCATCC		AGTCCCCCAAAAAGAAGTCC	

G	**Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; eta**		A	**Ywhah;eta/ (14-3-3;eta)**
R	Apoptosis; antiapoptotic			
L	102	R	[27]	
P	CAGGATGAAGAAGCAGGAGAA		AGTGATTGTGGCAAGGAAGA	
G	**tubulin, β3**		A	**TubB3**
R	Immature neurons			
L	136	R	[40]	
	CGATGAGCACGGCATAGA		AGGTTCCAAGTCCACCAGAA	
G	**Tumor necrosis factor *α***		A	**TNFa**
R	Proinflammatory cytokine; potential secondary damage even protection			
L	143	R	[20, 24, 25]	
P	TCCCTCCAGAAAAGACACCA		CCACAAGCAGGAATGAGAAG	

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