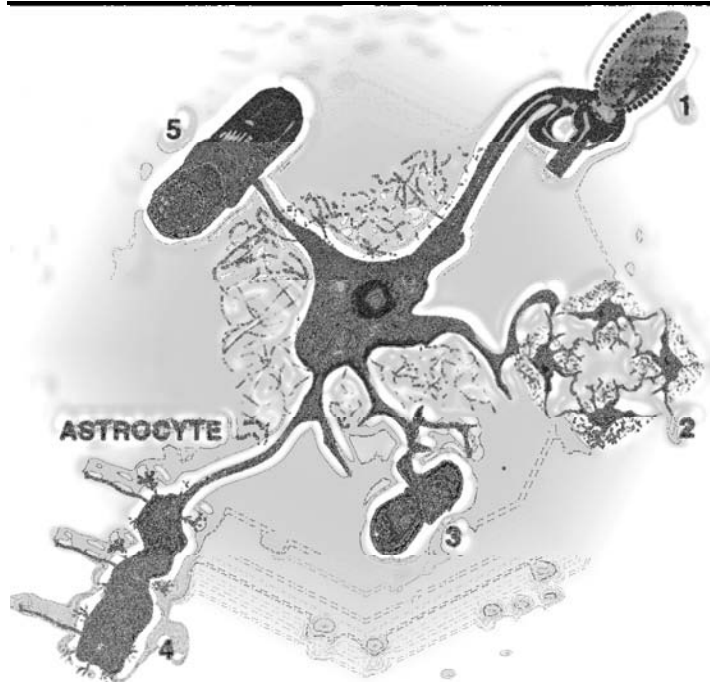




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Effect of IFN- γ , thrombin and Haloperidol on human primary astrocytes



Argiro Kalogeropoulou

Project work in Biology 2011

EXAMINERS

Μπαλατσός Νικόλαος

Λέκτορας Βιοχημείας
Τμήμα Βιοχημείας και Βιοτεχνολογίας
Πανεπιστήμιο Θεσσαλίας

Jazin Elena

Professor of Behavioral Genetics
Department of Development Genetics
Uppsala University

Γιαννούλη Σταματίνα

Διδάσκουσα
Τμήμα Βιοχημείας και Βιοτεχνολογίας
Πανεπιστήμιο Θεσσαλίας

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INTRODUCTION

Glia is a class of cells that with neurons are the basic cells of the central nervous system (CNS). The main difference between glia and neurons lies in their electrical excitability; neurons are electrical excitable cells whereas glia represents non-excitabile neural cells. Glia is consisted by the macroglia astrocytes, oligodendrocytes, chondroitin sulfate proteoglycan NG-2 positive cells and microglia (Freeman, 2010). Astrocytes are the most abundant cell type in the human brain but their function has been extensively studied only over the last decades.

Founders of glial research

The idea of the co-existence of excitable and non-excitabile elements in the brain was first promulgated in 1836 by the Swiss professor of anatomy and physiology Gabriel Gustav Valentin (1810-1883). The concept 'glia' was coined in 1858 by Rudolf Karl Virchow (1821-1902) who was one of the most influential pathologists of the 19th century. Virchow derived the term 'glia' from the Greek 'γλία' for something slimy and of sticky appearance. He initially defined glia as a 'connective substance' which forms in the brain and in the spinal cord. For Virchow, glia was a connective tissue, completely devoid of any cellular elements. The first image of neuroglial cell was obtained by Heinrich Muller in 1851 and in 1869 Jakob Henle published the first image of cellular networks formed by stellate cells (astrocytes) (Verkhratsky and Butt, 2007).

Camillo Golgi (1843) using microscopic techniques discovered a huge diversity of glial cells in the brain and found the contacts between glial cells and blood vessels. In 1893 Michael von Lenhossek proposed the term astrocyte (from the Greek for star, astro, and cell, cyte). At the end of 19th century several possible functions for glial cells were considered but until the 1980 glial cells were still regarded as passive elements of the CNS, bearing mostly supportive and nutritional roles (Golgi, 1903).

The first breakthrough discovery for astrocytes was made in 1984 when groups led by Helmut Kettenmann and Harold Kimelberg discovered glutamate and GABA receptors. Several years later, in 1990, Ann Cornell-Bell and Steve Finkbeiner found that astroglial cells are capable of long-distance communication by means of propagating calcium waves. Detailed analysis during the last two decades

demonstrated that astrocytes are capable of expressing practically every type of neurotransmitter receptor (Kettenman, 1984).

Phylogeny of glia

Glia appear early in phylogeny, even primitive nervous systems of invertebrates such as annelids and leeches, crustacea and insects, and molluscs and cephalopods contain clearly identifiable glial cells, and their study has provided a significant contribution to our understanding of glial cell physiology. Most strikingly, however, the evolution of the CNS is associated with a remarkable increase in the number and complexity of glial cells (Fig. 1A). In the leech, for example, the nervous system is organized in ganglia; each ganglion contains 20-30 neurons, which are coupled to one giant glial cell. The nervous system of the nematode *Cianorhabditis elegans* contains 302 neurons and only 56 glial cells. In drosophila, glial cells already account for ~20-25 per cent of cels in the nervous system, and in rodents about 60 per cent of all neural cells are glia (Oberheim, 2006).

In human brain, glial cells are certainly the most numerous as it is generally believed that glial cells are outnumber neurons in human brain by a factor of 10 to 50, although the precise number of cells in the brain of *Homo sapiens* remains unknown. Early estimates put a total number of neurons at ~85 billion, however, now we know that this number should be substantially larger as a cerebellum alone contains ~105 billion neurons. Therefore, the human brain as a whole may contain several hundred billion of neurons and probably several trillions of astrocytes. Morphological data for the cortex are more reliable and they show that human brain has the highest ratio among all species (0.3:1 in mice and about 1.65:1 in human brain) (Fig. 1B). Interestingly, however, the overall volume of the glial compartment remains more or less constant as they occupy about 50 per cent of the nervous system throughout the evolutionary ladder (Oberheim, 2006).

Not only does the human brain have the largest number of glia, but the glia cells in primates also show remarkable differences compared to non-primates. The most abundant astroglial cells type in human and primate brain are the protoplasmic astrocytes, which densely populate cortex and hippocampus. Human protoplasmic astrocytes are much larger and far more complex than protoplasmic astrocytes in rodent brain. The linear dimensions of human protoplasmic astroglial cells are about 2.75 times larger and have a volume about 27 times greater than the same

cells in mouse brain (Fig 1C, D). Furthermore, human protoplasmic astrocytes have about 40 main processes and these processes have immensely more complex branching than mouse astrocytes. As a result, every human protoplasmic astrocyte contacts and enwraps two million synapses compared to only 90.000 synapses covered by the processes of a mouse astrocyte (Oberheim, 2006).

Moreover the brain of primates contains specific astroglial cells, which are absent in other vertebrates. Most notable of these are the interlaminar astrocytes, which reside in layer I of the cortex, this layer is densely populated by synapses but almost completely devoid of neuronal cell bodies. These interlaminar astrocytes have a small cell body, several short and one or two very long processes which can be up to 1 mm long. Human brains also contain polarized astrocytes, which are uni- or bipolar cells which dwell in layers V and VI of the cortex quite near to white matter and they have one or two very long (up to 1 mm) processes (Colombo, 2004).

Most interestingly, the evolution of neurons produced fewer changes in their appearance. The shape and the dimensions of neurons have not changed dramatically over the phylogenetic ladder (Fig 1C). Thus, at least morphologically, evolution resulted in far greater changes in glia than in neurons, which most likely has important, although yet undetermined, significance (Oberheim, 2006)

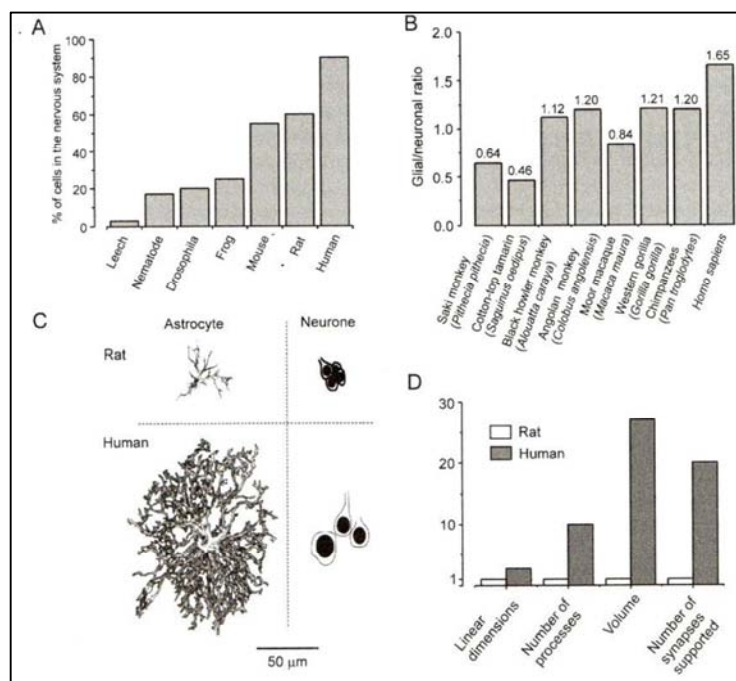


Figure 1: Phylogenetical advance of glial cells. (A) percentage of glial cells is increased in phylogenesis (B) the numbers of glia and neurons in cortex is more precisely quantified, and this graph shows the glia/neuron ratio in cortex of high primates (C) graphic representation of neurons and astroglia in mouse and in human cortex (D) relative increase in glial dimensions and complexity during evolution. (Modified from: Verkhatsky and Butt, 2007)

Astroglial morphogenesis and heterogeneity

All neural cells derive from the neuroepithelium, which forms the neural tube. These cells are pluripotent in a sense that their progeny may differentiate into neurons or macroglial cells with equal probability, and therefore the neuroepithelial cells may be defined as true neural progenitors. These neural progenitors give rise to neuronal or glial cells, neuroblasts and glioblasts respectively, which in turn differentiate into neurons or macroglial cells. For many years it was believed that the neuroblasts and glioblasts appear very early in development and that they form two distinct and non-interchangeable pools committed, respectively, to produce strictly glial lineages. It was also taken for granted that the pool of precursors cells is fully depleted around birth, and neurogenesis is totally absent in the mature brain. Recently, however, this paradigm has been challenged, as it appears that neuronal and glial lineages are much more more closely related than was previously thought, and that the mature brain still has numerous stem cells, which may provide for neuronal replacement. Moreover, it turns out that neural stem cells have many properties of astroglia (Goldman, 2003).

The modern scheme of neural cell development is as follows: At the origin of all neural cell lineages lie neural progenitors in the form of neuroepithelial cells. Morphologically, neural progenitors appear as elongated cells extending between the two surfaces of the neuronal tube. Very early in development, the neuronal progenitors give rise to radial glial cells, which are in fact the first cells that can be distinguished from neuroepithelial cells. These radial glial cells are the central element in the subsequent neurogenesis, because they act as the main neural progenitors during development, giving rise to neurons, astrocytes and some oligodendrocytes. Astrocytes are generated both from radial glia and later in development from glial precursors that also give rise to oligodendrocytes. Moreover, the radial glia cells and astrocytes that differentiate from them retain the

function of stem cells in the brain throughout maturation and adulthood (Fig. 2) (Verkhratsky and Butt, 2007).

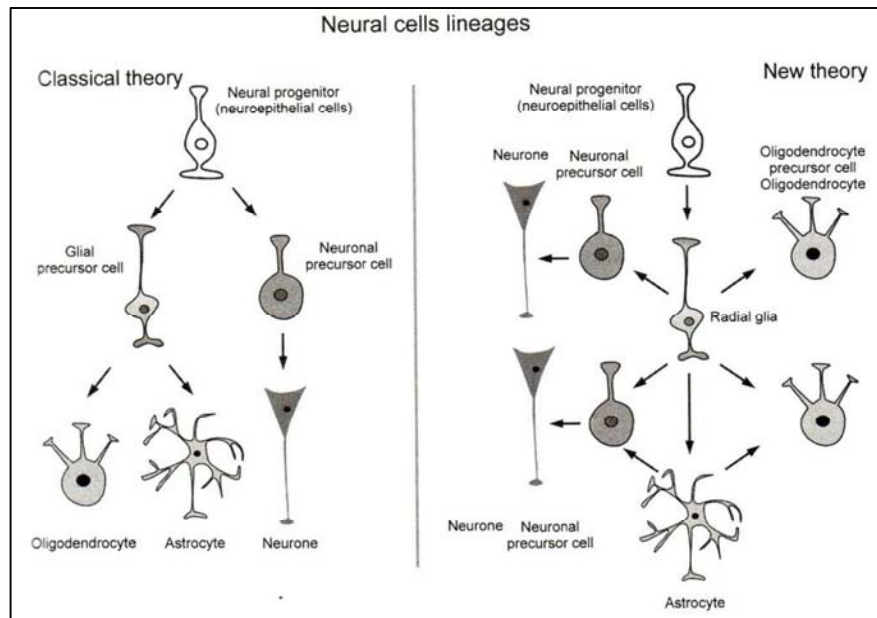


Figure 2: Modern views on pathways of neural cell development. (From: Verkhratsky and Butt, 2007)

Astrocytes are characterized by functional and morphological heterogeneity. Astroglial precursors are programmed differentially according to the brain region and the developmental stage and they give rise to diverse subtypes of astrocytes (Fig. 3) (Verkhratsky and Butt, 2007). Morphologically, the name astroglial cell is an umbrella term that covers several types of glial cell. The largest group are the ‘true’ astrocytes, which have the classical stellate morphology and comprise protoplasmic astrocytes and fibrous astrocytes of the grey and white matter, respectively (Fig 3 IV). The second big group of astroglial cells is the radial glia, which are bipolar cells with an ovoid cell body and elongated processes (Fig 3 II). In addition to the two major groups of astroglial cells, there are smaller populations of specialized astroglia localized to specific regions of the CNS, namely the velate astrocytes of the cerebellum, the interlaminar astrocytes of the primate cortex, tanycytes, pituicytes in the neurohypophysis and perivascular and marginal astrocytes. Finally, brain astroglia also include several types of cells that line the

ventricles or the subretinal space, namely ependymocytes, choroid plexus cells and retinal pigment epithelial cells.

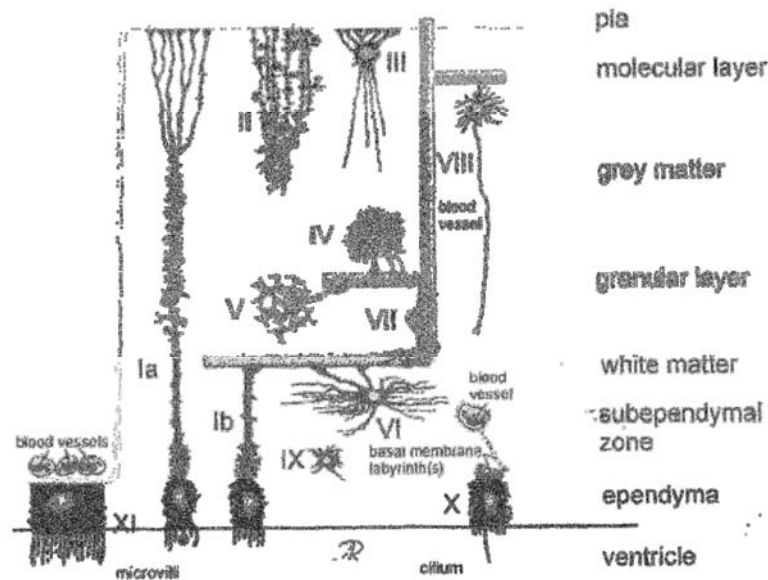


Figure 3: Morphological types of astrocytes. Ia-pial tanycyte, Ib-vascular tanycyte, II-radial astrocyte (Bergmann glial cell), III-marginal astrocyte, IV-protoplasmic astrocyte, V-velate astrocyte, VI-fibrous astrocyte, VII-perivascular astrocyte, VIII-interlaminar astrocyte, IX-immature astrocyte, X-ependymocyte, XI-choroid plexus cell. (From: Rechenbach A. and Wolburg H., 2005)

The lack of reliable astrocyte specific markers for distinguishing specific developmental stages and specific subtypes of astrocytes complicates their study. However, there are some basic criteria for astrocytes that are common among literature. First of all astrocytes are not excitable like neurons, they have a negative membrane potential, they express the Glial Acidic Fibrillary Protein (GFAP) and glutamate transporters, they contain glycogen granules and they communicate with adjacent astrocytes through gap junctions. Additionally, astrocyte processes are in contact with the blood vessels and the synapses (Kimelberg, 2010).

Astrocyte function in the CNS

Astrocytes cover a wide range of functions and therefore they are very important for the proper development and function of the brain. Astroglial cells form pathways for the migration of newborn neurons and control the formation of the synapses during development (Raponi et al., 2007). Astrocytes retain their

multipotency and give rise to new neurons and glial cells during adulthood (Raponi et al., 2007; Verkhratsky and Butt 2007). Moreover, they support the structure of the brain and they are the main cells that maintain the blood-brain barrier (Nag, 2011). Astrocytic processes surround the synapses and absorb the excess of glutamate, that preventing glutamate induced neurotoxicity (Krencik et al, 2011). At the same time they provide metabolic substrates to neurons (Rouach et al, 2008; Verkhratsky and Butt, 2007). Astrocytes can also release a variety of gliotransmitters such as 5'-triphosphate, glutamate and D-serine, and control synaptic plasticity (Zhang and Haydon, 2005). Finally, astrocytes control the ion-distribution, the pH and the water homeostasis through ion-channels and specific aquaporins (Verkhratsky and Butt, 2007).

In detail, neurogenesis in adult humans is restricted to the hippocampus and subventricular zone. In both locations, the stem elements that produce neurons are astroglia. These 'stem' astrocytes have the morphology, physiology and biochemical markers characteristic for astrocytes: they express GFAP, have negative resting membrane potentials and they are non-excitabile. 'stem' astrocytes different from mature astrocytes by specific expression of the protein nestin and some of them form cilia. Neurons born in the subventricular zone migrate to the olfactory bulb, whereas those produced in the hippocampus remain there and integrate themselves into existing neuronal networks. 'stem' astrocytes residing in the hippocampus and subventricular zone are multipotent, as they give birth to both neurons and glia (Mori et al, 2005).

In contrast to neurogenesis, gliogenesis occurs everywhere. New glial cells are born locally and the locality also mainly determines the type of glial cell produced, as it was referred previously. In the subcortical white matter most of the newly produced glial cells are oligodendrocytes, whereas in the spinal cord astrocytes and oligodendrocytes are produced roughly in the same quantities (Mori et al, 2005).

The first neural cells to develop are radial glia. After this, neural precursors in the ventricular zone (VZ) and subventricular zone (SVZ) immediately surrounding the lumen of the neural tube migrate to their final destinations and give rise to the enormously diverse range of neurons and glia found in the adult brain. An important function of foetal glial cells is to provide the scaffolding along which neural precursors migrate. Not all neurons migrate along radial glia, but a is

always the case where neurons are organized in layers such as the cerebellum, hippocampus, cerebral cortex and spinal cord. After neurons reach their final sites, they extend axons, which in some cases grow for considerable distances and have to cross the brain midline to reach their synaptic targets. Channels formed by astrocytes provide a mechanical and guidance substrate for axon growth. In the corpus callosum, for example, astrocytes form a bridge that connects the left and the right sides of the telencephalon. The ability of astrocytes to support axon growth decreases with age, embryonic astrocytes strongly support axon growth, whereas mature astrocytes inhibit axon growth (Alvarez-Buylla et al, 2001).

Protoplasmic astrocytes in the grey matter are organized in a very particular way, with each astrocyte controlling its own three-dimensional anatomical territory. Individual astrocytes establish contacts with blood vessels, neurons and synapses residing within their anatomical domain. Using clearly delineated anatomical territories, astrocytes divide the whole of grey matter into separate domains, the elements of which are integrated via the processes of protoplasmic astrocytes. The astrocytic processes provide for local signalling within the domain as their membranes that contact neurons, synapses and blood vessels are packed with receptors, which sense the ongoing activity (Allen and Barres, 2005).

Astrocytes are not very much involved in the blood-brain barrier function per se, but astrocytes are important in the regulation of the blood-brain interface as a whole. Astrocyte endfeet membranes are enriched with numerous receptors, transporters and channels which mediate glial-endothelial communication and regulate exchange through the glial-vascular interface. It appears that all astrocytes participate in the glial-vascular interface, through which the astrocytes maintain the exchange between blood and its own territory, thus establishing metabolically independent glia-neurone-vascular units. Astrocytes also play a central role in regulation of the local vascular tone, hence linking the metabolic demands of grey matter with local blood supply (Verkhratsky and Butt, 2007).

The notion that astroglial cells provide a metabolic connection between neurons and blood vessels was initially made by Camillo Golgi in the 1870s. Recent advances in in situ cellular imaging have clearly demonstrated that astroglial Ca^{2+} signals triggered by neuronal activity enter endfeet and initiate the release of vasoactive substances, which in turn affect the tone of small arterioles wrapped

by these endfeet. In fact, astrocytes are able to provide dual control over the neighbouring blood vessel: they may either induce vasodilation or vasoconstriction.

Maintenance of the extracellular ion composition is of paramount importance for brain function, because every shift in ion concentration profoundly affects the membrane properties of nerve cells and hence their excitability. Apart from preserving their own transmembrane ion homeostasis, astroglial cells are heavily involved in maintenance of extracellular ion concentrations. As neuronal activity inevitably associated with influx of Na^+ and Ca^{2+} and efflux of K^+ the extracellular concentration of these ions vary. Astrocytes help prevent the accumulation of extracellular K^+ , thereby stabilize neuronal activity (Kofuji and Newman, 2004).

Astrocytes regulate water exchange between blood and brain and within the brain compartments through numerous aquaporins, the latter being particularly concentrated in the endfeet enwrapping blood vessels. On a systemic level, water homeostasis in the brain is controlled by several neuropeptides produced and released by neurosecretory cells (Simard and Nedergaard, 2004).

The brain produces energy by oxidizing glucose. The brain receives glucose and O_2 from the blood supply. Neurones account for about 90 per cent of brain energy consumption, and glia cells are responsible for the remaining 10 per cent. Neurons require a continuous supply of energy to fuel their Na^+ - K^+ ATPases, which are constantly active to maintain ion gradients across neuronal cell membranes in the face of the continuous ionic fluxes during synaptic activity and action potential propagation. However, monitoring the distribution of glucose in the brain tissue has demonstrated that it is accumulated equally by neurons and astroglial cells. This implies the involvement of an intermediate product of glucose utilization, which is produced by astrocytes and subsequently transported to neurons. It turns out that astrocytes are ideally situated and have the biochemical machinery to provide metabolic support for neurons via the so-called 'astrocyte-neuron lactate shuttle (Auld and Robitaille, 2003).

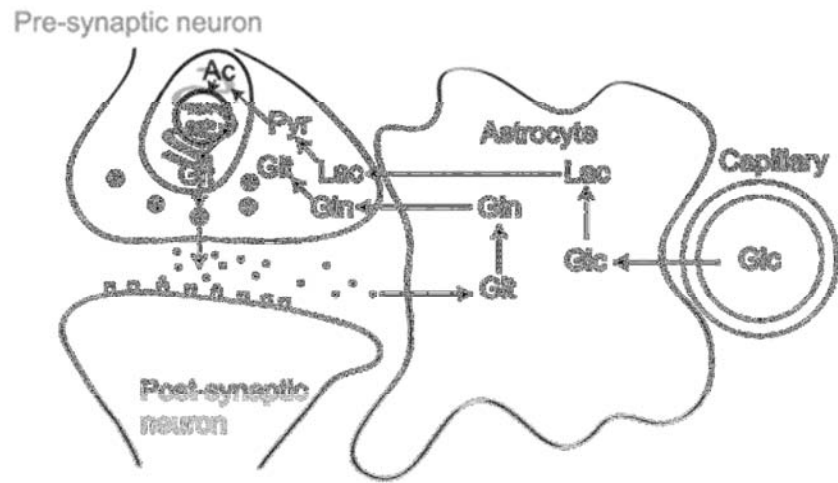


Figure 4: Schematic of the proposed mechanism of metabolic trafficking in the brain. Glucose from the vasculature is metabolized by astrocytes to lactate in the cytosol. Lactate is then exported into the extracellular fluid, and taken up by neurons, which utilize it via the TCA cycle. This first involves conversion (in the neuron's cytosol) of lactate to pyruvate, which is transported into the mitochondrion, and converted into acetyl-CoA, which then feeds into the TCA cycle. A portion of the TCA cycle intermediate α -ketoglutarate is converted into the spin-off product glutamate (From: Gallagher, 2009).

Astroglia affect neuronal synaptic transmission in several ways. First, astrocytes modulate synaptic strength by controlling the concentration of neurotransmitters in the cleft via glial transporters. This is the case for most synapses that use amino acid neurotransmitters, such as glutamate, GABA and monoamines, where astrocytes express specific transporters, depending on the synapse. Second, 'glial' transmitters released from astrocytes also affect synaptic transmission in neighbouring neuronal circuits. And third astrocytes are also capable of modulating synaptic transmission through the release of ATP (Auld and Robitaille, 2003).

Reactive astrogliosis

One of the most important role of astrocytes is that they have a specific mechanism to protect the brain. Astrocytes respond to different forms of CNS insults including brain trauma, ischemia, infection or neurodegenerative disease by the process called reactive astrogliosis. reactive astrogliosis is a defensive brain reaction which is aimed at (a) isolation of the damaged area from the rest of the CNS tissue (b) reconstruction of the blood-brain barrier and (c) facilitation of the remodelling of brain circuits in areas surrounding the lesioned region. (Verkhratsky and Butt, 2007). These main tasks are solved into two distinct ways: the reaction of

astrocytes close to the insult is very different from that in astroglial cells positioned at a distance from the primary lesion.

This reactive condition is divided into two stages (Fig. 5). During the first stage reactive astrocytes overexpress GFAP and become hypertrophic. Later, they release growth factors and cytokines (Verkhratsky and Butt, 2007; Sofroniew, 2009). The second stage of astrogliosis occurs only under severe insults and includes the glial scar formation. The scar is formed from newly proliferating cells which origin is not known yet. The role of the scar is to form a border between the healthy and the damaged tissue (Fig. 5).

Astrocytes located immediately around the damaged zone undergo a robust hypertrophy and proliferation, which ultimately ends up in complete substitution of previously existing tissue architecture with a permanent glia scar, this process is called anisomorphic astrogliosis. In astrocytes more distal to the lesion, the reactive changes are much milder and although astroglial cells modify their appearance and undergo multiple biochemical and immunological changes, they do not distort the normal architecture of the CNS tissue, but rather permit growth of neurites and synaptogenesis, thus facilitating the remodelling of neuronal networks. This type of astrocyte reaction is defined as isomorphic astrogliosis (Verkhratsky and Butt, 2007).

The primary signals, which trigger both forms of astrogliosis, derive from damaged cells in the core of the insult, and are represented by neurotransmitters, cytokines and adhesion molecules, growth factors and blood factors. The actual combination of these 'damage signals' and their relative concentrations most likely determine the type of astrogliosis experienced by astrocytes in different regions surrounding the initial zone (Verkhratsky and Butt, 2007).

On a cellular level, insults to the brain, be they ischaemia, trauma or inflammation, result in hypertrophy of the astroglial processes and a significant increase on the astrocyte cytoskeleton. The biochemical hallmark of astrogliosis is the upregulation of synthesis of GFAP. In animals with genetically deleted GFAP the astroglial scar is formed slower, it is much less organized and the healing of brain traumas is generally prolonged (Penky, 2005).

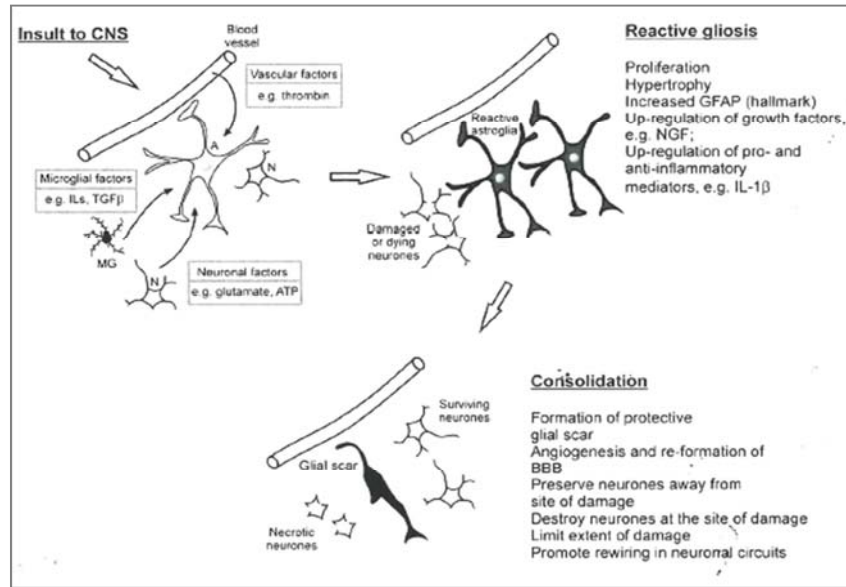


Figure 5: Stages of astrogliosis. First stage: innate factors that are released during brain insults trigger the activation of astrocytes, which become hypertrophic and proliferate intensively. Second stage: the damaged issue is replaced by a permanent glia scar. (From: Rechenbach and Wolburg, 2005)

(ILs: Interleukins, TGFβ: Transforming Growth Factor beta, NGF: Nerve Growth Factor, BBB: Blood Brain Barrier)

Role of astrocytes in the nervous system pathology

Because of the plethora of functions performed by astrocytes, particularly their protective role against brain insults, it is not surprising that these cells are involved in many diseases. On Table 1 are listed brain diseases in which astrocytes dysfunction is involved.

Table 1: Diseases in which astrocytes dysfunction is involved.

Brain diseases	Astrocytes pathology	Literature
Alexander's disease	Astrocytes express mutated GFAP and develop Rosenthal fibres	Brenner et al., 2009
Niemann-Pick disease Type C	Astrocytes express mutated NPC1 and cause neurodegeneration	
Alzheimer's disease	Astrocytes accumulate amyloid-β become	Rodríguez et al., 2009

	reactive and cause neuronal injury	
Parkinson	Astrocytes are activated and attack neurons in psecific brain areas	Sidoryk-Wegrzynowicz et al., 2011
Amyotrophic lateral sclerosis (Lou Gehrig's disease)	Astrocytes loose their function in glutamate clearance and cause toxicity	Seifert et al., 2006
Schizophrenia	Reduced number of astrocytes	McKenzie et al., 2001
Depression	Spreading depression waves cause astrogliosis	Verkhatsky and Butt, 2007
Stroke	Astrocytes are unable to provide metabolic substrates to neurons	Takano, 2009
Gliomas	Brain tumors developed by astroglia	Sontheimer, 2003

QKI- a new candidate gene for scizophrenia

Schizophrenia is a neuropsychiatric disorder that affects 1% of the population worldwide (Aberg, 2005). Twin and adoption studies have shown that heritability of the disease is 80%. This indicates that both environmental and genetic factors are involved in the development of the disease. Environmental factors that are already proposed to be involved in schizophrenia affect the development of the brain. For example maternal infections during pregnancy have toxic effects on embryo's neurons (Urakubo et al., 2001). Additionally, fetal hypoxic or ischemic damage that can be due to complications during pregnancy or delivery are also related to abnormal development of the CNS (Miyamoto et al., 2003). Although genetic factors are strongly involved in the etiology of Schizophrenia there is not a Mendelian inheritance as would be expected. The explanation for this is that more than one gene are responsible for the pathologic phenotype of the disease (Riley and McGuffin, 2000).

Association, linkage and expression studies suggest that *the quaking homolog KH domain RNA binding* or *QKI*, is a novel candidate gene for schizophrenia (Lindholm et al, 2004). *QKI* belongs to the STAR protein family (Signal transduction and Activation of RNA). The members of this family regulate post-transcriptionally the mRNA levels of various genes by directly binding to them in specific regions called QKI Response Element (QRE) (Galarneau and Richard, 2005). In humans, *QKI* is expressed in 4 isoforms with different size (*QKI-5*, *QKI-6*, *QKI-7*, *QKI-7b*), which derive from alternative splicing of the same transcript. In the adult nervous system, *QKI* gene is exclusively expressed in glial cells. To date, the functional studies of *QKI* focused mainly on its role in oligodendrocytes, since white matter alterations have been associated with schizophrenia (Lindholm, 2004). These studies revealed that *QKI* is necessary and sufficient to promote oligodendrocyte differentiation and myelination (Chen et al., 2007). Aberg et al. have found that the expression of *QKI-7* and *QKI-7b* is downregulated in SCZ patients. It has been further proposed that abnormalities in alternative splicing of the gene may explain disrupted expression of several myelin-related genes observed in the patients (Aberg et al., 2006)

Interestingly, *QKI* is also expressed in astrocytes and it has been shown that *QKI-7* specifically regulates the interferon related genes in human astrocytoma cells (Jiang, 2010). These results suggest that *QKI* has different function in oligodendrocytes and astrocytes. Further studies performed in this group revealed that *QKI* regulates expression of several structural and functional genes in human primary astrocytes (unpublished data). The physiological consequences of these *QKI*-driven expression changes in astrocytes are to be determined.

However, it cannot be ruled out that alterations of astrocytic *QKI* may contribute to the phenotype observed in SCZ patients. In fact, the nature of cells with reduced *QKI* expression is not known. Furthermore, a role of astrocytes in schizophrenia has already been proposed (Jiang et al., 2010).

Further studies are necessary to reveal the role of specific *QKI* splice variants in astrocytes. Since astrocytes are highly responsive to all types of brain insult, it is reasonable to investigate the role of *QKI* gene not only under normal physiological conditions, but also in the context of reactive astrogliosis that is a consequence of such insult.

Aim

The main purpose of this project was to develop an in vitro system that is suitable to study a specific function of QKI in human primary astrocytes under different physiological conditions. 1. We aimed to mimic astrocyte activation by treatment of human primary cells with thrombin and interferon- γ (IFN- γ) – potent inducers of astrocyte activation in rodents. Additionally, we treated cells with haloperidol, an antipsychotic drug commonly used in the treatment of schizophrenia. 2. We studied the effect of QKI depletion in normal, and activated human astrocytes.

MATERIALS AND METHODS

Cell cultures

Primary human astrocytes (HA) (ScienCell) were cultivated in the astrocytes medium (ScienCell) supplemented with 2% fetal bovine serum (FBS), 1% Astrocyte Growth Supplement (AGS) and 1% penicillin/streptomycin (PS) (ScienCell). Cells (2nd passage HA) were plated onto poly-L-lysined (ScienCell) 75 ml culture flasks (Costar). After reaching 80% confluence cells were removed from the flasks using trypsin and they were plated onto 6-well plates (Costar) at the density 5.000 cells/cm². No antibiotics were added to the medium after passaging. All cultures were maintained at 37°C and 5% CO₂.

Treatments

We treated the cells with three different reagents: IFN- γ , thrombin and haloperidol. IFN- γ was obtained from PeproTech reconstituted with MiliQ water and further diluted into working solution with PBS containing 0.1% bovine serum albumin (BSA). Cells were treated with 10 ng/ml IFN- γ for 6h and 24h. Thrombin was obtained from Sigma and it was reconstituted with 0.1% BSA solution. Cells were treated with 1U/ml thrombin for 6h and 24h. Haloperidol (Sigma) was reconstituted in the plating medium to final concentration 0.2 μ M, which is the same concentration detected in the blood plasma of treated patients. The duration of Haloperidol treatment was 6h and 24h.

RNA extraction and real time RT-PCR

RNA was isolated from the cells using the PureLink RNA MiniKit (Invitrogen) according to the manufacturer's instructions. The concentration and purity of the extracted RNA was measured using NanoDrop ND1000 Spectrophotometer. RNA integrity was monitored by agarose gel electrophoresis (two ribosomal bands 18S and 28S were intact in all samples). RNA samples were stored at -80°C until use for RT-PCR.

From each biological replicate, 200 ng of RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The final concentrations of reagents were: 1x RT Buffer, 5.5 mM MgCl, 0.5 mM dNTPs (each), 2.5 μ M random hexamers, 0.4 U/ml RNase inhibitor and 1.25 U/ μ l reverse

transcriptase in RNase free water to a final volume 10 μ l. The mix was incubated at 25°C for 10min. (primer annealing), 48°C for 1h (synthesis) and 95°C for 10min. (enzyme inactivation). cDNA samples were diluted with RNase-free water to a final concentration 2.5 ng/ μ l and stored at -20°C.

All real-time PCR experiments were performed in ABI Prism 7000 Sequence Detector System (Applied Biosystems) using 96-well optical plates (ABI). The reaction mix included 4 μ l of cDNA sample, 9.5 μ l of MiliQ water, 0,75 μ l (10 μ M) of forward primer , 0,75 μ l (10 μ M) of reverse primer and 10 μ l of Power SYBR ® Universal PCR Master Mix (ABI). Sequences of all primers are listed in Table 2. The thermal cycle was as follows: 50°C for 2min., 95°C for 1min., 40 cycles of 95°C for 15sec. and 60°C for 1min. each. A dissociation step was added for each gene run. Gene expression was quantified using standard curve method and normalized against two housekeeping genes, GAPDH (Glyceraldehyde 3-phosphate Dehydrogenase) and VIM (vimentin).

Table 2: Genes and primers used in RT-PCR

Gene	5'-Forward Primers-3'	5'-Reverse Primer-3'
GAPDH	GGAAGCTCACTGGCATGGC	TAGACGGCAGGTCAGGTCCA
B2M	GTGCTCGCGCTACTCTCTCT	TCTCTGCTGGATGACGTGAG
VIM	CGAAAACACCCTGCAATCTT	ATTCCACTTTGCGTTCAAGG
ACTB	GAGCTACGAGCTGCCTGACG	GTAGTTTCGTGGATGCCACAGGACT
GFAP	AAGAGGAACATCGTGGTGAAGAC	CTGCCTCACATCACATCCTTGT
GBP1	GCAGTTGCTAGAAAGAGCAAGAGA	TCCCTCTTTTAGTAGTTGCTCCTGTT
GLUL	CACCTCAGCAAGTTCCCACT	GAGCCATCGAAATTCCACTC
QKI-T	GCACCTACAGAGATGCCAACATTA	CTGGGCTGTTGCTGCAAGA
QKI-7	GCTACATCAATCCTTGAGTATCCTATTG	CAGGCATGACTGGCATTTC

Immunocytochemistry

Human astrocytes were cultured on poly-L-lysined coverslips inserted into 6-well plates. Following treatment, cells were fixed in 4% paraformaldehyde (PFA) for 15min. at room temperature. Each well was rinsed three times with PBS and then the blocking solution (0.25% Triton X-100, 0.25% BSA in PBS) was added for 1h.

Subsequently the cells were incubated with anti-GFAP chicken antibody (AbCam) diluted 1:1000 in blocking solution, and kept overnight at 4°C. After rinsing three times with PBS the secondary antibody Alexa647 goat-antichicken (Invitrogen) was applied (1:400) for 2h at room temperature. After rinsing with PBS the cells were stained with DAPI (Sigma). Finally the slides were mounted in DTG mounting solution. The slides were viewed and analyzed using Olympus microscope and images were finalized with the Image-J software.

RNA silencing

QKI gene was silenced in human primary astrocytes using stealth RNAi siRNA duplexes targeting all QKI splice variants (QKI-t) (Invitrogen). The sequence of the siRNA duplex was as follows: 5'-UAUAGAUUAAACCAGCUUCGGGCCC-3', 3'- AUAUCUAAUUUGGUCGAAGCCCGGG-5'. These siRNAs were targeting exon6 which is common for all QKI splice variants (Figure 3). As a control we transfected cells with scramble stealth RNAi siRNA (Invitrogen). All transfections were performed in biological duplicates using Lipofectamine 2000 (Invitrogen), following manufacturer's instructions. The final concentration of targeting and scramble siRNA in each well was 25 nM.

In order to study whether QKI silencing may modify the response of human astrocytes to IFN- γ - activation, we treated QKI-silenced and scramble cells with IFN- γ 24 h after transfection. Cells were then harvested 72h after transfection.

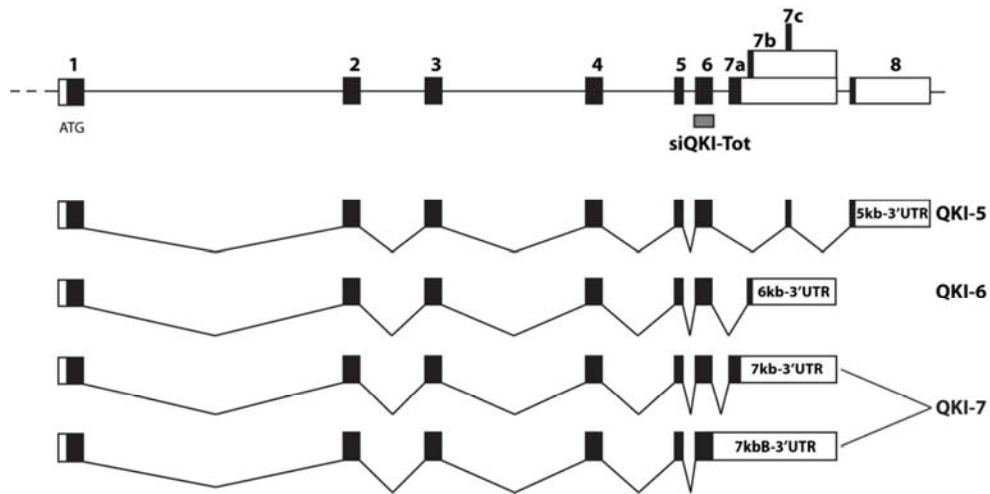


Figure 6: The structure of the quaking gene and the four isoforms in the human brain. Exon 1-6 are common for all splice variants. siQKI-t is targeting exon 6 and suppress the expression of all QKI splice variants. (Modified from: Jiang, et al. 2010)

Statistical analysis

Statistical analysis was performed using two-tailed t-tests. The significance level is indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ accordingly. Error bars indicate the standard error of the mean for three biological replicates.

RESULTS

Gene expression analysis of HA treated with thrombin.

To test the effect of thrombin on astrocytes we treated primary human astrocytes with 1 U/ml thrombin and measured gene expression after 6h and 24h using real-time PCR. After 6h of treatment only QKI-7 was affected and it was up-regulated although after 24h it showed a tendency for down-regulation. GFAP and GLUL have been significantly upregulated after 24h of treatment. All the other genes remained stable until the end of the treatment (Fig. 7).

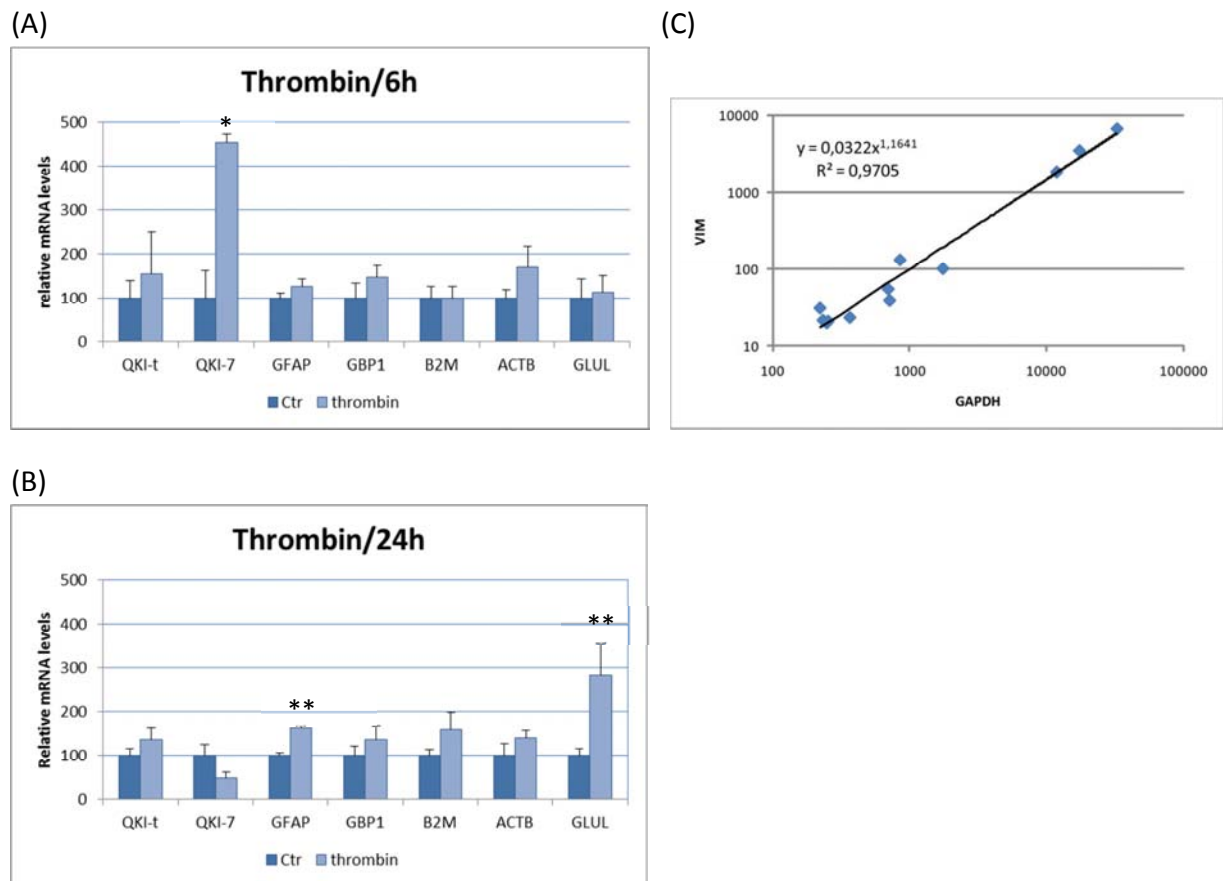


Figure 7: Gene expression analysis of primary human astrocytes treated with 1u/ml Thrombin for (A) 6h and (B) 24h. The mRNA expression levels of thrombin treated cells are relative to untreated cells (Ctrl) which mRNA level is defined as 100. (C) Regression plot of normalization genes after the treatment with Thrombin.

Gene expression analysis of HA treated with Haloperidol

Accordingly we treated primary human astrocytes with 0.2 μM of the antipsychotic drug Haloperidol and analyzed their gene expression. Gene analysis after 6h of treatment revealed an increase in the expression of GFAP and B2M and a tendency for increase in the expression of QKI-7 and GLUL. QKI-t, GBP1 and ACTB remained stable. After 24h the only significant change was the increase of GFAP. The rest of the tested genes were not affected by the treatment (Fig. 8).

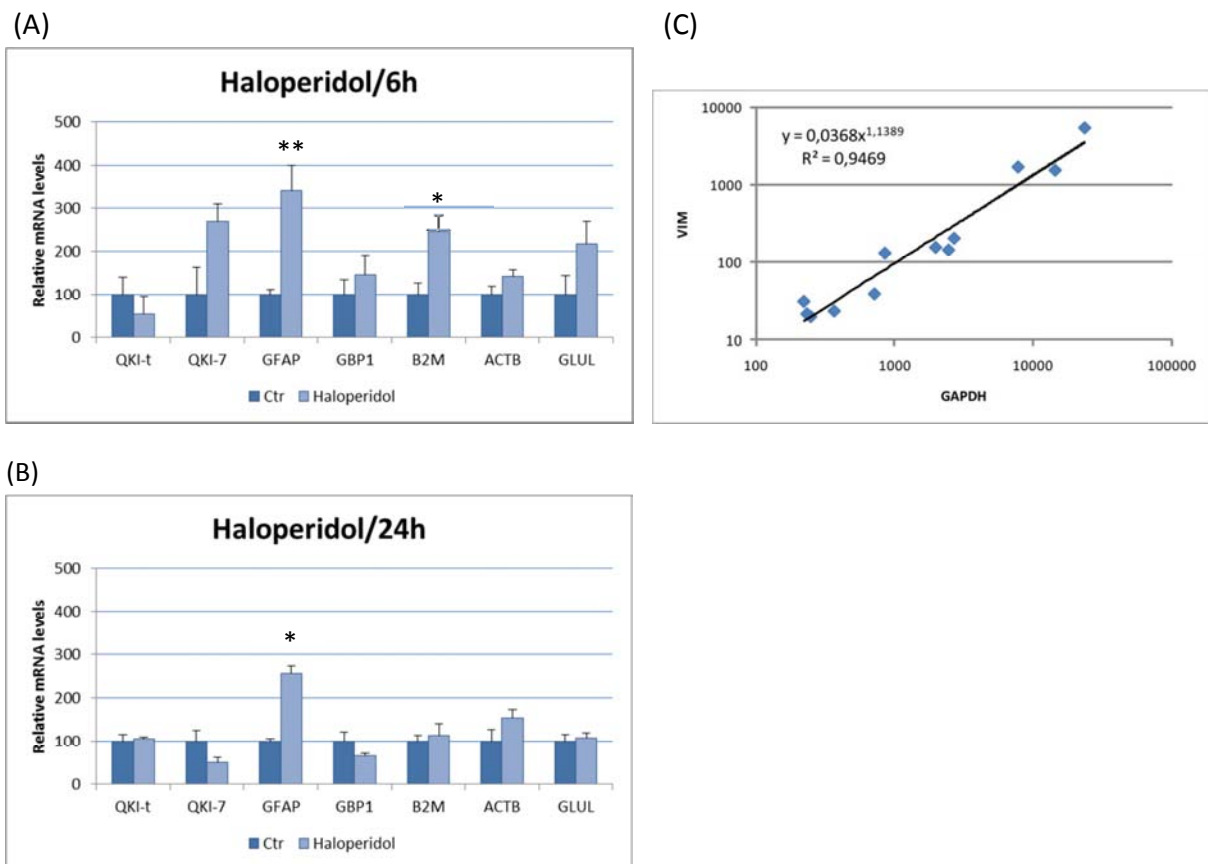


Figure 8: Gene expression analysis of primary human astrocytes treated with 0.2 μM Haloperidol for (A) 6h and (B) 24h. The expression levels are expressed relative to untreated cells (Ctr) which mRNA level is defined as 100. (C) Regression plot of normalization genes after the treatment with Haloperidol.

Gene expression analysis of HA treated with IFN- γ

We tested the effect of IFN- γ on human primary astrocytes. We treated primary cells with 10 ng/ml IFN- γ and analyzed gene expression with real-time PCR after 6h and 24h. We observed an upregulation of the interferon-related genes GBP1 and B2M after 6h of treatment. These changes became even more pronounced after 24h of treatment. On the other hand QKI-7 and GFAP were down-regulated after 6h and 24h. (Fig. 9).

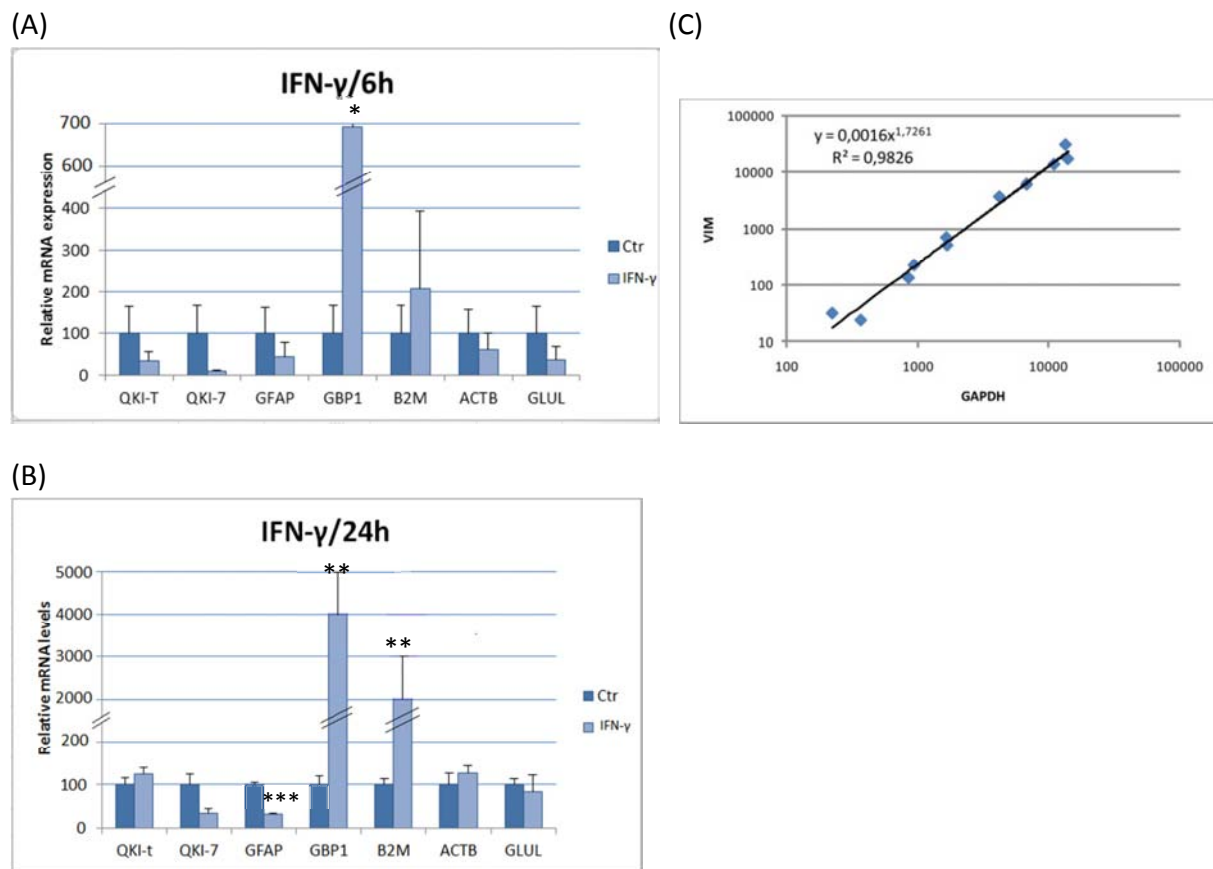


Figure 9: Gene expression analysis of primary human astrocytes treated with 10 ng/ml IFN- γ for (A) 6h and (B) 24h. The expression levels are expressed relative to untreated cells (Ctrl) which level is defined as 100. (C) Regression plots of genes which are used for normalization of the tested genes.

Changes in cell viability and morphology after treatment

At the end of each treatment the cultures were studied with a phase-contrast microscope. Although initially all cultures were plated at the same density, cells treated with IFN- γ for 24 h seemed to be more dense compared to the untreated cultures. The density of cultures that were treated with thrombin or haloperidol was similar to the control. The higher density of the IFN- γ treated cells suggests that IFN- γ may induce cell proliferation rate (Fig. 10)

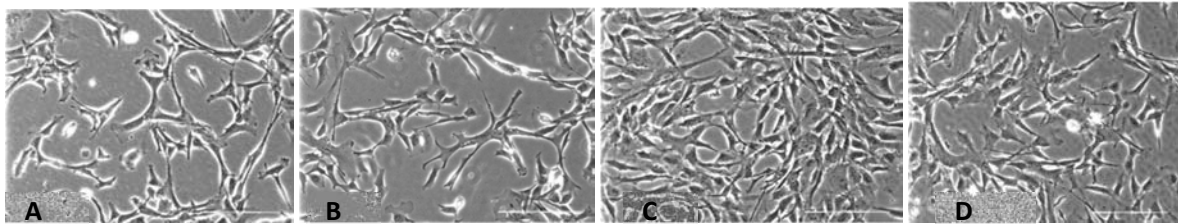


Figure 10: Phase contrast images of human astrocytes treated with different reagents for 24h. (A) Control culture of human astrocytes (B) Human astrocytes treated with 0.2 μ M Haloperidol (C) Human astrocytes treated with 10 ng/ml IFN- γ (D) Human astrocytes treated with 1 U/ml thrombin. Scale bar 20 μ m.

In order to examine morphological differences between the treated and control cells we performed immunocytochemical staining using antibodies against GFAP, a cytoskeleton protein commonly used as a specific marker for astrocytes. A control culture of primary human astrocytes was morphologically heterogeneous. After the treatment with IFN- γ the cell's morphology changed substantially and many longitudinal cells with thin long processes were observed. We also noticed that fewer processes were formed (Fig 11). On the other hand, the cells treated with thrombin or haloperidol seemed larger and had thicker processes. Furthermore these cells developed higher diameter processes (Fig. 11).

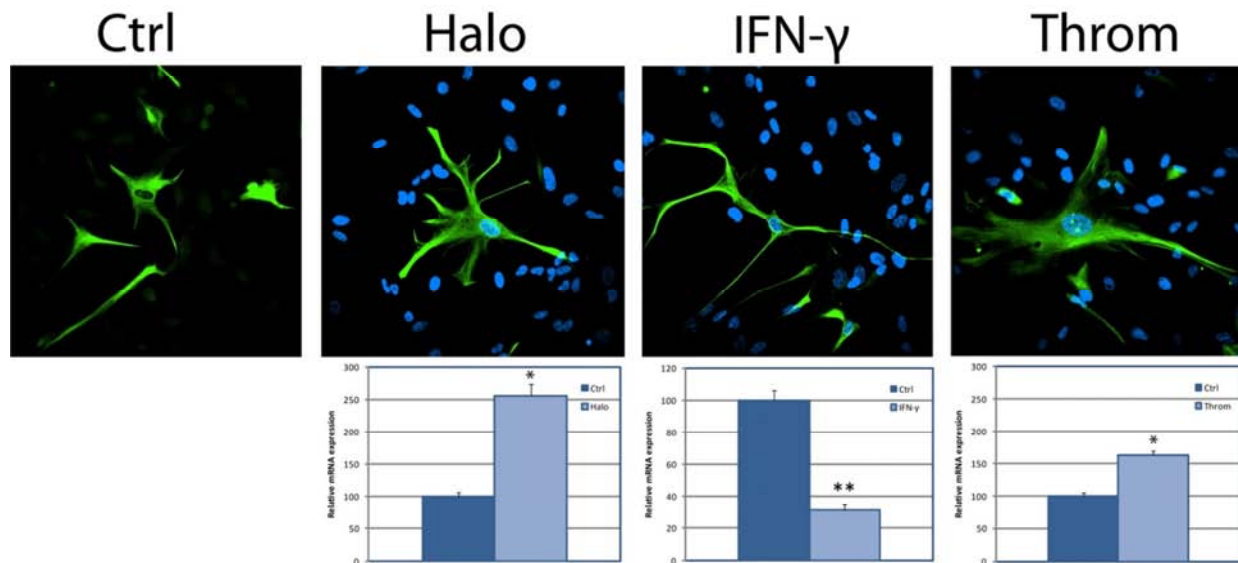


Figure 11: Changes in GFAP expression have an affect on astrocytes' morphology. Immunocytochemical staining of GFAP in astrocytes treated with three different reagents for 24h. The diagramms show changes in GFAP expression according to each treatment. The pictures were taken at 40x magnification.

Silencing the expression of QKI-t in HA treated with IFN- γ

In order to study the function of QKI in activated astrocytes we tested how primary human astrocytes react to stimulation with IFN- γ when QKI is not expressed. First we silenced the expression of the *QKI* gene using small interference RNA complementary to the QKI-t transcript and then we treated the cells with 10 ng/ml IFN- γ for 48h. After the treatment we analyzed the genes expression and the morpogological changes of the cells. At the end we had four groups of cells: Scram – transfected with scramble siRNA, untreated; siQKI-Tot – transfected with siRNA targeting QKI-t, untreated; Scram IFN- γ – transfected with scramble siRNA, treated with IFN- γ ; siQKI-Tot IFN- γ - transfected with siRNA targeting QKI-t and treated with IFN- γ .

The expression levels of QKI-t after 48h of transfection was at 50% of control level (Fig. 11) Other genes that were down-regulated after the transfection were GFAP, B2M and GLUL. In contrast, GBP1 was up-regulated and ACTB was stable.

The treatment with IFN- γ for 48h had an effect on gene expression as in the previous experiment. Briefly, GBP1 and B2M were overexpressed, QKI-7 and GFAP were decreased and QKI-t and ACTB were not affected. We next measured gene expression in the cells that were both transfected with siQKI-Tot and treated with IFN- γ we observe that the down-regulated genes after the IFN treatment (Scram IFN- γ) had recover. QKI-7 and GFAP were almost in control levels and ACTB and GLUL were slightly increased. On the other hand the interferon related genes remained highly expressed even after the transfection.

Phase contrast images also revealed that IFN- γ caused morphological changes in astrocytes that are not affected by the silencing. After the IFN- γ treatment the cells developed long and thin processes (black arrows in Fig. 13)

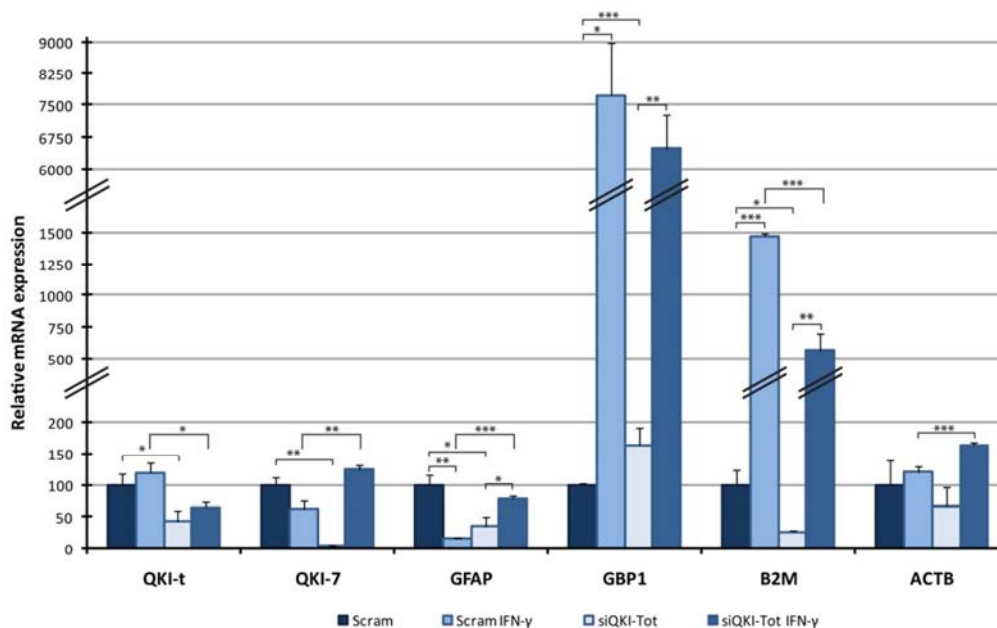


Figure 12: Gene expression analysis of primary human astrocytes transfected with siRNA targeting QKI-t and treated with 10 ng/ml IFN- γ for 48h. The diagram depicts the mRNA levels of untreated cells (Scram), cells treated with IFN- γ (Scram IFN- γ), cells transfected with QKI-Tot siRNA (siQKI-t) and cells transfected with QKI-Tot siRNA and treated with IFN- γ (siQKI-t IFN- γ). Error bars indicate the standard error of the mean for four replicates (two biological duplicates, two technical duplicates)

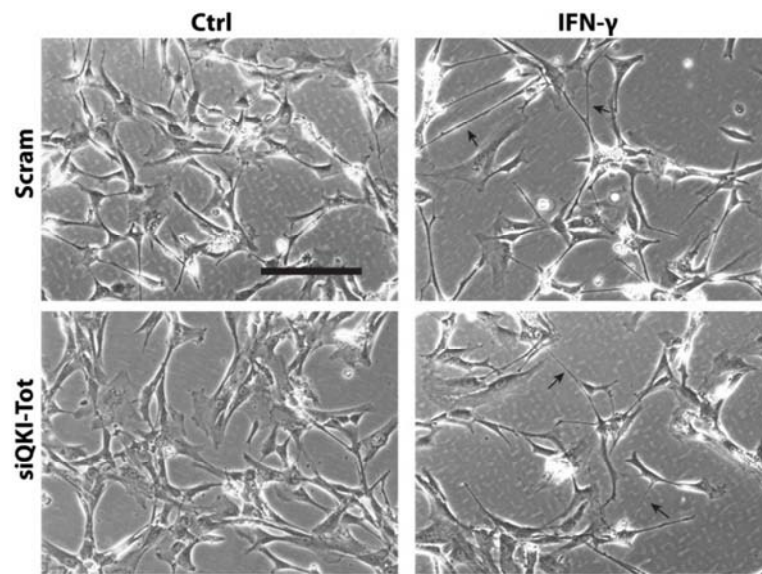


Figure 13: Phase contrast images of human astrocytes transfected with siQKI-Tot and treated with 10 ng/ml IFN- γ for 48h. The black arrows in the treated cells indicate the longitudinal processes that are developed after the treatment. Scale bar 20 μ m.

DISCUSSION

We treated primary human astrocytes with two reagents that are known from literature to trigger astrogliosis in the brain after treatment (Niego et al., 2011; John et al., 2003). We also treated astrocytes with an antipsychotic drug, which affects astrocytes (Jiang et al., 2009). We measured the expression of specific astrocytic markers, cytoskeleton genes and genes that are involved in immunological respond. It is already known that the expression of these three groups of genes is modified during astrogliosis (Verkhatsky and Butt, 2007). We also measured the expression of QKI-t and its splice variant QKI-7 in order to study if the expression of this gene changes under conditions that cause astrogliosis. Each treatment affected the astrocytes differently and changed the mRNA levels of the studied genes into different directions.

Thrombin is a trypsin-like serine protease which is known for its role in coagulation pathway. However, thrombin can also penetrate into the brain through the blood-brain barrier after hemorrhage or be produced directly in the brain during cerebral ischemia (Niego et al., 2011). It was found that thrombin in the brain causes changes in the morphology and in proliferation rate of astrocytes, the same as in activated cells (Shirakawa et al., 2010; Ubl and Reiser, 1997). We used primary cultures of human astrocytes which we treated with thrombin and we observed changes in the morphology and genes' expression. GFAP is a cytoskeleton protein and one of the hallmarks for astrogliosis and it was up-regulated after the treatment with thrombin. Furthermore, the up-regulation of GFAP caused morphological changes in the cells such as hypertrophy. Additionally, we saw an effect in the expression of GLUL which is a specific astrocytic marker. GLUL is responsible for the conversion of glutamate to glutamine, which in turns is shuttled to neurons when it serves as substrate for glutamate production. We also detect an up-regulation in QKI-7 expression which in contrast to the other affected genes was transient.

Our results reflect three basic characteristics of activated astrocytes which indicate that primary human astrocytes respond to thrombin accordingly to previous studies in rats' astrocytes (Suidan, 1997; Niego, 2011). Furthermore an effect of thrombin in QKI expression reveals that this gene has a function in reactive astrocytes. The fact that the increase of QKI-7 was preceded and that QKI is a member of the

STAR family indicates that QKI is involved in the beginning of astrogliosis by regulating the expression of other involved genes.

Haloperidol is a typical antipsychotic drug which is widely used among schizophrenic patients (Joy et al., 2001) and it was shown that one of its in vitro effects was to increase the expression of QKI in cells from patients' brain (Aberg et al., 2006). Further studies in human astrocytoma cells revealed that astrocytes are target cells for haloperidol which can cause overexpression of QKI-7 (Jiang, 2009). In this study we repeated the experiment of Jiang et al using primary human astrocytes and we observed a strong tendency for increase in the expression of QKI-7 after 6h of treatment which is in accordance with the previous results. Additionally we measured the expression of more genes and we detect that GFAP and B2M are also up-regulated although B2M is again in control levels after 24h. Subsequently, GFAP overexpression had an effect on the morphology of astrocytes which become hypertrophic and develop more processes than control cells.

These results indicate that haloperidol may activate astrocytes in a similar way with innate factors that cause astrogliosis. It was already known that QKI responds to haloperidol but now we can associate the overexpression of QKI with the activated astrocytes. Transient increase of QKI may play a role in the first stage of astrogliosis and its expression is not necessary in further activation. Further studies may reveal the relationship between haloperidol and astrogliosis.

Finally we studied the effect of IFN- γ in human astrocytes. IFN- γ is an inflammatory cytokine and is mainly produced by systematic lymphocytes such as T-cells and natural killer cells (John et al., 2003). It is involved in many different cellular programs such as antiviral, antibacterial and anti-tumor protection (Aschner, 1998). Recently it was proposed that IFN- γ is also involved in neuroinflammatory processes by stimulating glial cells to produce adhesion molecules, express MHC class I and II and secrete other proinflammatory cytokines (Dong and Benveniste, 2001). On the other hand genes that are stimulated by cytokines were up-regulated in schizophrenia brains supporting an inflammatory hypothesis of the disease (Saetre et al., 2007). Since astrocytes are one of the immunocompetant cell-type in the brain they may be involved in this new hypothesis. We observed that astrocytes respond to IFN treatment with a dramatic overexpression of GBP1 and B2M which both are interferon-induced

genes. Additionally, we detected a significant down-regulation of GFAP which caused morphological changes in the cells. After the interferon treatment the cells lost completely their star-like shape, they were longitudinal and developed a few thin processes. Furthermore, by comparing cell density after 24h between control and treated cultures we observed that IFN may affect the proliferation rate of astrocytes, however, this observation should be verified by a viability test.

The fact that the genes expression pattern of the cells is modified and inflammation related genes, which normally are not expressed, are expressed after the treatment, and that the proliferation rate of the cells is probably increased indicates that IFN- γ stimulates astrocytes. However, GFAP is decreased and astrocytes are hypertrophic, thus astrocytes are activated after the IFN treatment in a way different of astrogliosis. Astrocytic activation by cytokines has already been described in mice-models and it was proposed that such activation reflects the brains' response to infections (John et al., 2003).

Another characteristic of cytokine-activated astrocytes was that the expression of QKI-7 was decreased, indicating that the mechanism of this activation affects QKI. In order to study the function of QKI under this condition we combined the silencing of QKI with IFN treatment for 48h. As control we had cells in which we silenced QKI-t and cells which were treated with IFN- γ for 48h. These two cultures certified that QKI-t and QKI-7 expression was decreased after the transfection with the siRNA and that astrocytes have the same genes' expression pattern after 48h of treatment like after 24h, thus they were activated. Additionally we detected that when QKI was not expressed GFAP and B2M were down-regulated. According to literature we expected that these genes would be decreased but we also expected the same decrease for GBP1 (Jiang et al., 2010). It is possible that we did not observe any change in GBP1 expression because the expression of QKI was decreased at 50% and not more than 80% like previous studies. When we silenced the expression of QKI-t and subsequently treated the cells with IFN- γ we observed that the interferon related genes were still overexpressed but with a slight decrease and that QKI-7 and GFAP were expressed in control levels.

IFN- γ causes to astrocytes a reactive condition in which they could act like immune cells and in which the expression of QKI-7 and GFAP are low. However

QKI-7 is involved in the mechanism of this condition because IFN- γ recovers its expression after silence. These results are in agreement with previous studies which have shown that QKI-7 specifically regulates the expression of the interferon-related genes (Jiang et al., 2010). Thus, QKI-7 should be expressed during activation by cytokines because it may regulate the expression of the interferon-related genes which is essential for this condition.

However when QKI is silenced the effect of IFN- γ on astrocytes is lighter while IFN- γ causes also an increase in the expression of QKI-7 and GFAP. These results agree with previous studies which have shown that QKI-7 regulates the expression of GFAP and interferon-related genes. Furthermore these results indicate that QKI is eventually involved in the cytokine-activation of astrocytes.

CONCLUSION

Gene analysis and observation of the cell cultures indicate that factors which cause activation of astrocytes in the brain have the same effect when they are used in vitro. Thrombin and the antipsychotic drug haloperidol stimulate astrocytes and cause astrogliosis while IFN- γ affects them differently and causes immunologic activation. The in vitro activation of astrocytes mimic pathological conditions of the brain and offers cells that can be used for further studies of these conditions. It was common to our results that modifications in genes' expression did not reach the significance level probably because of the high standard error. A solution to this problem would be the repetition of the experiments with more biological replicates. Hence future research which will include more tests on cells and optimization of the variable parameters is needed in order to establish a fully reliable model of activated astrocytes.

From our analysis was shown that QKI is involved in both kinds of activation. We tested only the significance of QKI under the immunologic activation but it would be also interesting to study the relationship of QKI and astrogliosis in the future. When astrocytes are activated by IFN- γ QKI-7 is decreased indicating that its expression is not essential. However when the expression of QKI is modified IFN- γ reset the expression of QKI-7 in normal levels. Thus when the expression of QKI is disturbed IFN- γ is able to increase the expression of QKI-7 at the level which is essential for the immunologic activation of astrocytes. This observation was after 48h of treatment and does not show how the expression levels of QKI-7 are changing during the treatment. Time course observation could reveal if IFN- γ triggers the expression of QKI-7 directly or through other mediators. Future studies in the relationship between QKI and IFNs will be useful in the research of etiology for diseases which involve mental disorders and environmental insults.

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