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Effects of factors involved in the pathogenesis of hepatic encephalopathy and some phosphodiesterase inhibitors on neuroinflammation-related events in primary rat astrocyte cultures

PhD thesis

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1. List of abbreviations

ALF: acute liver failure

ANOVA: analysis of variance

AraC: cytosine β-D-arabinofuranoside hydrochloride

BPTES: Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide

BT: bacterial translocation

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CLD: chronic liver disease

CM-H_2DCFDA: a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate

cPSS: congenital portosystemic shunt

DAMP: danger-associated molecular pattern

DMSO: Dimethyl Sulfoxide

DON: 6-Diazo-5-oxo-l-norleucineú

EAAT: Excitatory amino acid transporters

ELISA: enzyme-linked immunosorbent assay

GFAP: glial fibrillary acidic protein

GLAST: L-glutamate/L-aspartate transporter

GTK: Glutamine transaminase K

GTL: Glutamine transaminase K

h: hour

HE: hepatic encephalopathy

Iba-1: Ionized Calcium-binding Adapter Molecule-1

IL: interleukin

KGA: kidney-type glutaminase

LDH: lactate dehydrogenase

LGA: liver-type glutaminase

LME: L-Leucine methyl ester

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinases

MEM: minimal essential medium

MHE: minimal hepatic encephalopathy

min: minute

Mn: manganese

MRI: Magnetic resonance imaging

mRNA: messenger ribonucleic acid

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: nuclear factor kappa B

NR: neutral red

PAMP: pathogen-associated molecular pattern

PDE: phosphodiesterase

PI: propidium iodide

PRR: pattern recognition receptor

RNOS: reactive nitrogen and oxygen species

RNS: reactive nitrogen species

ROS: reactive oxygen species

rpm: Revolutions per minute

S.E.M: standard error of mean

SIRS: Systemic inflammatory response syndrome

TNF-α: tumor necrosis factor alpha

α-KGM: α-ketoglutaramate

2. Summary

Astrocytes are the most abundant glial cell type in the brain. They were historically considered as a homogenous cell population; however an increasing body of evidence suggests that there is morphological and functional heterogeneity of this cell type, thus distinct astrocyte subtypes can be found in the brain. They are crucial for the physiological function of the nervous system, since they provide energy for neurons by releasing lactate in the extracellular compartment, coordinate neurovascular coupling, control synaptic transmission, have role in synapse formation, recycle the neurotransmitter glutamate and maintain ion homeostasis. Since astrocyte have many essential functions, not surprisingly, they also have pivotal role under pathological conditions. One of these conditions is hepatic encephalopathy (HE), a serious neurological complication of liver failure or portosystemic shunt. Astrocyte swelling represents the leading cause of cerebral edema during acute HE, while chronic HE can be characterised by the so-called Alzheimer Type II astrocytosis. The precise etiology of HE is still unknown, however there is a growing body of evidence indicating that hyperammonemia, cerebral accumulation of ammonia and manganese, alteration of neurotransmitter systems, bacterial translocation, systemic inflammation as well as oxidative stress have essential role in the pathogenesis of HE. Beside these events, in the last few decades neuroinflammation has attracted considerable attention. Microglia as resident macrophages, are the key cellular participants in neuroinflammation by producing inflammatory mediators including cytokines, reactive oxygen species and other small molecules, such as nitric oxide. Astrocytes have also been implicated in neuroinflammatory events; however their role has not been fully clarified yet, due to the fact that studies addressing this question, used different or even not appropriate models, thus it is doubtful whether the findings are attributable exclusively to astrocytes or not.

More recently, several studies established that phosphodiesterase (PDE) inhibitors have some effects not associated directly to the inhibition of PDE enzyme. PDE inhibitors have impact on neuroinflammation by changing phenotype polarization and cytokine production of microglia or attenuating astroglial reactivity. Moreover, a study found unexpectedly that one of the PDE inhibitors is able to inhibit the glutaminase enzyme as well. Since there is a growing body of evidence that glutaminase is required for some neuroinflammatory processes, it could be suggested that the effects of PDE inhibitors on neuroinflammation may be linked to their glutaminase inhibitory effect.

In my PhD work we aimed to study the effects of the most important etiological factors of HE on astrocytes. Cell viability, reactive oxygen species (ROS) production and cytokine secretion were measured *in vitro* after the treatment of the cells with hydrogen peroxide, ammonia, manganese and lipopolysaccharide. With the aim of better understanding the role of astrocytes related to both neuroinflammation and oxidative stress, we used primary rat astrocyte cultures, purified from microglia by using two different methods. These procedures resulted in highly enriched astrocyte cultures verified by immunocytochemistry. For the ROS quantification, we applied not only the two types of highly purified astrocyte cultures, but also mixed glial cultures, which contained significant amount of microglia as well. Thus, the extent of the oxidative stress evoked by the examined factors could be compared between mixed glial cultures and enriched astrocyte cultures.

In the current research we also studied, whether some PDE inhibitors could block the glutaminase enzyme in astrocytes, thereby clarifying their therapeutical significance during neuroinflammation and astrocyte swelling.

3. Introduction

Hepatic encephalopathy (HE) occurs both in humans and animals as well. Albeit the pathogenesis, nomenclature and symptoms show many similarities in human and animal patients, there are some relevant differences as well (1)

3.1. Hepatic encephalopathy in humans

HE is a neurological complication derived from liver disease or portosystemic shunt. The related brain disfunction manifests as a broad spectrum of neuropsychiatric symptoms ranging from subclinical form to coma (2). At present, the grading of the mental status is traditionally based on the West Haven Criteria, in which grade 0 indicates minimal HE and grade 4 represents coma (3). Minimal HE (MHE) is the mildest, subclinical manifestation of HE (4), albeit it is the preclinical stage of the more severe stages (5), it reduces the quality of life (6), furthermore, patients with MHE have higher rate of motor vehicle crashes compared with cirrhotic patients without MHE (7). MHE could not be established by routine physical and neurological examinations (8), only by using psychometric, neuropsychological tests (2),(8) or applying magnetic resonance imaging to measure the grey matter volume (8). Coma is a common outcome in patients with chronic liver disease (CLD) associated with HE (9) as well as in patients with acute liver failure (ALF) (10), in addition, hepatic coma was observed in all examined patients with late-onset hepatic failure (10), which is a subset of ALF (11). Based upon the underlying cause, HE is divided into three types: Type A is found in patients with ALF, type B is associated with portosystemic bypass, type C arising because of cirrhosis (12). Liver cirrhosis is a worldwide problem that is a significant global health burden, since the mortality level increased from around 676,000 in 1980 to over 1 million in 2010 (13). It has been found that the most frequent causes of cirrhosis were alcohol consumption, Hepatitis C virus infection and non-alcoholic fatty liver disease. Indeed, HE is a common complication of liver cirrhosis (14) and among more than 160 thousand patients with cirrhosis the overall incidence of HE was 11.6 per 100 patient-years.(12), in addition, the mortality for cirrhotic patients with HE grade 3-4 is higher than for patients with milder grade of HE (15). Compared to this, incidence of ALF is around 1–6 cases per million in developed countries (16) and the most frequent causes are the hepatotoxic drugs, such as a paracetamol (acetaminophen) and hepatitis viruses (16), (17).

3.2. Hepatic encephalopathy in animals

The occurrence of HE has already been described in many species, such as in horses (18), in cattle (19,20), in goats (21,22) and in dogs and cats (23–25)**.** Interestingly, HE was first described over a hundred years ago in dogs when experimentally created portacaval shunt led to neurobehavioral changes (26).

The etiological classification of HE in humans (type A, type B, type C) can be applied to dogs and cats as well, however all intrinsic hepatocellular disease and portal hypertension or acquired portal systemic shunting (PSS) are needed to be included in the definition of type C (27). The acute form (type A) of HE is very rare in dogs (10%), the most common form is the B type due to congenital portosystemic shunt (cPSS).

Being the major cause of HE in dogs and cats (27), PSS could be congenital or might develop due to portal hypertension, which leads to opening of collateral vessels. In a retrospective study it has been found that the most common etiological factor for HE in dogs was congenital PSS (28), and the annual diagnostic proportion of congenital PSS has increased between 1980–2002, furthermore the most affected breeds were Yorkshire Terrier and Miniature Schnauzer (29), which finding was supported by others as well (28). The HE is a syndrome occurring with mostly neurological signs but can also be presented with gastrointestinal or urinary tract symptoms as well. The neurological signs fall into four categories and including lethargy, circling, compulsive walking, behavioral changes, seizures, head pressing, stupor, obtundation, ataxia, temporary blindness and in the serious stages 3-4 the semicoma and coma (27,28,30,31). The stages 3-4 are linked to increased cranial pressure with brain edema due to astrocyte swelling with hyperammonaemia (details see later).

4. Pathophysiology of HE

4.1. Hyperammonemia

Ammonia elimination predominantly takes place in the liver, and as detoxification capacity of the liver is impaired during HE, it leads to hyperammonemia (32). Ammonia mainly originates from glutamine, although intestinal bacteria also produce ammonia from urea. Glutamine is mostly synthetized from ammonia in the skeletal muscle and the brain, while glutamine will be catabolized in enterocytes and kidneys resulting in ammonia (32) *(Fig. 3.)*.

Ammonia is thought to be the main etiological factor in the pathogenesis of HE, elevation of plasma ammonia level has been described in human patients and also in dogs with ALF after the development of HE (33), in cirrhotic patients with HE (34) and also in HE due to congenital portosystemic shunts (35). Since, ammonia can cross the blood brain barrier as an ion and gas as well (36), it is not surprising that ammonia level increases not only in the blood plasma but also in the brain, which was demonstrated in acute HE (37) and in also in cirrhotic patients with HE as well (38). In addition, ammonia can lead to cerebral edema, that develops in patients with acute-on-chronic liver failure with HE (39), cirrhotic patients with HE (14) as well as in ALF (34), as verified by Magnetic Resonance Imaging (MRI). In accordance with this, arterial ammonia is found to be elevated in patients who died from cerebral herniation (33).

The brain is anatomically encased in a non-dilatable capsule, the skull, thus an increase in brain volume (brain edema) will lead to an increase of intracranial pressure and consequently brain herniation, which subsequently causes death. This process stands as a central complication of ALF (33), however an intracranial hypertension rarely occurs in CLD.

Brain edema can develop as a consequence of either cytotoxic or vasogenic mechanisms. Although this aspect received limited attention, recently increasing body of evidence showed that the disruption of blood brain barrier occurs during ALF and it might be a potential underlying mechanism of vasogenic edema (41). In mice with ALF, transforming growth factor ß1 is released from the liver into the blood circulation and subsequently it disrupts the blood brain barrier (42).

Being exclusively relevant for the present examinations, cytotoxic edema is generated a result of swelling of astrocyte, i.e., the main affected cell type in HE. Having key role in the maintaining brain homeostasis, astrocytes represent the most abundant glial cell type in the central nervous system (43). The main reason for the vulnerability of astrocytes in HE is that glutamine synthetase, an enzyme responsible for ammonia-removal, is mainly located in this cell type, at least within the brain (44).

This enzyme is responsible for the conversion of glutamate and ammonia to glutamine, the reaction that comprises the most important step in the elimination of brain ammonia. Summing up, during HE, hyperammonemia leads to astrocyte swelling by synthesizing glutamine, i.e., the main underlying mechanisms of cerebral edema. The fact that glutamine synthesis may be detrimental is shown by both *in vivo* and *in vitro* studies. For instance, by mean of brain microdialysis technique it has been reported, that an increase in the extracellular brain glutamine concentration closely correlates with the elevation of intracranial pressure in patients with ALF (45). In addition to this, in cirrhotic patients the cerebral glutamine concentration is paralleled by the severity of HE, and glutamine level obviously decreases in those who recovered from the HE episode (46).

Several aspects of the pathological relevance of glutamine accumulation have already been clarified by *in vitro* experiments as well. Ammonia-induced astrocyte swelling could be blocked by inhibition of glutaminase, the enzyme that catalyses the hydrolysis of glutamine to produce glutamate and ammonia *(Fig. 1.)*, thereby resulted in intracellular increase of glutamine (47). Furthermore, inhibition of either glutamine synthetase or mitochondrial glutamine transport (see later) also reduced ammonia-induced astrocyte swelling (48). To explain the background of astrocyte swelling, there are two hypotheses. One of the theories, proposed in 1986, is that glutamine may act as an osmolyte and thus contributes to water-influx in astrocytes, resulting in their swelling *(osmolyte hypothesis* (49).

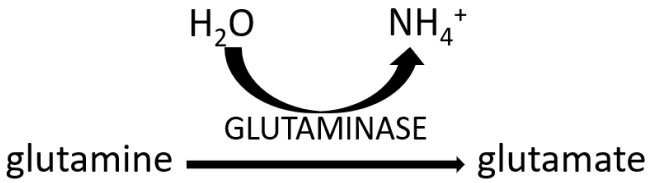


Figure 1. Conversion of glutamine to glutamate by the action of glutaminase enzyme.

Another, recently proposed mechanism represents the so-called „Trojan horse hypothesis", which postulates that glutamine, after its accumulation in the cytoplasm of astrocytes by the action of *glutamine synthetase*, is transported into mitochondria where it is converted to ammonia and glutamate by *phosphate-activated glutaminase (Fig.2.)*. The increased concentration of ammonia in mitochondria will subsequently lead to oxidative stress and astrocyte swelling by impaired energy metabolism (50), which is associated with the induction of mitochondrial permeability transition (51).

This hypothesis is based on the study according to which increases in intracellular concentration of glutamine caused cell swelling only at a later time point (47), and antioxidants attenuated ammonia-mediated astrocyte swelling suggesting the osmotic effect of glutamine accumulation being not the only cause of cell swelling (52).

It is worth mentioning that not only glutamine accumulation, but other mechanisms might be also involved in the astrocyte swelling, such as the ammonia-evoked elevation of aquaporin-4 protein expression (53), however it could be not detected in mixed glial cultures containing significant number (30%) of microglia (54). Furthermore, it has been described that ammonia-exposure leads to reversible mitochondrial fragmentation and impairs glyocolysis in astrocytes, which could affect the energy metabolism (55).

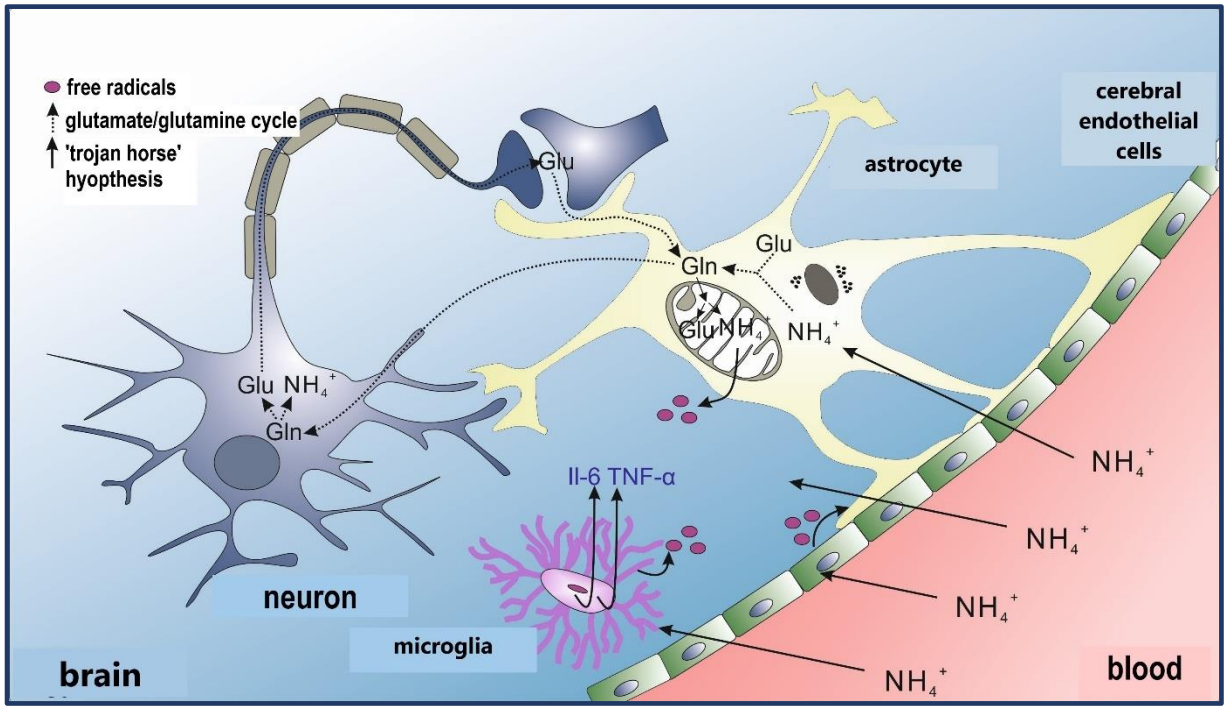


Figure 2. Glutamate/glutamine cycle under physiological and pathological conditions.

However, ammonia detoxification occurs predominantly in astrocytes by glutamine synthesis; both *in vitro* an *in vivo* findings provide evidence that inhibition of glutamine synthesis increases ammonia incorporation in alanine, thereby cerebral alanine formation means an alternative pathway for ammonia-scavenging (56).

Besides, it should be mentioned that astrocyte is affected not only in ALF, but also in CLD. In this condition, Alzheimer Type II astrocytes can be detected in the brain, which have large swollen nuclei, prominent nucleoli, and the chromatin pattern is marginated (57).

Taken together, inhibition of the glutamine synthesis could be a potential therapeutical target in the therapy of HE. Besides, inhibition of glutaminase could provide also therapeutic interest for prevention of astrocyte swelling, since according to the 'trojan horse' hypothesis, blocking of glutaminase activity leads to the reduction of ammonia-induced astrocyte swelling (47). This hypothesis is supported by findings showing that glutaminase is present in the astrocytic mitochondria (50). Glutaminase has two isoenzymes: kidney-type isozyme (KGA) and liver-type isozyme (LGA), moreover in both cases two transcripts (isoforms) also have been described (58). It was shown that KGA is present in the mitochondria, whereas one of the LGA transcript (GAB) was detected in both mitochondria and nucleus in primary mouse astrocyte cultures (59). In order to exploit the therapeutic benefits of blocking the glutaminase activity one may have several candidates. 6-Diazo-5-oxo-l-norleucine (DON) is one of the earliest known glutaminase inhibitor (60,61) and it inhibits both forms of glutaminase (KGA, LGA) (60). Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) is another glutaminase inhibitor, and unlike DON, it selectively blocks the KGA (60). Although, at present specific glutaminase inhibitors do exist, these have only been tested *in vitro*.

More recently, it was demonstrated that some other drugs have glutaminase inhibitor activity, however these effects fall outside of their licensed indications. For example, zaprinast does not only act as a phosphodiesterase (PDE) 5 inhibitor, but it turned accidentally out to also block the enzyme glutaminase (62). In addition, THDP17, a thiourea derivative and metformin, an anti-diabetic drug are also able to inhibit glutaminase activity in the intestines (63,64). Therefore, it is suggested that there are some potential drugs that might be effective *in vivo* in the therapy of HE as well, either by reducing astrocyte swelling or glutamine production by enterocytes (see earlier) via glutaminase inhibition.

Although hyperammonemia has long been incriminated in the development of HE, it is recognized that some dog patients with cPSS and consequent HE have normal plasma ammonia concentration (35). Similarly, another study demonstrated that almost 20% of human patients with ALF and HE had arterial ammonia levels within normal range (65). These findings suggest that other factors are likely to be important in the pathogenesis of HE.

4.2. Oxidative/nitrosative stress

Oxidative stress is defined as *"an imbalance between oxidants and antioxidants in favour of the oxidants"* (66). In the case of this process, there is an overproduction of reactive oxygen species, ROS (i.e. superoxide anion radical, hydrogen peroxide, etc.) or reactive nitrogen species, RNS (i.e. peroxynitrite anion, nitric oxide, etc.) compared with antioxidants (superoxide dismutase, glutathione peroxidase, catalase and non-enzymatic antioxidants such as glutathione, ascorbic acid, etc.) which results in oxidative/nitrosative stress (67,68). In rats with ALF, oxidative stress develops in the brain, verified by the increased lipid peroxidation and protein oxidation as well as the decreased level of antioxidant enzymes in both the cerebral cortex and cerebellum (69). This finding is line with the result of another study, which showed that rats with MHE have elevated cerebral oxidative stress (70). Increased cerebral oxidative stress was measured in human cirrhotic patients with HE as well, and interestingly, it was associated with HE, not with cirrhosis itself (71).

Oxidative stress could be detected not only in the brain, but also in the blood during HE. Human patients with chronic liver disease and HE have higher level of oxidative stress biomarkers in the plasma than healthy subjects (72). *In vitro* experiments have also demonstrated the role of oxidative stress in the pathogenesis of HE. Ammonia-exposure leads to increase of astrocyte volume by generation of oxidative and nitrosative stress, since scavenging of both nitric oxide and superoxide radicals blocked astrocyte swelling (73). The precise underlying mechanism of the oxidative stress-mediated cell swelling has not been fully clarified yet, however it has been shown that ammonia increases phosphorylation of mitogen-activated protein kinases (MAPK) in an oxidative stress-dependent manner and inhibition of MAPK blocks astrocyte swelling (52). In addition to the involvement of MAPK in the oxidative stress-mediated astrocyte swelling, oxidative/nitrosative stress was also implicated in the ammonia-induced activation of transcription factor p53, which participates in the process of astrocyte swelling as well (74).

It is important to keep in mind that not only the generation of Reactive Nitrogen and Oxygen Species (RNOS) triggers astrocyte swelling, but also cell swelling in turn induces RNOS formation (75).

4.3. Systemic inflammation

Systemic inflammatory response syndrome (SIRS) may develop after infectious and non-infectious insults (76). SIRS is associated with ALF, moreover it is an independent predictor of renal failure in patients with non-paracetamol-induced ALF (77). SIRS occurs also in patients with alcoholic liver cirrhosis and HE, and also it is an independent predictor of death within 30 days after visiting the emergency department (78).

Examination of dogs with cPSS has also revealed that SIRS predicted the occurrence of HE (35). In patients with liver cirrhosis, TNF-α level was higher in the portal blood and IL-6 peripheral blood concentration had predictive role in HE (79). One important cause of systemic inflammation during liver failure/HE is bacterial translocation (BT) (80). BT is defined as the passage of bacteria or bacterial products from the intestinal tract to the mesenteric lymph nodes and systemic circulation (81,82). Some bacterial components, the so-called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) are recognized by pattern recognition receptors (PRRs), like the toll-like receptors on immune cells of the innate immune system, and thereby induce inflammatory response.

This above mechanism occurs frequently in decompensated cirrhosis (80), however BT has been detected in experimental ALF as well (83). Another underlying process of inflammation could be the releasing of danger-associated molecular patterns (DAMPs) by dying hepatocytes, which finally causes systemic inflammation (80). It has been shown that HMGB1, (a typical DAMP) released by necrotic cells or secreted by immunocompetent cells induces inflammatory cytokine production and BT in ALF, thereby HMGB1 contributes to the development of systemic inflammation (84).

4.4. Manganese

Manganese (Mn) is an essential element functioning as a cofactor for several enzymes. Important sources of Mn include whole grains, beans, leafy vegetables, etc. It is excreted by hepatobiliary system into the gastrointestinal tract (*Fig.3*.) and finally eliminated via faeces (85).

Elevated level of Mn in the blood was detected in dogs with cPSS (86), in human patients with cirrhosis (87) and with HE (88) as well; moreover, elevated Mn concentration in the blood could also predict death in patients with HE (88). Related to liver failures, Mn concentration is increased not only in the blood, but also in brain as it was demonstrated by a study in which significant Mn deposition was detected in the brain of human cirrhotic patients as well as in rats with either experimental cirrhosis or portacaval-shunt (89). Interestingly, ALF has not been associated with higher Mn level in the brain (89).

Considering these data, Mn has also been implicated in the development of HE, since cirrhotic patients with HE have higher Mn level in the blood plasma than in those without HE (87). On the contrary, blood Mn concentration did not decrease in dogs with cPSS after resolving the clinical signs of HE, indicating that other factors also contribute to these complex mechanisms leading to HE.

Again, quite interestingly, manganese itself is also able to elicit both astrocyte swelling in a dose-dependent manner (90) and oxidative stress in the astrocytes (91,92), moreover it has been described that the Mn-provoked swelling of astrocyte is caused by oxidative stress and mitochondrial transition pore (93).

4.5. Neuroinflammation

Neuroinflammation is defined as an inflammatory response in the central nervous system, and it is now recognized as a hallmark of HE. *In vitro* studies on astrocyte/microglia cultures and cell lines have shown that glial cells are main players in neuroinflammation and more recently the crosstalk between microglia and astrocytes has been implicated (94). Neuroinflammation is mainly initiated by the activation of microglia, which leads to morphological changes as well as the production of different pro-inflammatory cytokines, such as TNF-α, Il-1ß, etc. (95).

Although microglia are the major resident immune cells in the brain, astrocytes represent the most abundant glial type in the central nervous system (CNS). Cytokine and chemokine secretion of astrocytes can be elicited not only by different factors, such as at high glucose concentration (96), after both mechanical insult and ischemic injury (97), by vasoactive intestinal peptide (98), angiotensin-II (99) or different cytokines (100), after treatment with lipopolysaccharide (101,102), with hydrogen-peroxide (103) or after chronic exposure with paracetamol (104), but even also under physiological conditions (101).

Albeit numerous studies were published regarding the neuroinflammatory role of astrocytes, the overwhelming majority of them was carried out on cultures not (105) or inadequately purified from microglia (106,107) or on human astrocyte cell line deriving from astrocytoma (108). Evaluation of these data might be even more puzzling, once we consider evidences about the existence of astrocyte populations expressing no glial fibrillary acidic protein (GFAP), (so-called: GFAP-negative astrocytes), the most basic marker to identify astrocytes (109,110).

Thus, it is not offensive to state that the exact inflammatory role of astrocytes could not be established on the basis of these cultures, since on one hand, the results originate from distinct models, on the other hand, astrocyte cultures may contain other cell types (such as microglia), which makes the result regarding the cytokine production uncertain. The reason for the latter is that microglia produce considerable amount of cytokines and these cytokines may induce the formation of reactive, neurotoxic astrocytes type A1 (111), which have a noxious pro-inflammatory genomic profile (112).

The significance of the presence of microglia in the astrocyte cultures is supported by a study, in which TNF-α production was detected in astrocyte-microglia mixed culture, but not in pure astrocyte culture generated by neural stem cell differentiation (113). Interestingly, it has also been described that there is a sex-mediated difference in connection with TNF-α and IL-10 production in primary rat astrocyte cultures (114).

Cerebral microglial activation and increased protein levels of Il-1ß, IL-6, TNF-α in the cerebrospinal fluid were measured in rats with ALF at coma stage (115). Furthermore, the increased expression of different pro-inflammatory cytokines could be measured also in the brain. Rats with acute HE have higher cerebral protein level of Il-1ß and TNF-α (116), moreover, in the frontal cortex of mice with ALF, increased IL-1ß and TNF-α mRNA expression were observed at coma stage (117). Rats with experimental chronic liver injury also have elevated microglial activation and cerebellar IL-1ß protein level (118). Interestingly, neuroinflammation may contribute to the development of brain edema – which represents the main complication of ALF –, since mice with TNF-1-and IL-1-deficiency shows lesser degree of cerebral edema compared to wild type mice (117). In addition, neuroinflammation is suggested to have role in cognitive and motor alterations that occur during HE (118,119). Obviously, the precise pathogenesis of the neuroinflammation is not clarified yet, however it has been revealed that hyperammonemia *per se* induces microglial activation, increases IL-1ß level in the brain (118) and causes astrocyte activation as well (119). Bioactive molecules can also attenuate some processes involved in neuroinflammation: ibuprofen decreases, microglia activation and reduces cerebellar IL-1ß level (118); sulforaphane – an isothyocianate present mainly in broccoli – normalizes IL-1β and increases the anti-inflammatory cytokine, IL-10 level in the cerebellum (119), furthermore erythropoietin reduces IL-1ß level increased upon traumatic axonal injury (120).

Recently, it has been found that PDE inhibitors may also have beneficial effect against neuroinflammation. This class of drugs originally inhibits PDE enzymes, which are responsible for degradation of cGMP and/or cAMP at different neural connections. Thereby, PDE inhibitors may cause intracellular increase of cGMP and/or cAMP level. A PDE-5 inhibitor, sildenafil reduces mRNA levels of Il-1ß, IL-6 and TNF-α in the hippocampus of a mouse model for Alzheimer's disease (121), it also normalizes the protein levels of IL-1ß and TNF-α in the hippocampus in rats with HE (122).

Another PDE inhibitor, ibudilast have been also found to reduce neuroinflammation by decreasing of TNF-α, IL-6, and IL-1β mRNA expression in the striatum of a mouse model for Parkinson's disease (123).

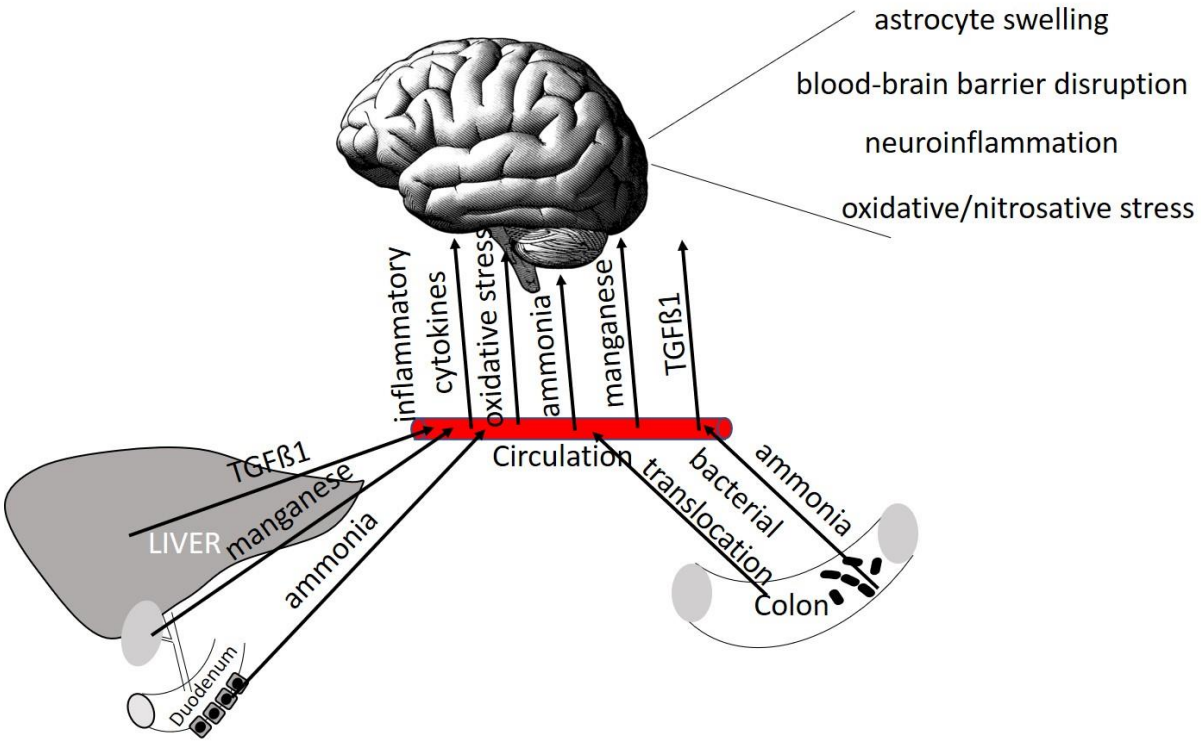


Figure 3. Schematic presentation of the pathophysiology of hepatic encephalopathy (HE).

4.6. The linkage between neuroinflammation and glutamine metabolism

Most recently, the enzyme glutaminase has been implicated in the development of neuroinflammation. Transgenic mice with overexpression of glutaminase C both in hippocampus and cortex show astrocyte and microglia activation and an increase of TNF-α and IL-1ß gene expression. These data suggest that glutaminase C is required for some relevant neuroinflammatory processes (124). Beyond this, it has been also clarified that glutaminase mediates the TNF-α-induced extracellular vesicle release from mouse astrocytes (125). Most recently, the key role of glutaminase in extracellular vesicle release was also demonstrated *in vivo* (126). These extracellular vesicles serve a communication pathway between cells by transferring different proteins, mRNAs, etc. (127), and could contain pro-inflammatory cytokines as well (128). Extracellular vesicle release is not the only way glutaminase may mediate neuroinflammation through, since it has been described that in focal cerebral ischemic brains of rats glutaminase 1 is involved in the microglia activation (126).

4.7. The linkage between neuroinflammation and oxidative stress

As previously described, oxidative stress is a key element in astrocyte swelling, ultimately leading to cerebral edema, which is one of the main causes of death in ALF. In addition, numerous studies have implicated the pathogenic role of neuroinflammation in HE. The two mentioned events are tightly linked to each other, proven by several findings. It has been shown, for instance, that TNF-α treatment induces intracellular ROS generation in mouse astrocytes, a process mediated by glutaminase activity (125). Moreover, an anti-inflammatory cytokine, IL-13 also triggers oxidative stress in microglia, mediated by NADPH oxidase (129).

The connection between inflammation and oxidative stress is bidirectional, i.e., not only the inflammation can elicit oxidative stress, but also ROS generation can contribute to inflammatory events. It has been described that angiotensin II-induced IL-6 production of astrocytes is mediated by the nuclear factor kappa B (NF-κB)-signalling pathway, which is required for the ROS generation (99). It is in line with another study indicating that ROS can cause NF-κB-activation in rat spinal cord astrocytes (130). Chronic paracetamol-treatment can induce IL-1β and TNFα-production in mouse astrocytes, which is suggested to be mediated by nuclear factor kappa B (NF-κB) (104). These findings have also been confirmed on human astrocytes, since it has been demonstrated that in resting and activated state, most of the cytokines produced by human astrocytes are direct targets of the transcription factor NF-kB (89). In summary, oxidative stress mediates the inflammatory role of astrocytes via activation of NF-κB, a master regulator of inflammatory processes. NF-κB is located in cytoplasm in an inhibitory kB (iκB)-bound form, from which, after the phosphorylation of iκB, gets released and translocates to the nucleus where it binds to specific sequences to promote transcription of different genes involved in inflammation including the production of different inflammatory cytokines (104,131). Other neuroinflammatory signalling pathway may also be attributed to the pathogenesis of HE, since some evidences have been risen that the production of the proinflammatory cytokine (TNF-α and IL-1β) in microglia is mediated by the p38 MAPK signalling pathway (132).

All these above findings highlight that HE has a complex pathogenesis not fully understood and in this introduction, we only described those processes which are necessary for explaining our research.

5. Aims of the study

The aim of our study was to examine the neuroinflammation-and related processes during HE in different *in vitro* models. We hypothesised that some causative factors in HE, such as ammonia, Mn, ROS and bacterial LPS elicit the pro-and anti-inflammatory cytokines' production and the ROS generation in primary rat astrocyte cultures. We aimed to highlight the potential differences regarding the cytokine and ROS production between cultures prepared by distinct methods of microglia elimination. Our second hypothesis was that some PDE-inhibitors may block the enzyme glutaminase and thereby have beneficial effect on the astrocyte mediated processes of neuroinflammation. Therefore, we aimed to measure the glutamate and glutamine concentrations in the intra-and extracellular compartment as well after the treatment with different PDE inhibitors and/or ammonia.

6. Materials and methods

6.1. Materials

Dulbecco's Modified Eagle's Medium (D5796), Cytosine-ß-D-arabino-furanoside (C1768), L-Leucine methyl ester hydrochloride (L1002), Triton-X (T8787), anti-GFAP (ABIN115304), Manganese(III) acetate dihydrate (215880), Ammonium chloride (A9434), hydrogen peroxide (H1009), Lipopolysaccharides from Escherichia coli O111:B4 (L4391), Interleukin 6 (RAB 0311), Tumor necrosis factor- α (RAB 0479), KCl (P-5405), KH_2PO_4 (P-5655), NaCl (S-5886), Na_2HPO_4 (S-5136), Neutral Red (N4638), BPTES (SML0601), Zaprinast (Z0878), DON (D2141) were purchased from Sigma. LDH Cytotoxitity Detection Kit (11644793001) was obtained from Roche, Ionized Calcium-binding Adapter Molecule-1 (Iba-1, ab 178846) and Glutamate Assay Kit (ab83389) were purchased from Abcam. CM-H_2DCFDA (C6827) was obtained from Invitrogen. Propidium-iodide was purchased from ThermoFisher., Glutamin Assay Kit (KA1627) were purchased from ABNOVA.

6.2. Preparation of astrocyte cultures

The preparation of astrocyte cultures is based on our previous experiments concerning culturing of cerebellar granule cells (133). Astrocyte cultures were prepared from 2-day-old Sprague-Dawley rat pups as described earlier (134). Following decapitation, meninges were removed, followed by mechanical dissociation and enzymatic digestion of the cells. Cells were grown in Petri-dishes, maintained in Modified Eagle Medium, which was exchanged on the first two days daily and then twice a week. Cultures were incubated in a humidified incubator at 37 °C with 5% CO_2. After achieving confluency at about 14 days, cells were transferred to 96-well plates. One day later, microglia were removed by using two distinct methods: either incubating the cultures with cytosine β-D-arabinofuranoside hydrochloride (AraC) and L-leucine methyl ester (LME) (later: chemical elimination) or shaking (later: mechanical elimination). In some studies LME is used alone (135,136) or in combination with AraC with different concentrations (137),(138,139).

In our experiments, cultures were incubated with 10 μM AraC for 3 days, followed by 25 mM LME for 1 hour as previously described with some modifications (138).

The antimitotic agent, AraC blocks the proliferation of microglia (140,141), whereas the lysomotropic agent, LME is internalized by microglia, wherein it causes disruption of lysosomes followed by apoptosis (142). After the treatment with AraC and LME, the cultures were allowed to recover for 24 hours. In case of mechanical elimination, glial cultures were placed on an orbital shaker at 180 rpm for 30 min. Having removed the supernatants, cultures were further shaken at 240 rpm for 6 hours as described by others (143,144).

Several protocols are known in the literature, in some studies, a single cycling of shaking is used in order to eliminate microglia (145,146), but in our experiments we performed two cycles of shaking, because the first shaking serves to remove microglia and the second shaking procedure is responsible for removing oligodendrocyte precursor cells (147).

6.3. Immunocytochemistry

In order to assess the purity of astrocyte cultures, cell cultures were characterized by immunofluorescence labelling for the astroglial marker GFAP and the microglia marker Iba-1 as it was described earlier (136,148). For determination of GFAP expression, monolayers were fixed for 30 min with methanol, followed by three washes with 0.1 M PBS. After blocking non-specific binding sites with PBS containing 3% normal goat serum (NGS) for 40 min, cells were washed out three times with PBS. Afterwards, rabbit anti-GFAP monoclonal antibody (ABIN115304, 1:2000) was added in PBS containing 0.2% Triton-X-100 and monolayers incubated for 2 hours at room temperature. Unbound antibody was washed out three times with PBS and cells were incubated with Alexa Fluor 488-conjugated secondary antibody in PBS containing 0.2% Triton-X-100 for 90 min at room temperature in dark, and after three washes with PBS, mounted in medium containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to detect cell nuclei.

Microglia were detected by expression of Iba-1 as above, except that monolayers were fixed for 15 min, blocked with PBS containing 20% NGS and incubated with rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1; ab178846, 1:2000; Abcam, USA). Secondary antibody was diluted in PBS containing 0.3% Triton-X-100.

Images were acquired using an inverted fluorescence microscope (Zeiss Axio Vert.A1). Images were analysed using Image J software (NIH Image, USA). Determination of purity was performed by counting the number of Iba-1- and GFAP-positive cells in at least four fields in four separate coverslips.

6.4. Treatments

Astrocyte cultures were treated with different concentrations of H_2O_2, LPS, Mn and ammonia for various incubation time. In each experiment 3-4 replicates were performed. The treatment protocols for ROS measurements *(Table 1.)* and cell viability tests *(Table 2)* can be seen below. Other treatment protocols are attached to the particular figures in section ‘results’.

Examined factor	Concentration	Incubation time
H_2O_2	50 µM	18 h
	100 µM	
	150 µM	
	200 µM	
	300 µM	
	400 µM	
LPS	100 ng	1 h
		3 h
		6 h
	1000 ng	1 h
		6 h
Mn^{3+}	20 µM	1 h
		3 h
		6 h
Mn^{3+}	100 µM	1 h
		6 h
NH_4Cl	5 mM	1 h
		3 h
		6 h

Table 1. Treatment protocol for intracellular ROS measurements in primary rat astrocyte cultures after chemical microglia elimination. H_2O_2: hydrogen peroxide, LPS: Escherichia coli O111:B4 lypopolisaccharide, Mn3+: Manganese(III) acetate dihydrate, NH_4Cl: ammonium-chloride. In cultures after mechanical microglia elimination and in mixed astrocyte-microglia cultures, all of these treatments were performed as well, in addition, there were also a 3-hour treatment group in case of 1000 ng LPS and 100 µM Mn^{3+}

Examined factor	Concentration	Incubation time	
H_2O_2	5 µM	18 h	24 h
	10 µM		
	100 µM		
	1 mM		
	5 mM		
	10 mM		
LPS	1 ng	18 h	24 h
	10 ng		
	25 ng		
	50 ng		
	100 ng		
	1000 ng		
	5000 ng		
Mn^{3+}	5 µM	18 h	24 h
	50 µM		
	100 µM		
	500 µM		
NH_4Cl	10 µM	18 h	24 h
	100 µM		
	500 µM		
	2 mM		
	5 mM		

Table 2. Treatment protocol for Lactate dehydrogenase-activity and Neutral red uptake measurements in primary rat astrocyte cultures. H_2O_2: hydrogen peroxide, LPS: Escherichia coli O111:B4 lypopolisaccharide, Mn3+: Manganese(III) acetate dihydrate, NH_4Cl: ammonium-chloride

6.5. Analysis of cell viability

Firstly, cell death was confirmed by propidium iodide (PI) staining after the treatments with the test compounds. It is a nucleic acid stain, and the increased uptake of PI by the cells indicates elevated membrane permeability. Cultures were incubated with PI diluted with minimal essential medium (MEM) (1:50) for 5 minutes, followed by washing with PBS. Finally, MEM was added to the cultures and imaging was performed on a Zeiss Axio Vert.A1 using a Texas Red filter. Besides the membrane integrity detection by PI staining, two separate methods of cell viability were applied. On one hand, cytotoxicity of the test compounds was analyzed by measuring lactate-dehydrogenase (LDH)-activity in the cell culture medium according to the manufacturer's instructions.

LDH is found in all living cells and the release of this enzyme into the media is a result of damaged membrane and a marker of cell death. LDH activity was assayed using a standard colorimetric technique and it was normalized with respect to maximal LDH release after cell lysis with Triton X.

On the other hand, the neutral red (NR) uptake assay was used, as earlier described using commercial kit (149). This method is based on the ability of viable cells to incorporate and bind the dye NR. The dye is then extracted from the viable cells and the absorbance of the solubilized dye is quantified using a spectrophotometer.

6.6. Measurement of intracellular formation of reactive oxygen species (ROS)

Changes in ROS levels were determined by CM-H_2DCFDA as described in details elsewhere (150). For this measurement, cells in 96-well plates were first incubated with 10 µM CM-H_2DCFDA in serum-free loading medium for 30 min at 37°C, then washed twice with PBS (0.1M; pH 7.4; 37°C), and afterwards exposed to different concentrations of H_2O_2, ammonia, LPS and Mn *(Table 1).* Immediately, after the end of incubation period, fluorescence was measured with excitation at 485 nm and emission at 538 nm.

6.7. Enzyme-linked immunosorbent assays (ELISA)

IL-6, IL-10, IL-1ß and TNF-α were measured either from the culture medium or from cell lysate. Cell culture supernatants were collected, and the cytokines released into the culture medium were analysed using commercially available ELISA kits according to the manufacturer's instructions. Briefly, standards and samples were added into the appropriate wells, followed by incubation at room temperature for 2,5 h with gentle shaking. The solutions were discarded, and washed. Afterwards, the prepared cytokine antibody was added to the corresponding wells and after the incubation time (1 h, gentle shaking), the solutions were removed, washed and "*HRP-Streptavidin*" solution was added to each well. After the incubation period (45 min, gentle shaking), reagents were discarded, wells were washed and "*ELISA Colorimetric TMB Reagent"* was added into the wells for 30 min (dark, gentle shaking). Finally, stop solution was added and the absorbance was detected at 450 nm by a Microplate Reader. For calibration, absorbance of standards with known amounts of the given cytokine were measured and the values were converted into absolute concentrations in pg/ml.

6.8. Glutamate and glutamine assays

Intracellular (IC) and extracellular (EC) glutamate and glutamine concentration were quantified in order to examine the potential glutaminase inhibitor effect of some PDE inhibitors, such as theophylline and zaprinast.

Cells were homogenized in assay buffers, placed in a centrifuge (Eppendorf, Mini Spin plus) and centrifuged for 5 min at 14500 rpm. The optical density of supernatant was detected at 450 nm in a microplate reader. Using colorimetric assays, the EC level of glutamate and glutamine were measured in the cell culture medium, while the IC concentrations of these metabolites were detected in the cell lysate.

7. Statistical analysis

Experimental data were analysed using ANOVA design with Dunnett post-hoc comparison. Significant statistical difference was established at $p < 0.05$. All data are presented as mean ± S.E.M. and all statistical analysis was performed using Prism software (version 5.0; GraphPad Software).

8. Results

8.1. Characterization of the primary rat astrocyte cultures

After around the 2nd-3rd weeks the cultures reached confluence *(Fig 4. A,B,C,D).*Cell density increased gradually, followed by morphological alterations as well. At the beginning, the density of the cells was low, thus stellate morphology could be seen, while later the higher density was associated with a cobblestone-like appearance of the cells *(Fig. 4D).*

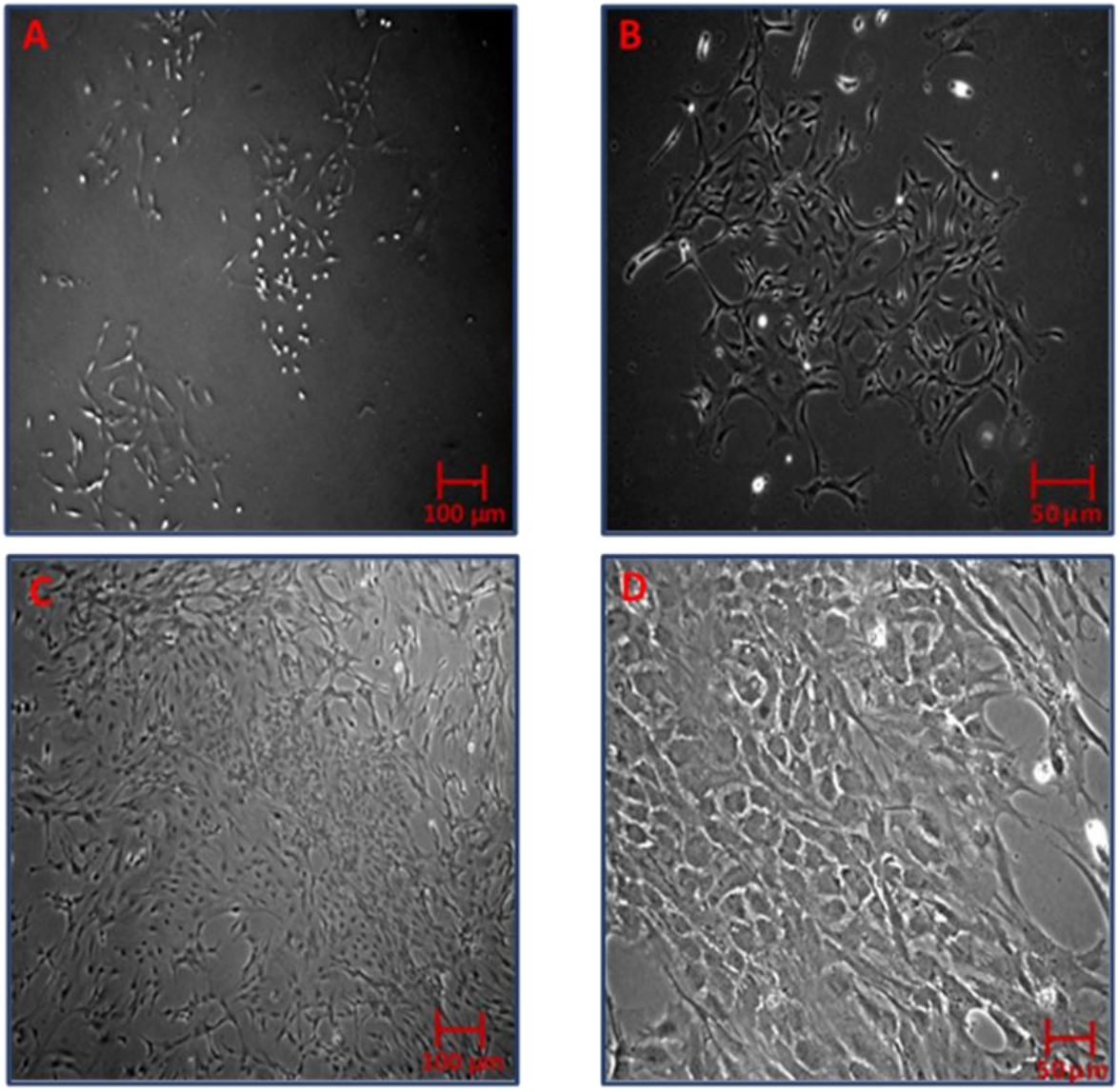


Figure 4. Light microscopic images. A: *7-day-old primary rat astrocyte culture, B: 7 -day-old primary rat astrocyte culture, higher magnification, C: 14 -day-old primary rat astrocyte culture, D: 14 -day-old primary rat astrocyte culture, higher magnification.*

Having treated with 10 μM AraC, cultures were incubated with LME of either 15 mM *(Fig.5B.)* or 25 mM *(Fig 5C.)* for 1 hour. The immunostaining for GFAP has shown that both protocols result in high purity of astrocyte cultures *(Fig. 5B, 5C.),* furthermore we verified that 25 mM LME is more efficient than 15 mM LME, thus we used 25 mM LME after the AraC-treatment for further experiments.

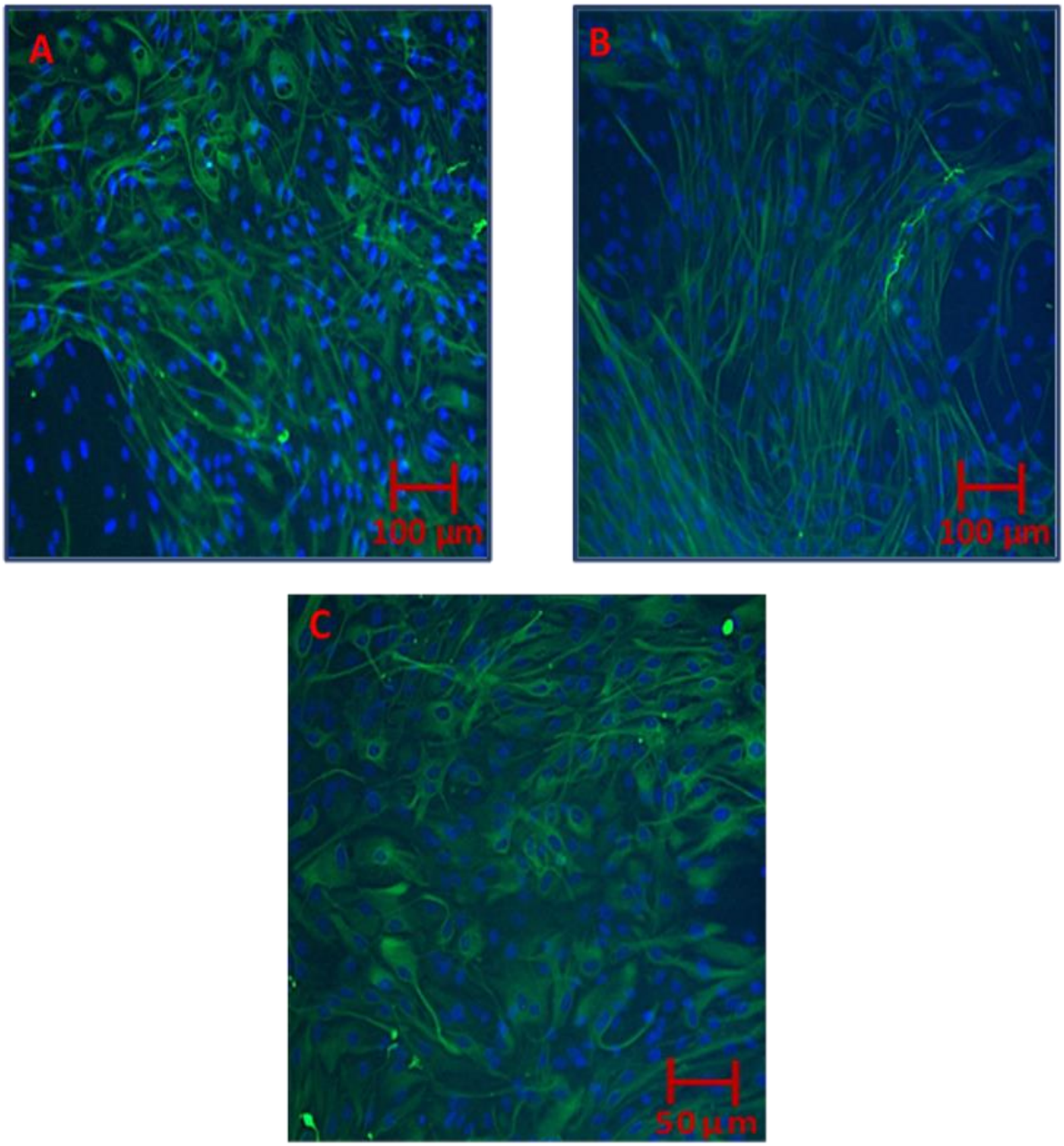


Figure 5. Primary rat astrocyte cultures without microglia elimination (A), after microglia elimination with the combination of 10 μM AraC for 3 days and 15 mM LME for 1 hour (B), after microglia elimination with the combination of 10 μM AraC for 3 days and 25 mM LME for 1 hour (C). Astrocytes show immunolabeling for GFAP (green), the cells were counterstained with DAPI (blue).

Although more than 90% of the cells were positive for the main glial marker, GFAP after the chemical microglia elimination, the remaining number of microglia had to be identified by a microglia-specific marker in order to directly confirm the success of the reduction of microglia number. As *Fig. 7.* shows, depleting microglia from the astrocyte cultures by AraC+LME-treatment resulted in highly purified astrocyte cultures, verified by immunostaining for microglia marker, Iba-1.

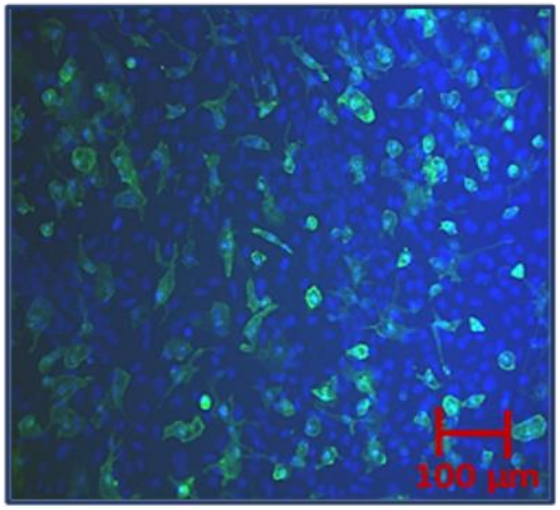


Figure 6. Primary rat mixed astrocyte-microglia cultures. Microglia show immunolabeling for Iba-1 (green), the cells were counterstained with DAPI (blue).

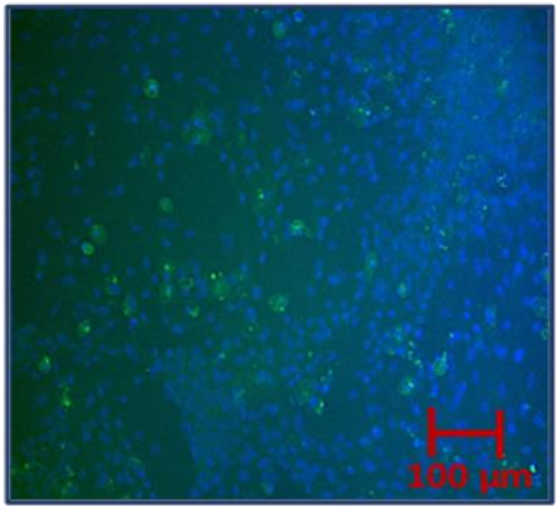


Figure 7. Primary rat astrocyte cultures after microglia elimination with the combination of 10 µM AraC for 3 days and 25 mM LME for 1 hour. Microglia show immunolabeling for Iba-1 (green), the cells were counterstained with DAPI (blue).

During the mechanical microglia elimination, the cultures were shaken with an orbital shaker for 2 cycles and we have shown that this procedure causes higher level of the microglia number reduction than chemical elimination. Using microglia marker Iba1, we have found that the purity of mixed astocyte-microglia cultures was 14% *(Fig. 6.)*, while 11% of the cells were positive for Iba1 after the chemical microglia elimination (10 µM AraC+25 mM LME). After shaking, significantly higher purity was exceeded ($p<0,01$), since only 6% of the cells were microglia verified by Iba1-staining (*Fig.8*.).

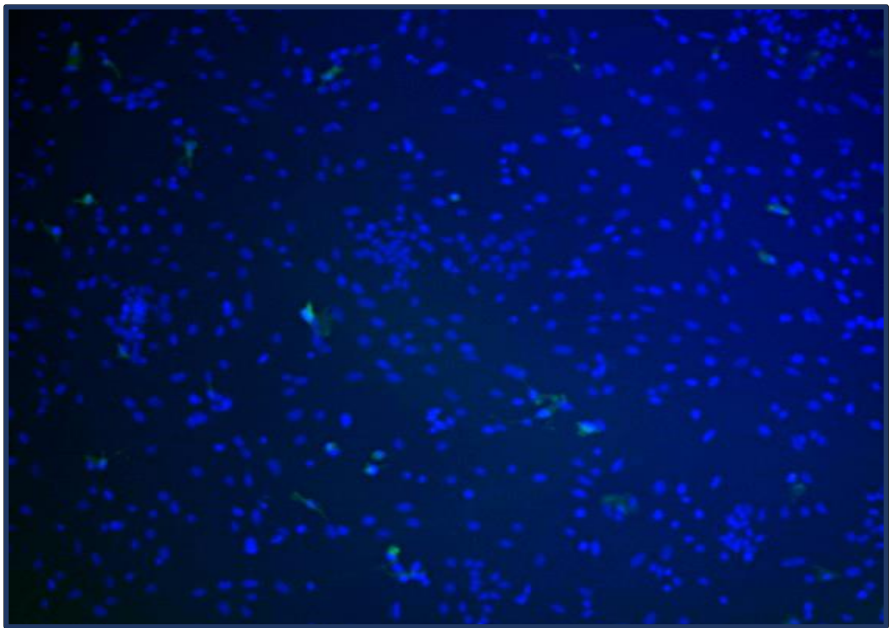


Figure 8. Primary rat astrocyte cultures after microglia elimination with shaking (orbital shaker at 180 rpm for 30 min, having removed the supernatants, cultures were further shaken at 240 rpm for 6 hours). Microglia show immunolabeling for Iba-1 (green), the cells were counterstained with DAPI (blue). The purity of the cultures exceeded 94% purity (p<0,01).

8.2. Test for membrane integrity and cell viability

First of all, PI staining was applied as an alternative method for evaluating the cytotoxicity of the test compounds in AraC+LME-treated astrocyte cultures. We demonstrated that neither of the compounds elicit significant cell death in primary rat astrocyte cultures, however 400 µM H_2O_2 caused significant cell death after 1 hour-treatment. Although, 200 µM H_2O_2 promoted cytotoxicity to a lesser degree than 400 µM, it also has considerable cytotoxic effect *(Fig. 9.; 10.).*

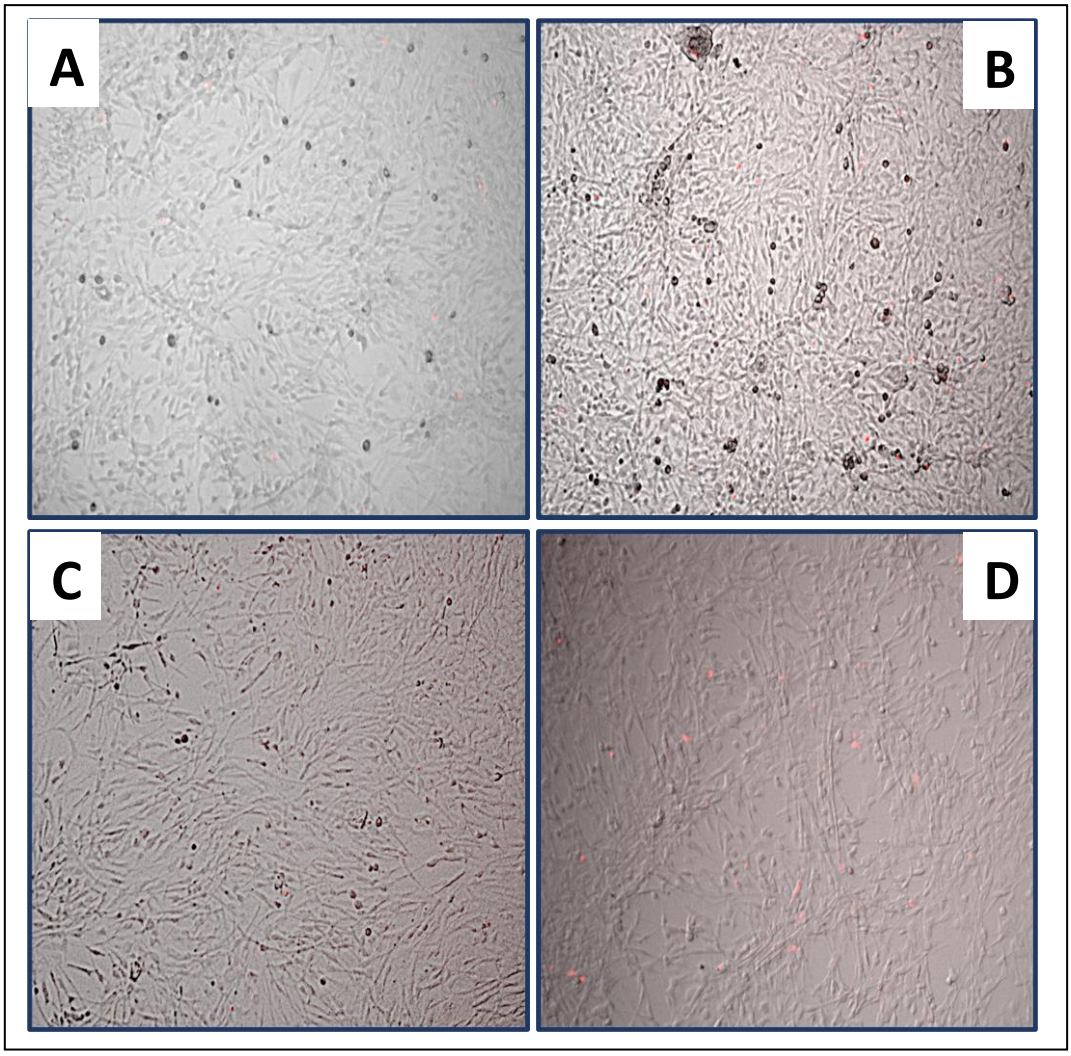


Figure 9. Ammonia-, manganese- and LPS-induced cell death in primary rat astrocyte cultures after AraC+LME treatment (chemical microglia elimination). Propidium-iodide nuclear staining was applied for the examination of cytototoxicity, images were obtained using a Zeiss inverted microscope (AxioVert), image analysis was performed by ImageJ software. Fig. 9A: : untreated control, Fig. 9B: manganese (100 µM, 18 h), Fig. 9C: ammonia (20 mM, 18h), Fig. 9D: LPS (1000 ng, 18h).

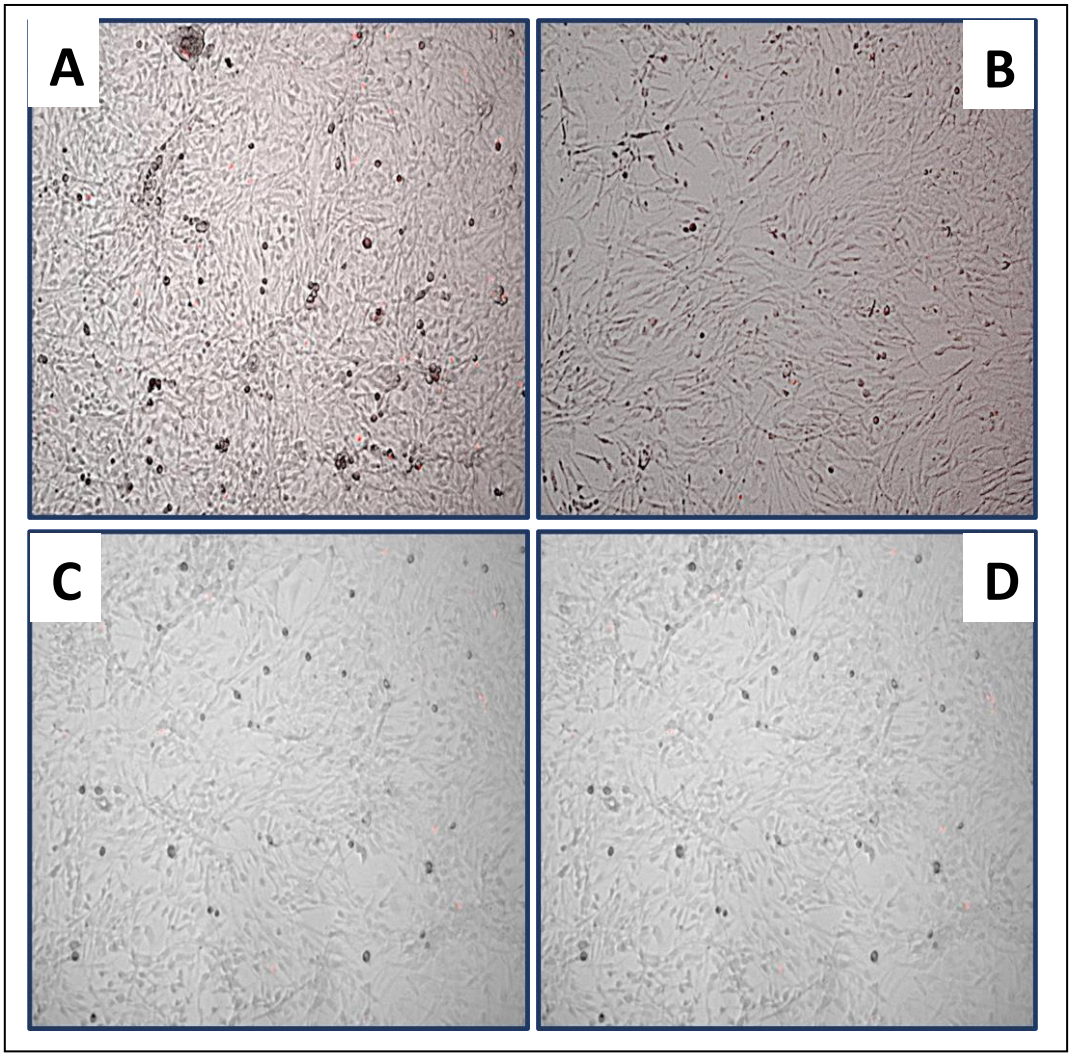
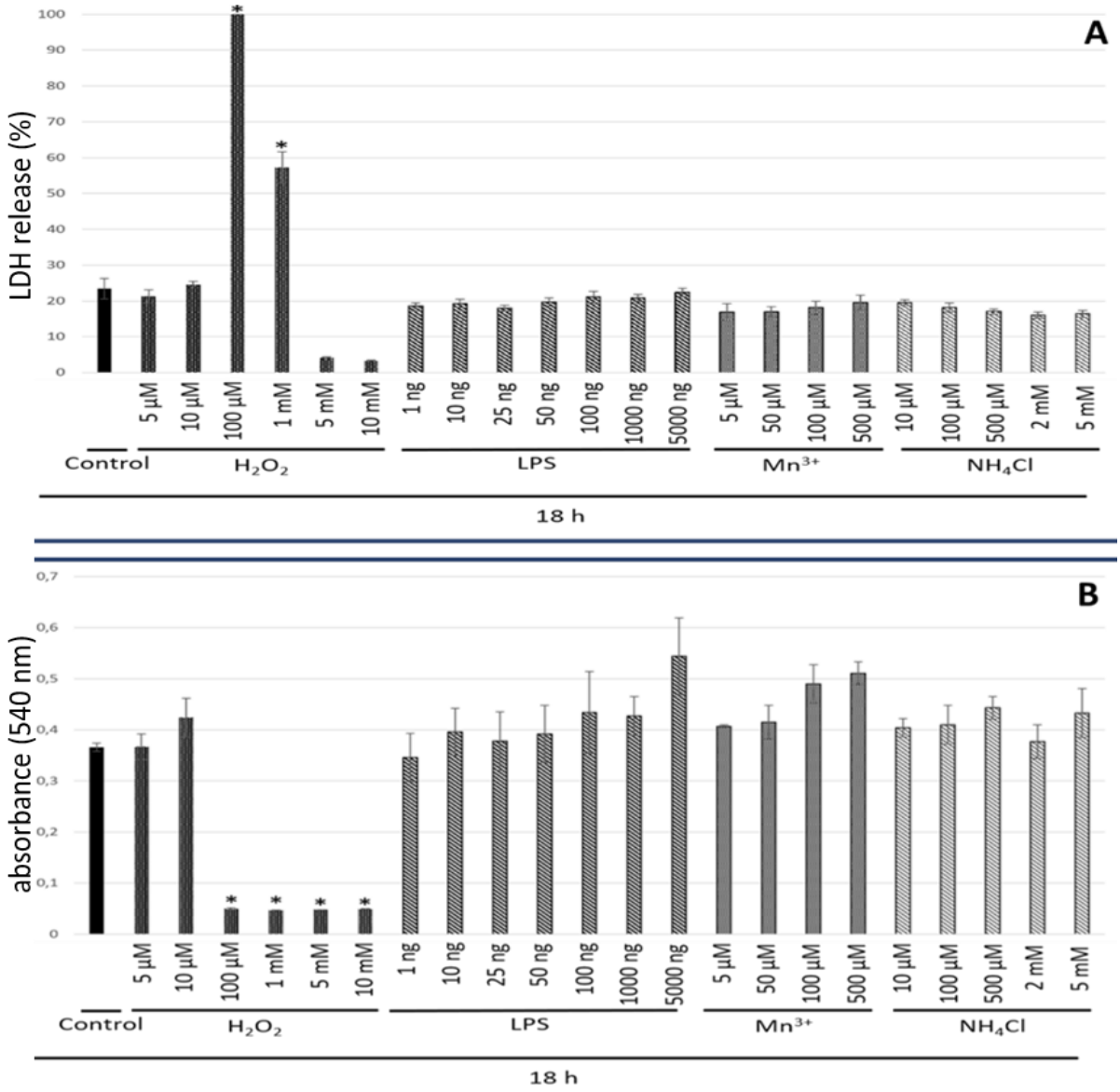


Figure 10. Hydrogen-peroxide (H_2O_2) induced cell death in primary rat astrocyte cultures after AraC+LME treatment (chemical microglia elimination). Fig. 10A: untreated control, Fig. 10B: 100 µM H_2O_2, Fig.10C: 200 µM H_2O_2, Fig. 10D: 400 µM H_2O_2 . Propidium-iodide nuclear staining was applied for the examination of cytototoxicity, images were obtained using a Zeiss inverted microscope (AxioVert), image analysis was performed by ImageJ software.

Although, PI staining was found to be an effective and simple tool for determining cell death, we examined cell viability also by measuring LDH activity in culture medium and by NR uptake assay. While nuclear staining with PI and LDH assay show the number of necrotic cells, NR uptake assay reflects the number of viable cells. Both methods were performed in mixed astrocyte-microglia cultures as well as in cultures after mechanical microglia elimination (shaking).

In astrocyte cultures after shaking, 100 µM and 1 mM H_2O_2 increased the LDH-activity in the medium after 18 hours *(Fig. 11A)*.

In the same system, the NR uptake was decreased after the incubation with 100 µM, 1 mM, 5 mM and 10 mM H_2O_2 respectively, for 18 hours *(Fig. 11B).* These data indicate that both methods are appropriate for establishment of cytotoxicity, moreover only H_2O_2 elicits cell death in astrocyte cultures after mechanical microglia elimination. 5 and 10 µM H_2O_2 did not increase astrocytic LDH release after 18 h, thus these concentrations were suggested to be safe for further investigations. After 24 h incubation period the same concentrations of H_2O_2 (100 µM and 1 mM) elicited cell death as in case of 18 hours-treatment, furthermore 10 µM H_2O_2 also promoted death of the cells after 24 h *(Fig. 11C).* The findings of NR uptake assay regarding the cytotoxicity of H_2O_2 were totally equal (100 µM, 1 mM, 5 mM and 10 mM H_2O_2 decreased the NR uptake), albeit 1 ng LPS and 100 µM ammonia also expressed cytotoxic effect after 24 hours *(Fig. 11D).*



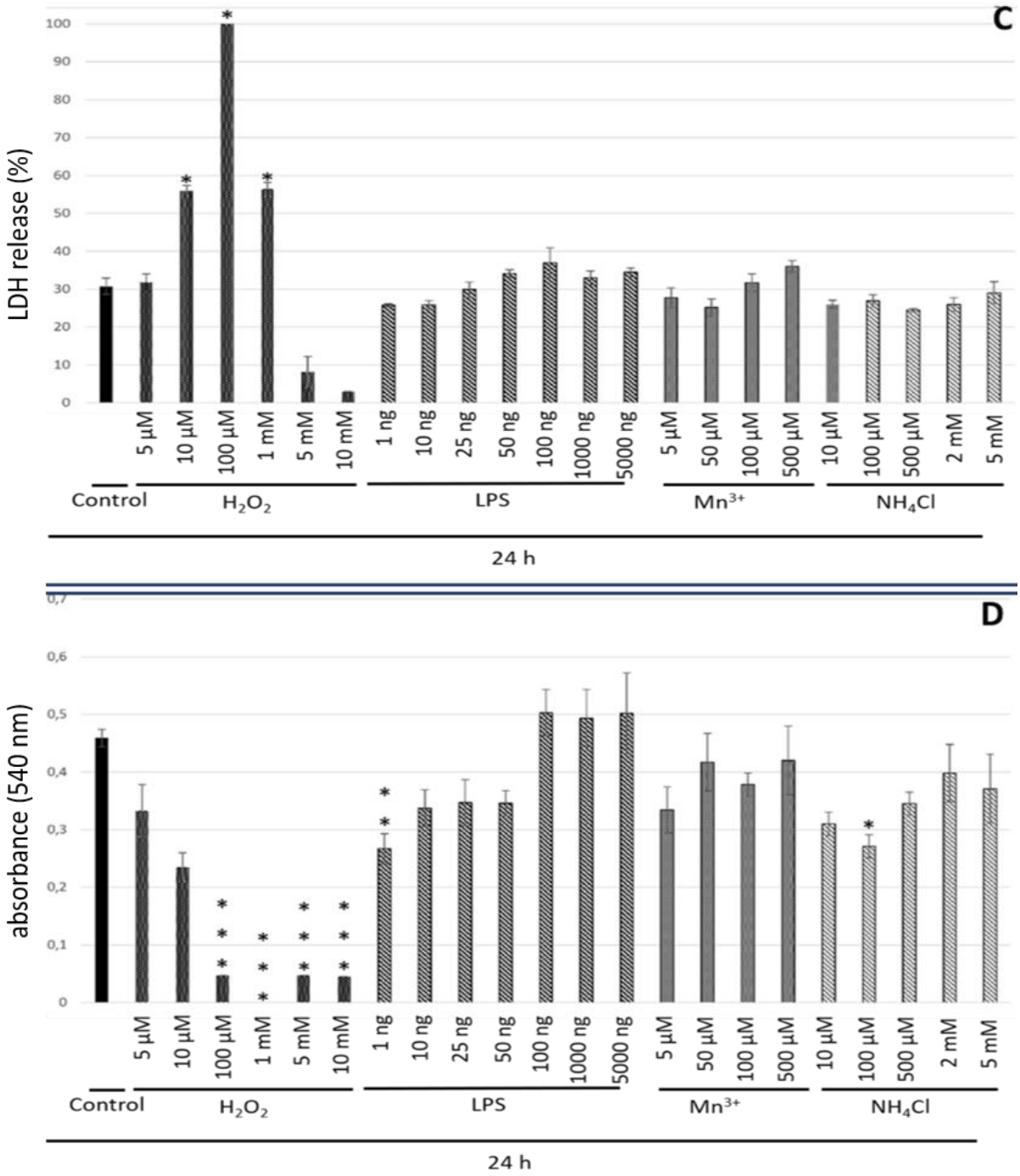
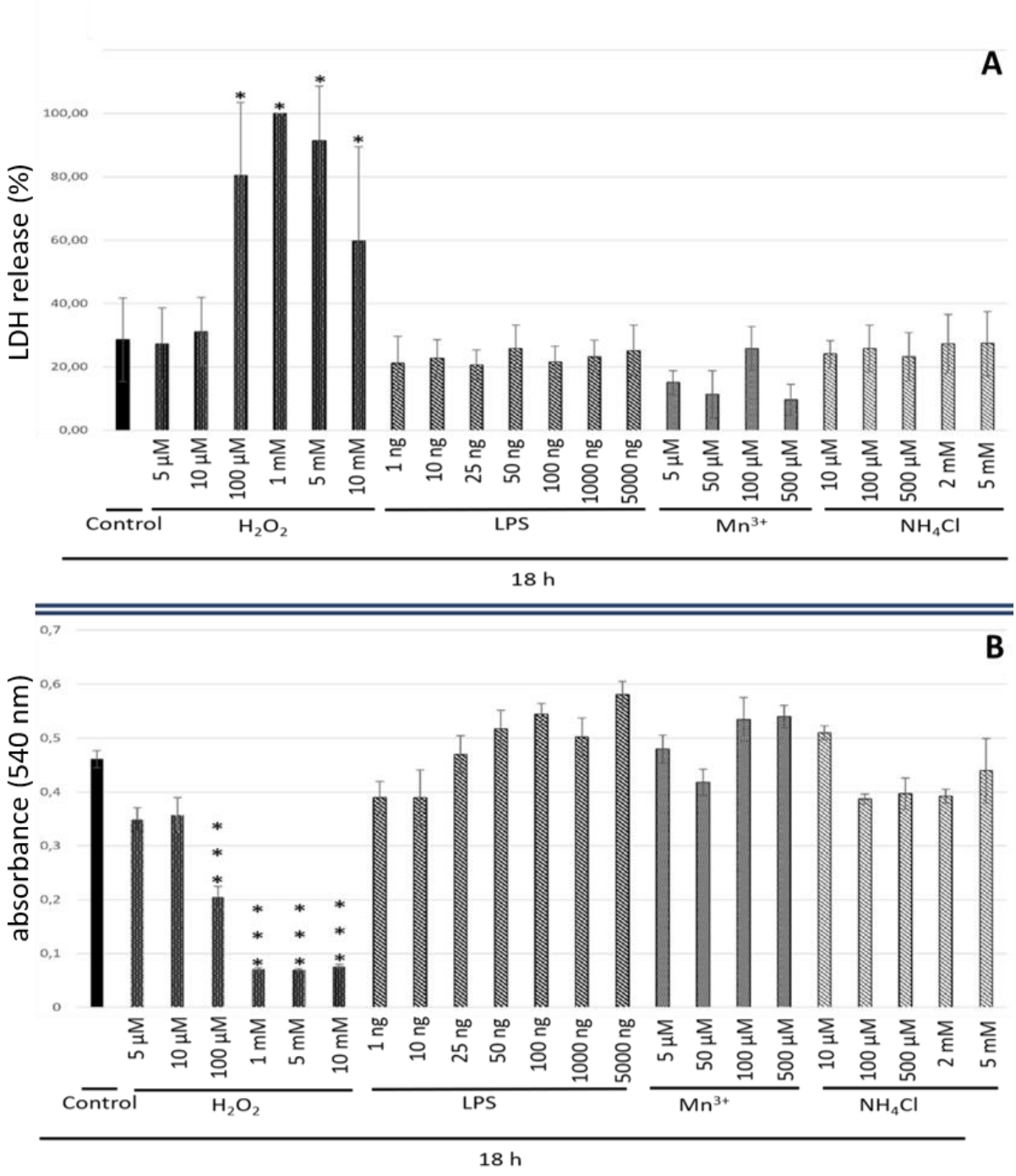


Figure 11. Lactate dehydrogenase (LDH) release assay (A,C) and neutral red uptake assay (B,D) in primary rat astrocyte cultures after microglia elimination by shaking. Cultured rat astrocytes were incubated with medium alone (control) or with different concentrations of H_2O_2, lipopolysaccharide, manganese and ammonia for 18 hours (A,B) and 24 hours (C,D). Both the LDH-activity (A,C) and neutral red uptake (B,D) were measured. The results were expressed as percentages of LDH released from lysed cells after the treatment with TritonX. Data show mean ± SEM of the experiments and analyzed by one-way ANOVA. Each experiment was replicated three times. *p<0,05; **P<0,01; ***P<0,001

Both LDH-activity measurement and NR uptake assay were performed not only in astrocyte culture after shaking, but also in mixed astrocyte-microglia cultures. The results of the both methods after the treatment with the different test compounds for 18 h, are fully equivalent with each other, since only 100 µM, 1 mM, 5 mM and 10 mM H_2O_2 caused cell death in cultures and there were no any other compounds which provoked toxic effect *(Fig. 12A, B).* In case of the 24 h treatment period, the findings of the LDH-release measurement were the same as in case of 18 h incubations *(Fig. 12C),* while the 24 h NR uptake assay resulted in different findings as the 18-hour-NR uptake assay. We have demonstrated that all concentrations of H_2O_2, the lower concentrations of LPS (1 ng, 10 ng, 25 ng), manganese (5 µM and 50 µM) and all concentrations of ammonia, except the lowest dose (10 µM) elicit cytotoxic effect in astrocyte cultures after 24 hours *(Fig. 12D).*


A
LDH release (%)
100,00
80,00
60,00
40,00
20,00
0,00
5 µM
10 µM
100 µM
1 mM
5 mM
10 mM
1 ng
10 ng
25 ng
50 ng
100 ng
1000 ng
5000 ng
5 µM
50 µM
100 µM
500 µM
10 µM
100 µM
500 µM
2 mM
5 mM
Control
H2O2
LPS
Mn3+
NH4Cl
18 h
B
absorbance (540 nm)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Control
H2O2
LPS
Mn3+
NH4Cl
18 h

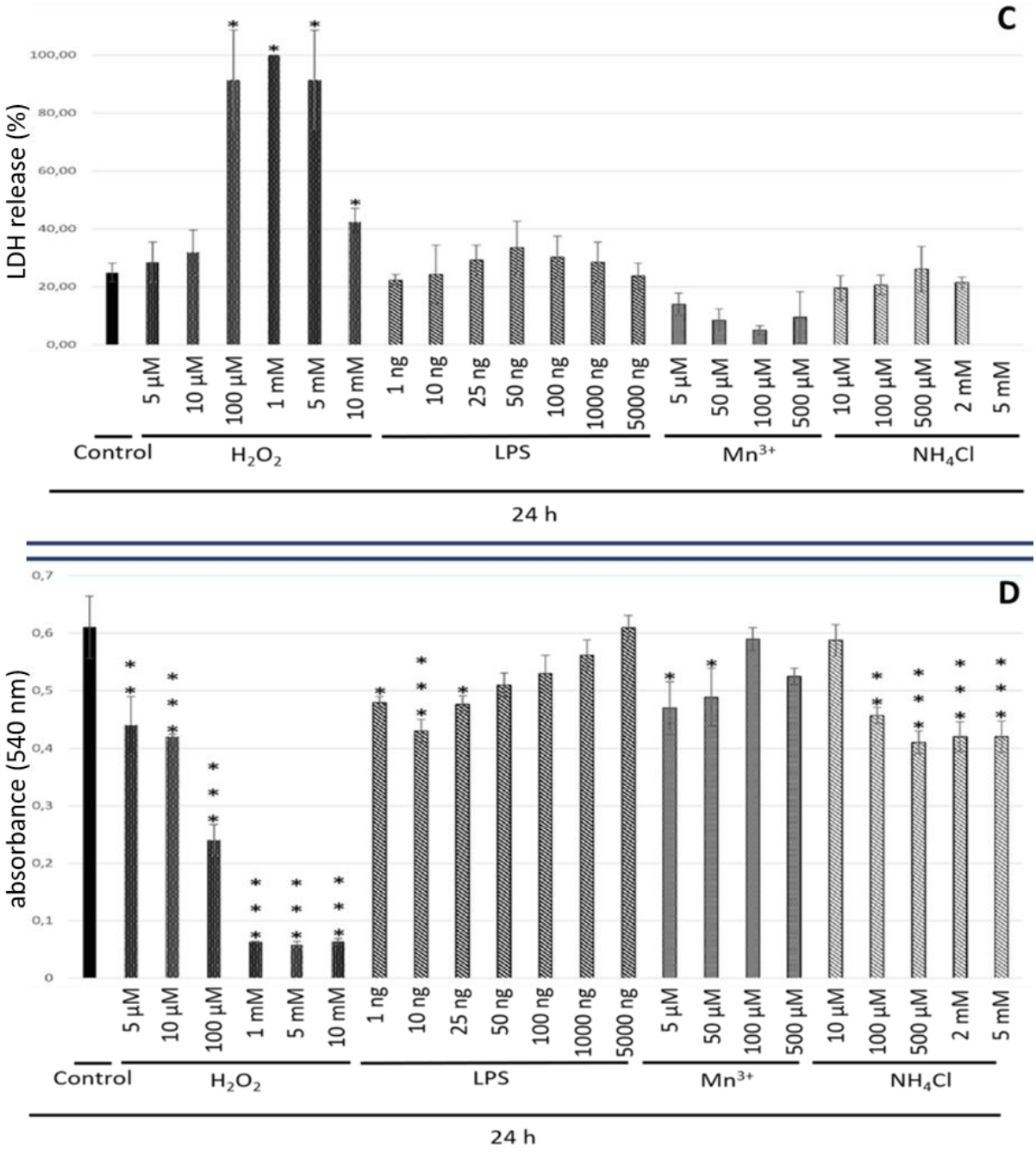


Figure 12. Lactate dehydrogenase (LDH) release assay (A,C) and neutral red uptake assay (B,D) in primary rat mixed astrocyte-microglia cultures*. Cultured rat astrocytes were incubated with medium alone (control) or with different concentrations of H_2O_2, lipopolysaccharide, manganese and ammonia for 18 hours (A,B) and 24 hours (C,D). Both the LDH-activity (A,C) and neutral red uptake (B,D) were measured. The results were expressed as percentages of LDH released from lysed cells after the treatment with TritonX.* Data show mean ± SEM of the experiments and analyzed by one-way ANOVA. Each experiment was replicated three times. *p<0,05; **P<0,01; ***P<0,001

8.3. Intracellular ROS measurement

Intracellular ROS formation was examined in primary rat astrocyte cultures treated with ammonia, Mn, LPS, and H_2O_2. The measurements were performed in cultures after chemical microglia elimination, after shaking as well as in mixed astrocyte-microglia cultures. In AraC+LME-treated cultures, after the exposure of Mn, applied with a concentration of 20 μM for 3h and 6h and with 100 μM for 6h, ROS generation was significantly increased over that of the controls. Furthermore, Mn at 20 μM elicited significant elevation of oxidative stress in time-dependent manner. In ammonia-, LPS- and H_2O_2-exposed astrocytes there was no elevation in intracellular ROS formation when compared to control, however both LPS- and ammonia-exposure lead to a notable, but non-significantly increasing of ROS production *(Fig 13).*

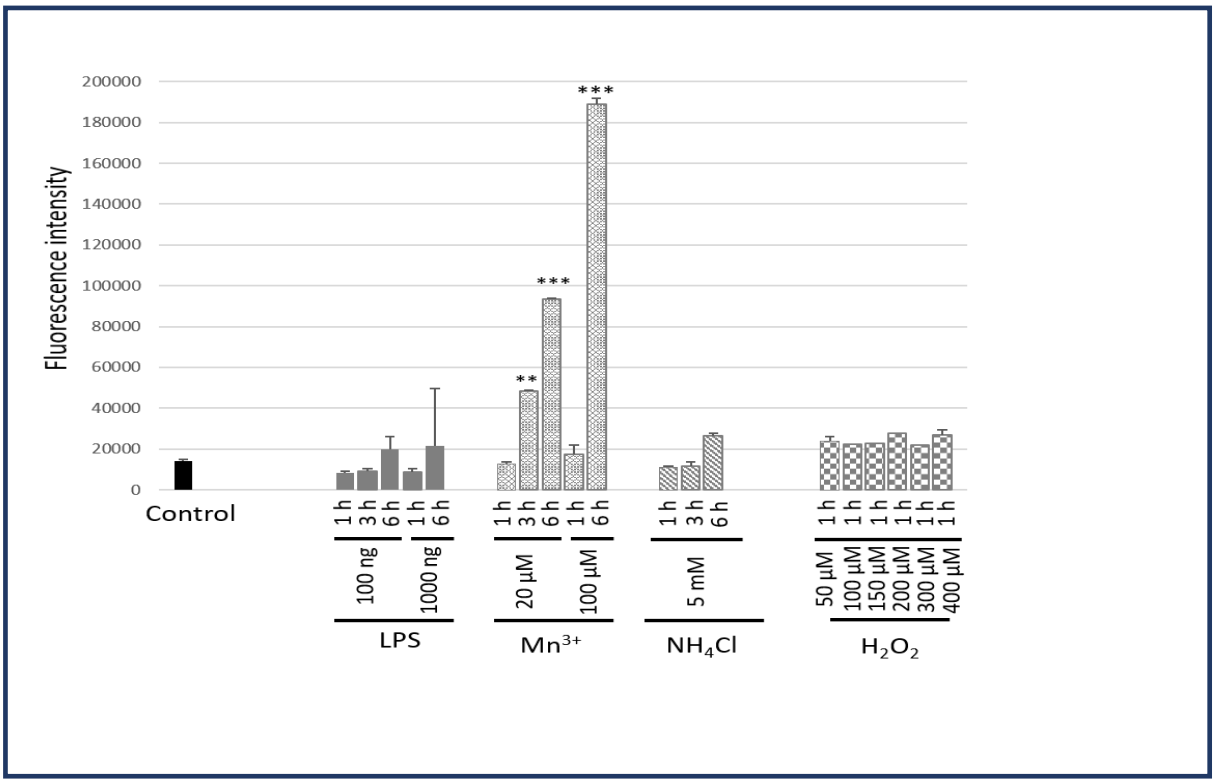


*Figure 13. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on intracellular ROS accumulation in highly purified primary rat astrocyte cultures by AraC+LME-treatment. Data show mean ± SEM of the experiments and is analyzed by one-way ANOVA. Each experiment was replicated four times. **P<0,01, ***P<0,001*

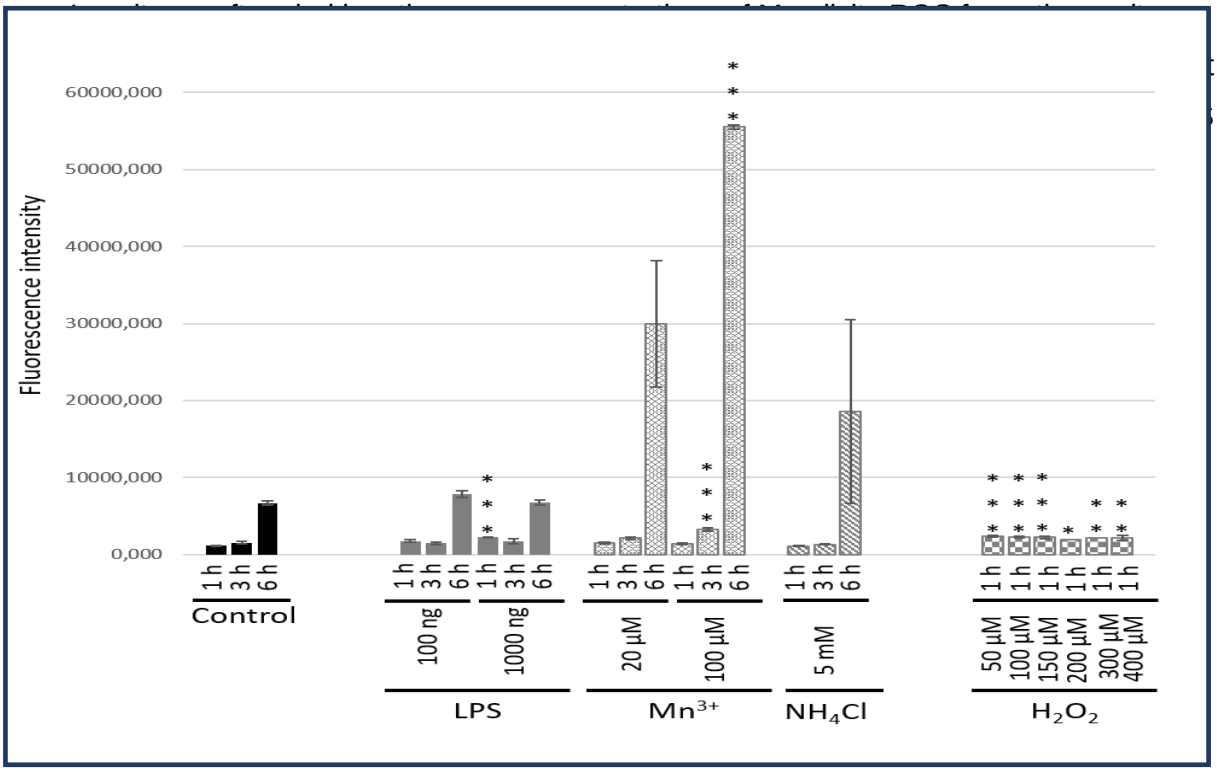


*Figure 14. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on intracellular ROS accumulation in highly purified primary rat astrocyte cultures by shaking. Data show mean ± SEM of the experiments and analyzed by one-way ANOVA. Each experiment was replicated four times. **P<0,01, ***P<0,001*

In mixed glial cultures, all concentrations of H_2O_2, 1000 ng LPS after 1 hour and 100 µM Mn after 3 and 6 hours provoked ROS formation, thus it is suggested that the astrocyte-microglia mixed cultures are more sensitive to the H_2O_2 regarding the oxidative stress, than cultures after microglia elimination *(Fig. 15.).*

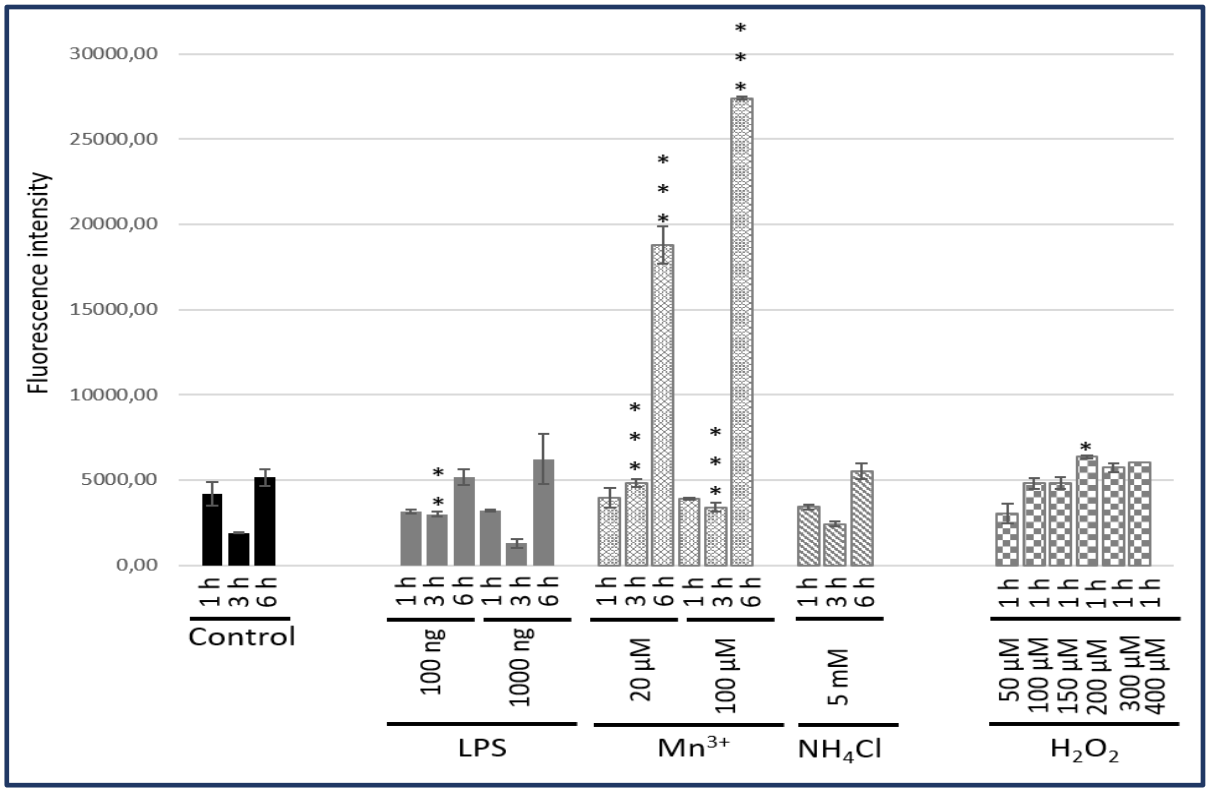


*Figure 15. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on intracellular ROS accumulation in mixed primary rat astrocyte-microglia cultures. Data show mean ± SEM of the experiments and analyzed by one-way ANOVA. Each experiment was replicated four times. **P<0,01, ***P<0,001*

8.4. IL-6 production in primary rat astrocyte cultures

The pro-inflammatory cytokine IL-6 was undetectable in the medium at resting state (untreated control) after mechanical microglia elimination, moreover even the test substances could not elicit IL-6 production (data not shown). In cultures, after chemical microglia elimination the non-stimulated resting astrocytes were able to produce IL-6, however neither of the compounds induced further significant increasing of IL-6. Interestingly, H_2O_2-treatments could not provoke IL-6-production *(Fig. 16.)*, albeit it has been demonstrated by others that H_2O_2 activates NF-κB, which led to increased IL-6 expression in prostate carcinoma cells (151). Since, in our experiments, H_2O_2 did not abolish the production of other cytokines (see later), it is suggested that this result is not associated with cell death. The Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway is required for the IL-6 production in astrocytes (152), thus in our investigations, the undetectable level of IL-6 after the H_2O_2-treatment might be caused by the potential inhibitory effect of H_2O_2 on this pathway.

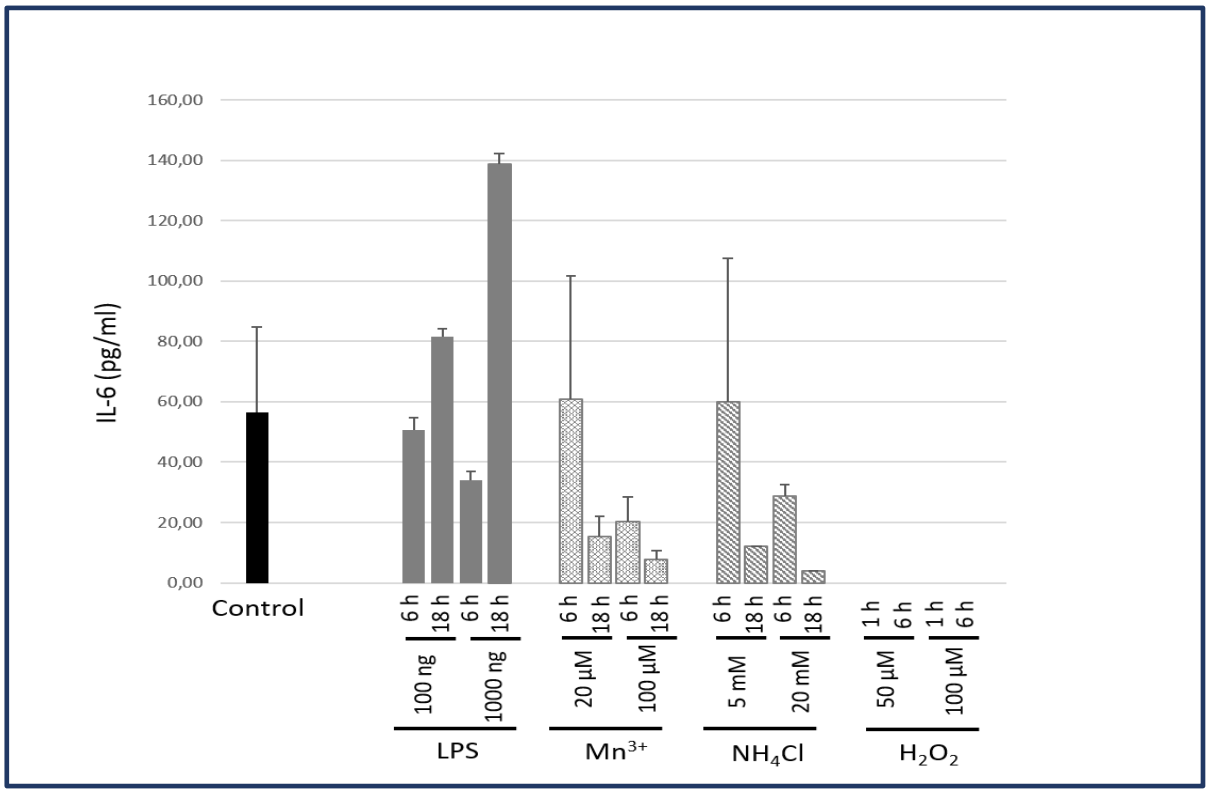


Figure 16. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on IL-6 production in primary rat astrocyte cultures after chemical microglia elimination (AraC+LME treatment). Data were expressed as mean ± SEM and analyzed by one-way ANOVA. Each experiment was replicated four times.

8.5. TNF-α production in primary rat astrocyte cultures

After shaking, resting astrocytes were able to produce TNF-α at resting state, in addition after both the 18-and 24 hour-exposure of 100 ng and 1000 ng LPS, the TNF-α protein expression was significantly increased in the medium *(Fig. 17.).*

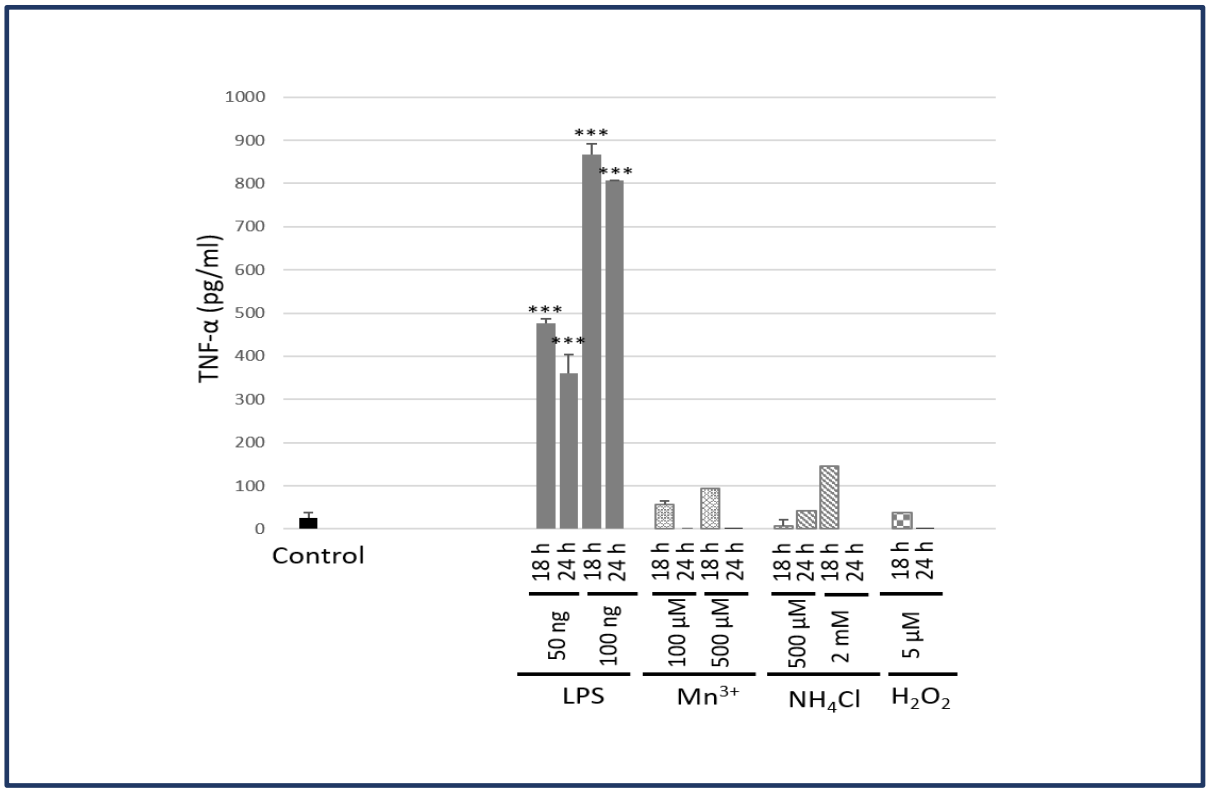


*Figure 17. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on TNF-α-production in primary rat astrocyte cultures after shaking. Data were expressed as mean ± SEM and analyzed by one-way ANOVA. Each experiment was replicated four times. ***P<0,001*

TNF-α could be detected not only in the medium of non-stimulated astrocytes after shaking, but also in case of chemical microglia elimination, however in contrast to the previously described findings, neither of the test compounds were able to increase the release of TNF-α *(Fig. 18.).*

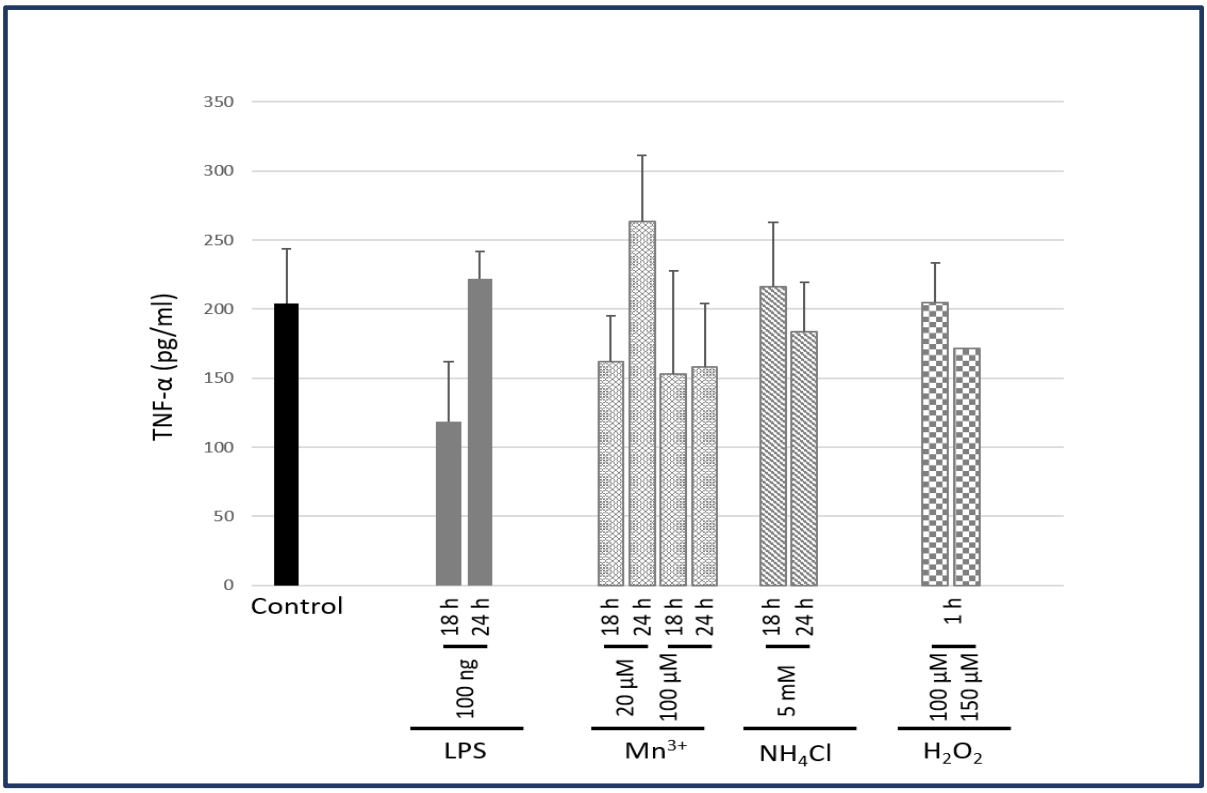


Figure 18. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on TNF-α-production in primary rat astrocyte cultures after chemical microglia elimination (AraC+LME treatment). Data were expressed as mean ± SEM and analyzed by one-way ANOVA. Each experiment was replicated four times.

8.6. IL-1ß-production in primary rat astrocyte cultures purified by shaking

IL-1ß secretion was quantified intracellularly in primary astrocyte cultures purified by mechanical microglia elimination. IL-1ß protein expression could be detected in the cells under baseline condition, moreover LPS, at all of the applied conditions as well as both concentrations of Mn and ammonia after 24 h provoked significant IL-1ß production, which was measured also after the lysis of the cells. Interestingly oxidative stress could not increase the IL-1ß production *(Fig. 19.).*

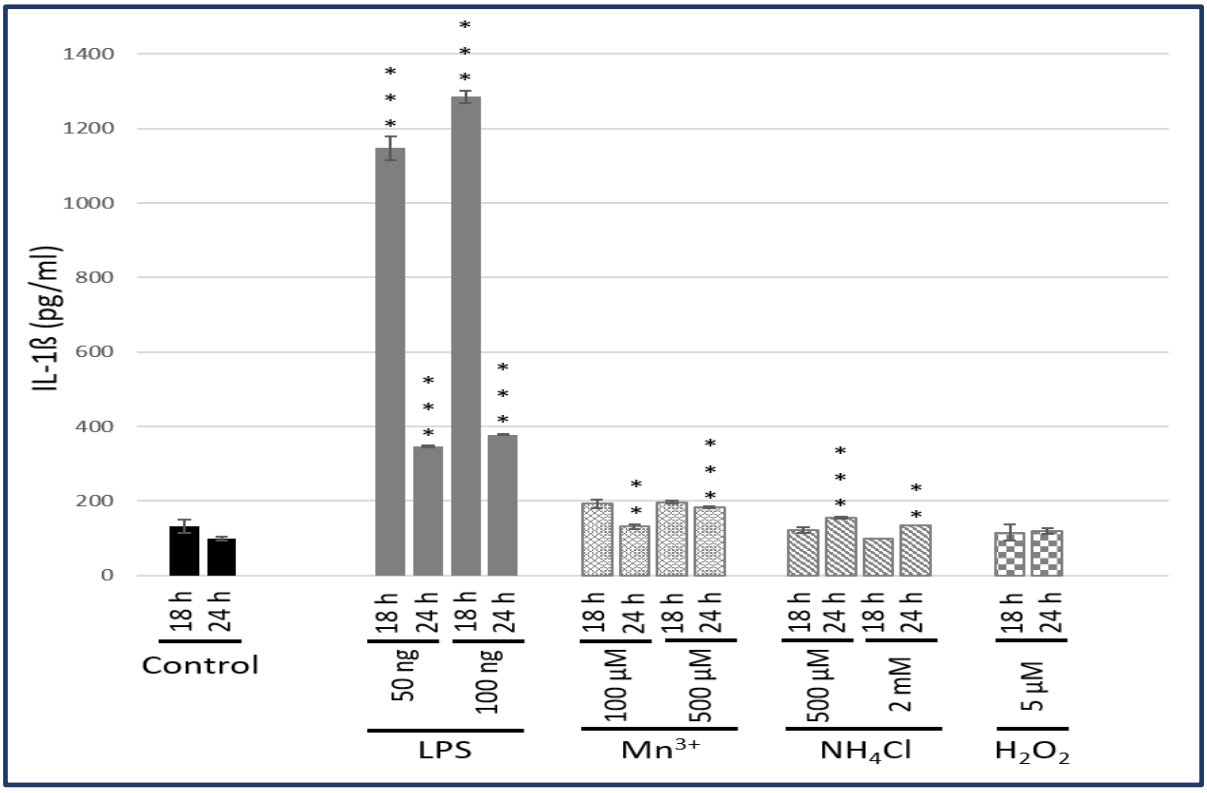


Figure 19. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on TNF-α-production in primary rat astrocyte cultures after chemical microglia elimination (AraC+LME treatment). Data were expressed as mean ± SEM and analyzed by one-way ANOVA. Each experiment was replicated four times.

8.7. IL-10 production in highly purified astrocyte cultures

IL-10 secretion was also quantified in both cultures and we have revealed that both astrocyte cultures are able to produce the anti-inflammatory cytokine IL-10 under basal condition. In cultures after shaking, no treatment could provoke an increase of IL-10 release *(Fig. 20.)*, however in AraC+LME-treated cultures significant IL-10 was detected after the incubation with LPS *(Fig. 21.).*

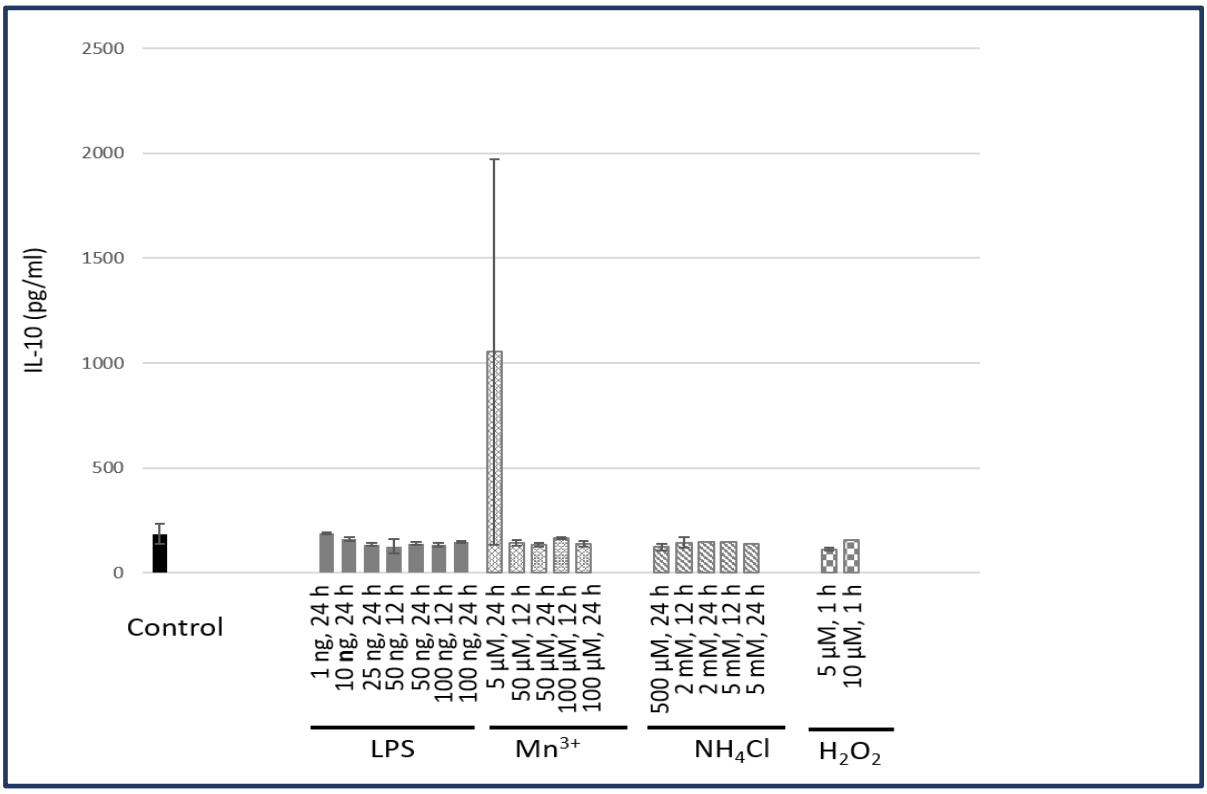


Figure 20. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on IL-10-production in primary rat astrocyte cultures after shaking. Data were expressed as mean ± SEM and analyzed by one-way ANOVA. Each experiment was replicated four times.

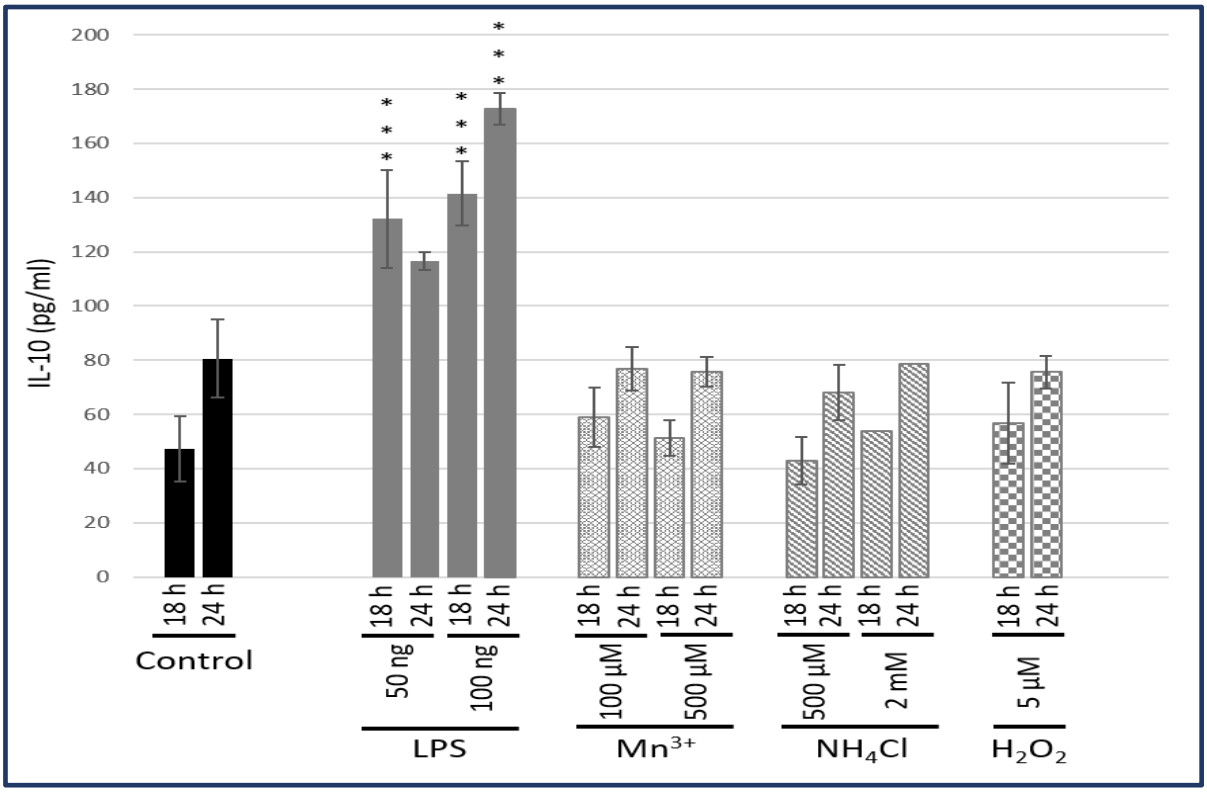


*Figure 21. Effect of ammonia, manganese, hydrogen-peroxide and lipopolysaccharide on IL-10-production in primary rat astrocyte cultures after chemical microglia elimination (AraC+LME treatment). Data were expressed as mean ± SEM and analyzed by one-way ANOVA. Each experiment was replicated four times. ***P<0,001*

8.8. Effect of PDE-inhibitors on glutamate/glutamine metabolism

Beside cytokine production of astrocytes, we examined the glutamate/glutamine metabolism in astrocyte cultures as well. Both glutamate and glutamine concentrations were measured in the medium and also intracellularly. While the extracellular (EC) glutamate level significantly changed in almost all of the treatment groups *(Fig. 22.)*, the intracellular (IC) level of glutamate remained unchanged in most of the treated groups *(Fig. 23.)*. The EC glutamate concentration significantly increased after the treatment with different glutaminase inhibitors (BPTES, DON) and also after the incubation with theophylline and zaprinast, while the IC glutamate level did not change in the BPTES-treated atrocytes, at the same time DON significantly increased that. Theophylline caused elevated glutamate concentration in the IC compartment only at the lowest dose, while zaprinast elicited the same effect at the higher concentration.

The EC and IC glutamine levels could be detected under basal conditions, furthermore both of them were changed significantly after the incubation with the test compounds, however the results were not consistent *(Fig. 24-25.).*

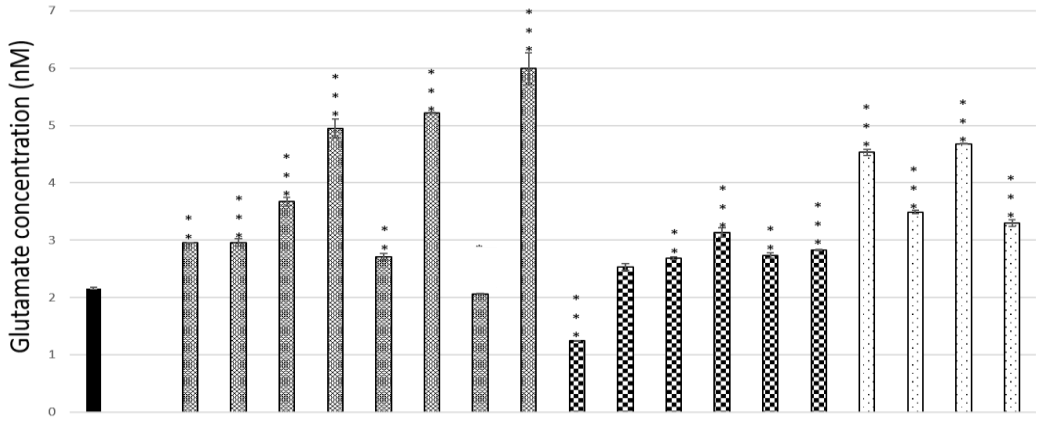
While the EC glutamate level *(Fig. 22.)* was significantly changed in almost all of the treatment groups, the IC level of glutamate *(Fig. 23.)* remained unchanged in the most treated groups. The EC glutamate concentration was significantly increased after the treatment with the different glutaminase inhibitors (BPTES, DON) and also after the incubation with theophylline and zaprinast, while the IC glutamate level did not changed in the BPTES-treated atrocytes, however DON significantly increased that. Theophylline caused elevated glutamate concentration in the IC compartment only at the lowest dose, while zaprinast elicited the same effect at the higher concentration.

The EC glutamine concentration was increased after the treatment with BPTES and theophylline at 150 µM, while all of the other treatements evoked significant decreasing of EC glutamine level *(Fig. 24 A).*

When we compared the potential synergistic effect of ammonia and the different PDE- and glutaminase inhibitors, we have found that the combination of theophylline and ammonia in all the applied concentration led to increasing of IC glutamate level relative to ammonia-treatment, while the combination of BPTES/DON/zaprinast and ammonia cauld not elicit this result (Fig. 23.B).The different concentration of these chemicals and ammonia decreased the glutamate concentration in the EC compartment relative to the ammonia-treated group *(Fig. 24 B).*

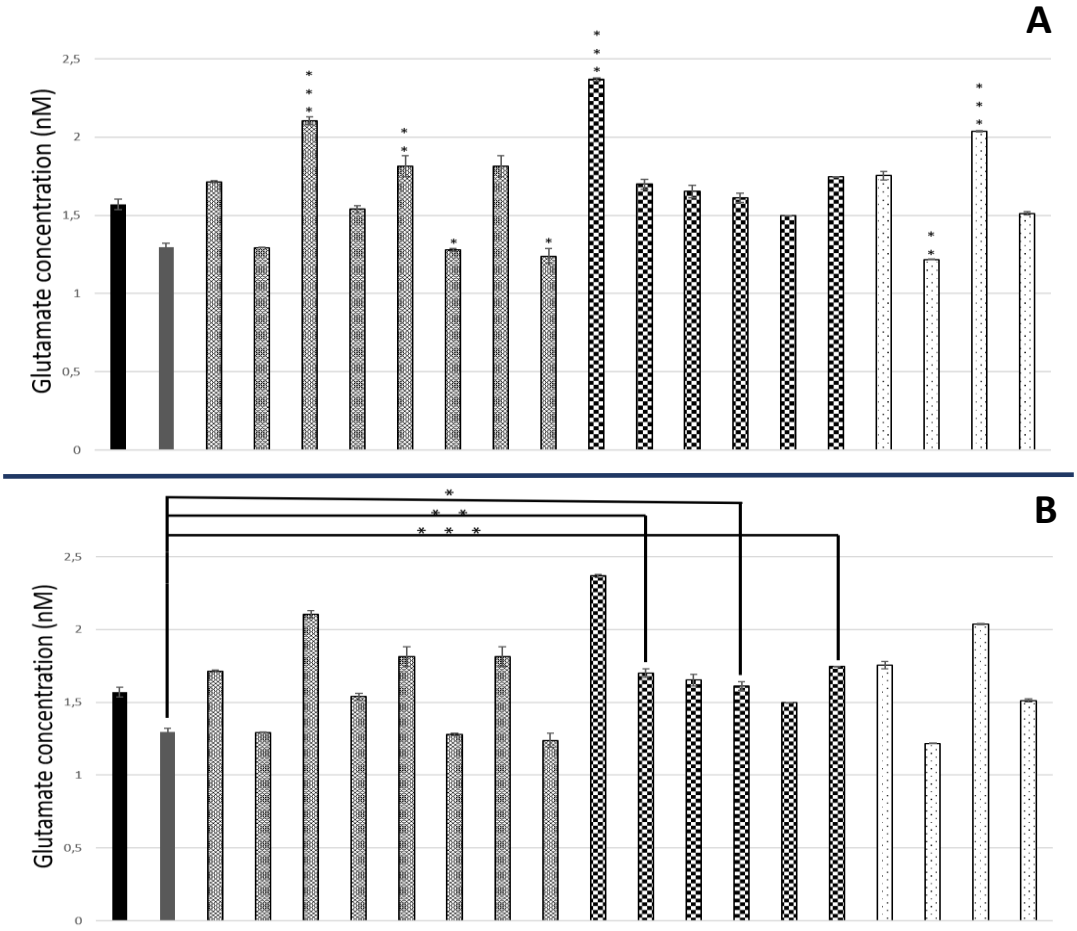
The combination of BPTES / all of the examined concentrations of DON / the lower concentrations of both theophylline and zaprinast and ammonia caused decreasing of EC glutamine level compared to ammonia-treatment alone *(Fig. 24 B).*

IC glutamine level was also decreased after the incubation with either of the test substances (except for the lowest concentration of theophylline) relative to untreated control *(Fig. 25 A).*, moreover, the combination of ammonia and DON/zaprinast/theophylline also decreased the IC level of glutamine relative to ammonia-treatment (except for 300 µM theophylline+ammonia) *(Fig. 25 B).*



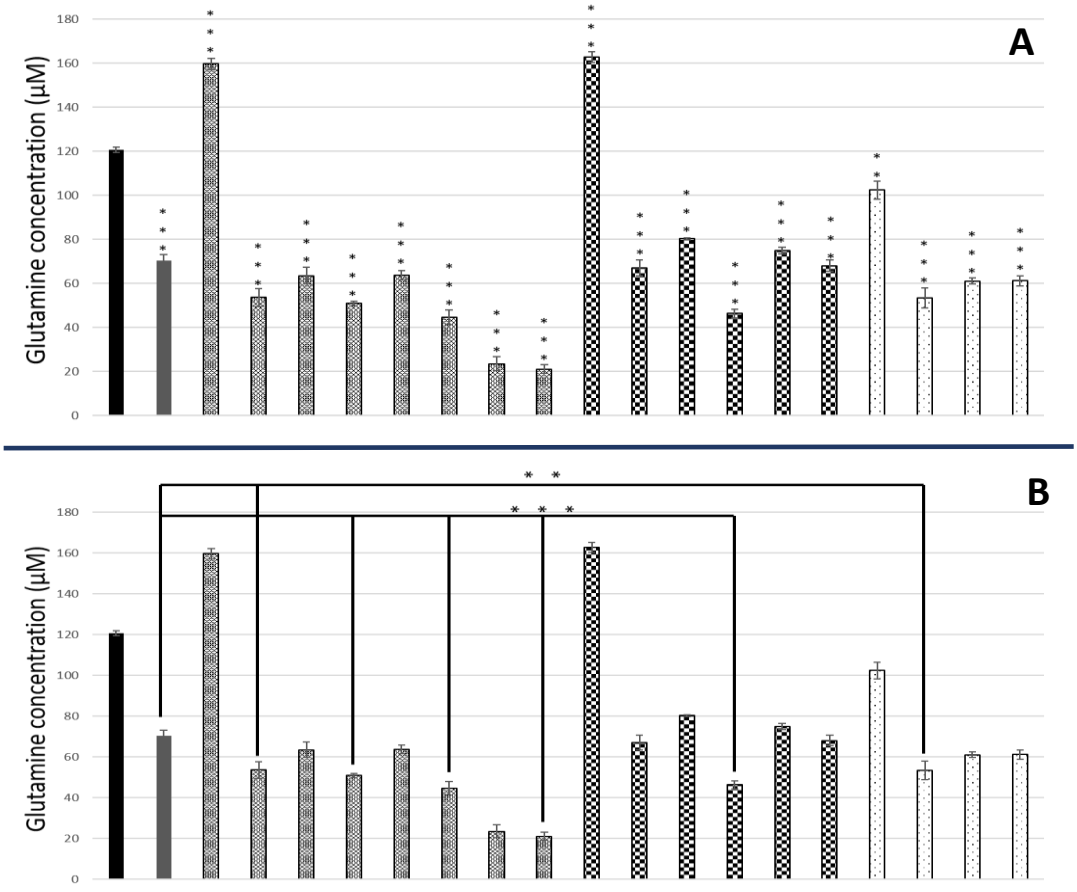
Control	+																		
ammonia: 5mM		+		+		+		+		+		+		+		+		+	+
BPTES: 10 µM			+	+															
DON: 50µM					+	+													
DON: 100µM							+	+											
DON: 1000µM									+	+									
Theophylline: 150µM											+	+							
Theophylline: 300µM													+	+					
Theophylline: 500µM															+	+			
Zaprinast: 100µM																	+	+	
Zaprinast: 300µM																			+	+

Figure 22. Effect of glutaminase inhibitors (BPTES, DON), phosphodiesterase inhibitors (theophylline, zaprinast), ammonia and the combination of these chemicals and ammonia on the ***extracellular glutamate*** *concentration in primary rat astrocyte cultures purified by mechanical microglia elimination (shaking). Cells were pretreated with BPTES/DON/theophylline/zaprinast 1 h before ammonia-exposure (1 h). Data are mean ± SEM from 4 independent experiments and analyzed by one-way ANOVA. **P<0,01, ***P<0,001*



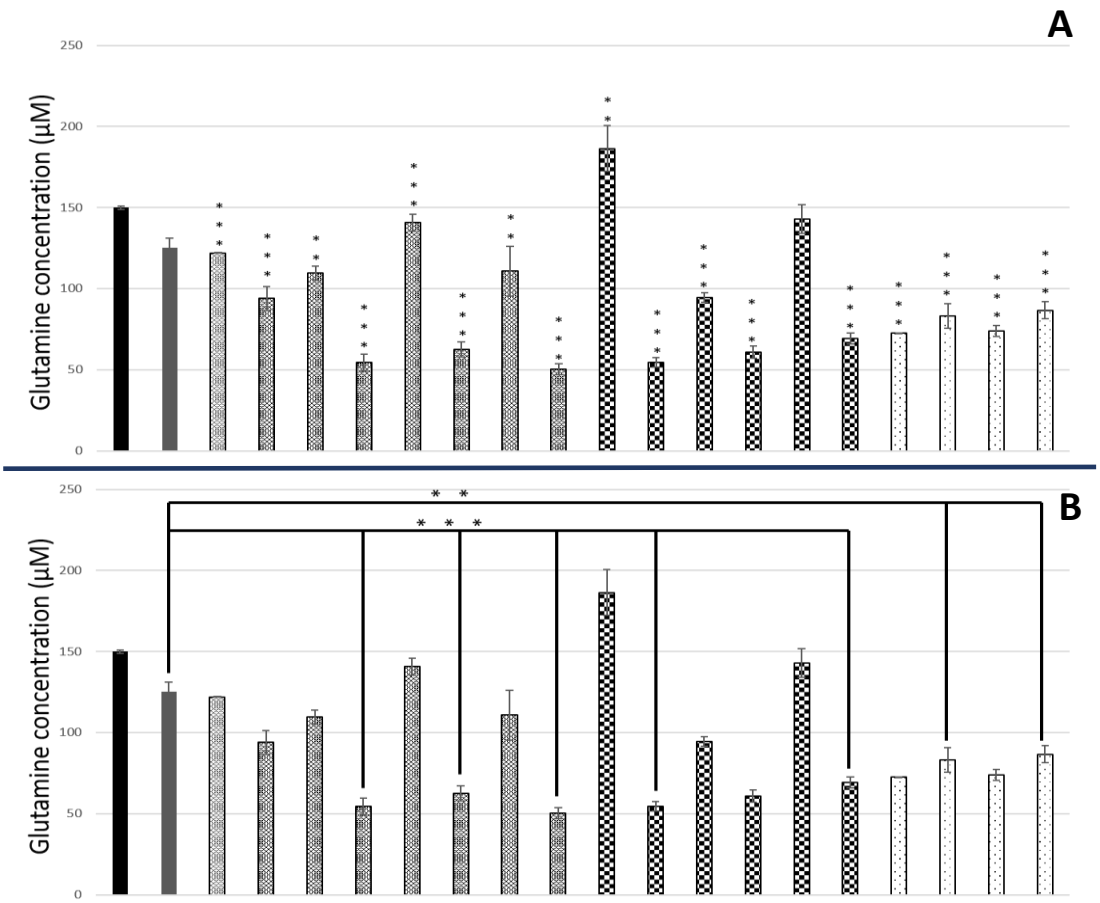
Control	+																		
ammonia: 5mM		+		+		+		+		+		+		+		+		+		+
BPTES: 10 µM			+	+															
DON: 50µM					+	+													
DON: 100µM							+	+											
DON: 1000µM									+	+									
Theophylline: 150µM											+	+							
Theophylline: 300µM													+	+					
Theophylline: 500µM															+	+			
Zaprinast: 100µM																	+	+	
Zaprinast: 300µM																			+	+

Figure 23. Effect of glutaminase inhibitors (BPTES, DON), phosphodiesterase inhibitors (theophylline, zaprinast), ammonia and the combination of these chemicals and ammonia on the ***intracellular glutamate*** *concentration in primary rat astrocyte cultures purified by mechanical microglia elimination (shaking). Cells were pretreated with BPTES/DON/theophylline/zaprinast 1 h before ammonia-exposure (1 h). Cell lysis was performed by centrifugation for 5 min at 14500 rpm. Data are mean ± SEM from 4 independent experiments and analyzed by one-way ANOVA. A: control group=untreated cultures ('Control'). B: control, group= ammonia-treated cultures *p<0,05; **P<0,01, ***P<0,001*



Control	+																		
ammonia: 5mM		+		+		+		+		+		+		+		+		+	+
BPTES: 10 µM			+	+															
DON: 50µM					+	+													
DON: 100µM							+	+											
DON: 1000µM									+	+									
Theophylline: 150µM											+	+							
Theophylline: 300µM													+	+					
Theophylline: 500µM															+	+			
Zaprinast: 100µM																	+	+	
Zaprinast: 300µM																			+	+

Figure 24. Effect of glutaminase inhibitors (BPTES, DON), phosphodiesterase inhibitors (theophylline, zaprinast), ammonia and the combination of these chemicals and ammonia on the ***extracellular glutamine*** *concentration in primary rat astrocyte cultures purified by mechanical microglia elimination (shaking). Cells were pretreated with BPTES/DON/theophylline/zaprinast 1 h before ammonia-exposure (1 h). Data are mean ± SEM from 4 independent experiments experiments and analyzed by one-way ANOVA. A: control group=untreated cultures ('Control'). B: control, group= ammonia-treated cultures. **P<0,01, ***P<0,001*



Control	+																		
ammonia: 5mM		+		+		+		+		+		+		+		+		+		+
BPTES: 10 µM			+	+															
DON: 50µM					+	+													
DON: 100µM							+	+											
DON: 1000µM									+	+									
Theophylline: 150µM											+	+							
Theophylline: 300µM													+	+					
Theophylline: 500µM															+	+			
Zaprinast: 100µM																	+	+	
Zaprinast: 300µM																			+	+

*Figure 25. Effect of glutaminase inhibitors (BPTES, DON), phosphodiesterase inhibitors (theophylline, zaprinast), ammonia and the combination of these chemicals and ammonia on the **intracellular glutamine** concentration in primary rat astrocyte cultures purified by mechanical microglia elimination (shaking). Cells were pretreated with BPTES/DON/theophylline/zaprinast 1 h before ammonia-exposure (1 h). Cell lysis was performed by centrifugation for 5 min at 14500 rpm. Data are mean ± SEM from 4 independent experiments and analyzed by one-way ANOVA.*

*A: control group=untreated cultures ('Control'). B:control, group= ammonia-treated cultures **P<0,01, ***P<0,001*

9. Discussion

9.1. Characterization of the primary rat astrocyte cultures

We used highly purified primary rat astrocyte cultures as well as astrocyte-microglia mixed cultures. In order to eliminate the residual microglia from the astrocyte cultures, we applied two distinct methods. On one hand, microglia were removed by treatment with the combination of AraC and LME. In our experiments, we tested both 15 mM and 25 mM LME (1 hour) after the incubation with AraC (3 days). On the other hand, highly purified astrocyte cultures can be reached by mechanical removing of microglia (shaking) too, which could be performed in several ways (147,153).

According to our immunocytochemical examinations, both the mechanical and the chemical purification procedure led to high purity of the astrocyte cultures.

In the chemical elimination method, we have found that the higher concentration of LME (25 mM) is more efficient. While comparing the chemical and mechanical methods, it turned out that shaking represents a more efficient method for microglia elimination as, we have exceeded 94% purity in contrast to the AraC+LME-treatment, where 11% of the cells were microglia. This is similar to a former result (146), however, it is worth to mention that in many studies the purity of cultures was confirmed only by GFAP staining (154,155).

9.2. Cell viability

In our experiments, we tested the potential cytotoxicity of the examined compounds by three different methods: PI staining, LDH release and NR uptake assay.

9.2.1. PI staining

PI staining represents an appropriate method for verifying the loss of membrane integrity in astrocytes (155,156). Our results show that the test compounds at the examined concentrations did not cause significant cell death. Since false positive events might occur during PI staining in a significant number (157), we further assessed cell death with both LDH release and NR uptake assays as well.

9.2.2. LDH assay

In primary rat astrocyte cultures prepared by shaking method as well as in mixed glial cultures, the LDH release assay showed that only H_2O_2 elicits significant cell death (independently from the incubation time).

It is in line with another study, which proved that H_2O_2 causes cell death in both astrocyte and microglia, furthermore astrocytes seem to be quite sensitive to H_2O_2 as 1h long incubation in 1 mM concentration is able to damage 72% of them (158), moreover it has been revealed that 72% of astrocytes were dead after treatment with 1 mM H_2O_2 for 1 h (159). It is well established that H_2O_2 provokes cell death in astrocytes, verified by several methods (145,160), which could be mediated not only by direct oxidation of different macromolecules in the cells, but also through activation of signalling pathways leading to alteration of cytoskeleton and membrane properties (145). In our study, on the highly purified astrocyte cultures, after 24 h treatment period with 10 μM H_2O_2 provoked significant cell damage, while in 100 μM concentration after 18 h was required for the same effect. Similarly to the purified astrocyte cultures, in primary mixed astrocyte-microglia cultures, exclusively H_2O_2 could cause cell death, in addition, this effect occurred at the same concentrations in both examined incubation periods.

Interestingly, in astrocyte cultures purified by shaking, LDH release remained under the control value after the 18 h-treatment with the highest concentrations of H_2O_2. This result is in line with a previous finding where ROS inhibited LDH activity (161), which occurred probably due to the H_2O_2-evoked diminishing of LDH activity in a concentration-dependent manner (162). Taken together, although both methods rely on a suitable tool for assessing the cytotoxicity, the application of LDH method should be considered carefully when testing cytotoxic effect of H_2O_2.

9.2.3. NR assay

NR uptake assay could be used for the determination of cytotoxicity under baseline condition in astrocyte cultures (163), after H_2O_2-exposure in cerebellar granule neurons (164) or after paraquat-treatment in both astrocytes and neurons (165).

In astrocyte cultures purified by shaking (18 and 24 h), as well as in mixed astrocyte-microglia cultures (18 h), the same concentrations of H_2O_2-exposure resulted in a decreased uptake of NR. Moreover, there were no other test compounds which could have provoked significant change in NR uptake, although it occurred in some cases after 24. In our experiment, in mixed astrocyte-microglia cultures, all concentrations of ammonia (except for the lowest dose), led to significant decrease of NR accumulation after 24 h. For all we know, there is no data regarding the measurement of ammonia-evoked cell viability reduction by NR uptake assay.

Both LPS- and Mn-exposure led to NR decrease in the mixed glial cultures after 24 h, however the highest concentrations of these compounds did not cause reduction of NR accumulation.

The alteration of cell viability after LPS-exposure was examined in astrocytes by others and it has been found that even the highest dose of LPS (30 μg/ml) did not lead to decrease of NR uptake (166).

9.2.4. Comparing the result of LDH and NR uptake assay

In astrocyte cultures after shaking as well as in mixed astrocyte-microglia cultures, we have demonstrated that both LDH and NR uptake assays are appropriate for confirming cytotoxicity, however NR uptake assay is more suitable to the establishment of H_2O_2-and ammonia-evoked cell death/reduction of cell viability. Taken together, both methods provided similar results about the cytotoxicity.

9.3. Intracellular ROS measurement

ROS formation was performed in enriched astrocyte cultures purified by mechanical or chemical microglia elimination as well as in mixed glial cultures. In our experiments related to quantification of oxidative stress, ammonia did not lead to ROS formation in either of the astrocyte cultures, even though we used higher concentration of ammonia for longer incubation time than others did (73,167). There is abundant evidence that ammonia elicits oxidative stress in astrocyte cultures (50,73,75), thus the reason for this discrepancy is not fully understood.

In contrast to ammonia, Mn-treatment elicited oxidative stress in all three types of cultures. It should be noted that in astrocyte cultures after chemical microglia elimination, ROS generation was found to be increased in a time-dependent manner after exposure of 20 μM Mn for 1, 3 and 6 hours. Our findings are in agreement with other studies demonstrating that Mn is able to cause oxidative stress at various concentrations, albeit these studies detected the ROS formation in less purified cultures or in cultures without any immunostaining for microglia marker (168,169). The time-dependent redox state changes after Mn-treatment has also been confirmed by others (169). In astrocyte cultures after chemical microglia elimination, we measured a slight, nonsignificant ROS production after the incubation with H_2O_2, conversely, we observed significant ROS generation at all the examined concentrations of H_2O_2 in mixed astrocyte-microglia cultures. The H_2O_2-evoked increasing of ROS level in astrocyte cultures has already been described by others (154,170), furthermore the graded concentrations of H_2O_2 provoked a dose-dependent rise of ROS formation in astrocytes, (150). It should be elucidated, why H_2O_2 did not elicit significant oxidative stress in AraC+LME-treated astrocyte cultures, even though H_2O_2 provoked ROS production in astrocyte-microglia mixed cultures.

We suppose that oxidative stress develops in a greater extent or more rapidly in microglia than in astrocytes. It is supported by the literature, since it has been shown that the methylmercury-provoked ROS production occurs at lower concentration of this compound and earlier, compared to astrocyte (171), in addition, it has been reported that while low-dose H_2O_2 significantly increased ROS levels in microglia, it did not occur in astrocytes (158). In our experiment, it is possible that the presence of microglia may have increased the sensitivity of astrocytes to the H_2O_2-evoked oxidative stress, however an opposite effect was revealed, as per which, since the activated microglia provoked the upregulation of antioxidative enzymes in astrocytes, a higher resistance to oxidative stress induced by H_2O_2 was developed (172). It is well known, that there is a crosstalk between microglia and astrocytes, via secreted mediators and extracellular vesicles (173), thus we may strongly suggested that the interactions between microglia and astrocytes contribute to our distinct results regarding the H_2O_2 -provoked ROS production in the two types of cell cultures, even if the precise mechanism is not fully understood. One possible underlying cause might be the different sensitivity of A1 and A2 astrocytes to oxidative stress, although it should be proven.

Finally, in our study, 100 ng LPS after 3 hours in astrocyte cultures purified by shaking and 1000 ng LPS after 1 hour in mixed primary rat astrocyte-microglia cultures led to significant ROS production. It is in line with a previous study, in which it has been demonstrated that 1000 ng LPS induced significant ROS production after both 4 and 24 h in primary astrocyte cultures without microglia-elimination (174), however it is not known, why the higher concentration of LPS did not cause oxidative stress in cultures purified by shaking.

9.4. TNF-α-production in highly purified astrocyte cultures

TNF-α-release was quantified in the medium of enriched astrocyte cultures generated by either chemical or mechanical microglia elimination. Both cultures were observed to secrete TNF-α without adding separate test substances, and importantly, cultures after the chemical treatment produced much more (around 10-fold more) TNF-α than cultures that underwent shaking. It is well established that both microglia and astrocytes are able to produce TNF-α (108,175–177), thus we assume that a minimal difference in the number of residual microglia in the two cultures could cause the distinct pattern of TNF-α-release. It is also supported by a study, which demonstrated that microglia-free astrocyte cultures do not produce TNF-α under baseline conditions (113). Another underlying mechanism could be caused by cathepsins, which are proteases located in the lysosomes. They not only act as mediators of apoptosis (178), but also might have a role in inflammatory processes, since cathepsin X inhibition significantly reduced LPS-induced production of the pro-inflammatory cytokines IL-6 and TNF-α from microglia (179).

L-Leucyl-LME provokes the release of cathepsins into the cytosol (180), thus we suggest that the LME-treatment can contribute to the neuroinflammatory events in our cultures.

We have found that in AraC+LME-treated cultures, no substances could rise the TNF-α secretion. On the other hand, both applied concentration of LPS at each incubation time increased the TNF-α production in cultures purified by shaking. It has been described earlier that adult astrocytes are able to produce TNF-α that can be increased by LPS, although the cultures contained significant amount of microglia (5%) (181). Interestingly, TNF-α could not be detected in LPS-treated human astrocyte cell line (182) and in microglia-free astrocyte cultures after LPS-treatment (113). LPS induces ROS production in astrocytes (174) and it has been described that ROS leads to reduced level of inflammatory mediators, such as inducible nitric oxide synthase in interferon-gamma-induced microglia culture (159). Thus, we suppose that the LPS-induced oxidative stress could influence the cytokine production in the minimal amount of residual microglia in our cultures.

9.5. IL-6-production in highly purified astrocyte cultures

In astrocyte cultures purified by shaking, IL-6 was undetectable in the medium, both in treated and untreated groups, groups. In contrast, basal IL-6 could be measured in cultures purified by AraC+LME treatment, although it remained unchanged after the incubation with all the different test compounds. There are increasing evidences showing that astrocytes secrete IL-6, although these results should be interpreted with caution due to the heterogeneity of the applied *in vitro* models, which does not allow comparing of the results. Enriched neonatal cortical astrocyte cultures were able to secrete IL-6, however secretion was much higher after 7 days than after 1 day (183). It has been described that LPS-treated astrocytes secrete IL-6, however it was measured on human astrocyte cell line (182). In other studies, IL-6 release could also be observed from activated astrocytes, albeit the purity of the astrocyte cultures were not described (97,184), thus it is suggested that microglia may have also contributed to the IL-6 secretion in these astrocyte cultures. Moreover, it has been described that the AraC-treatment leads to the development of stellate morphology and increased GFAP-level in primary astrocyte cultures, indicating an astrocyte activation (185).

Taken together, we hypothesise that the fact that we did not detect IL-6 in the supernatant of astrocyte cultures purified by shaking, but the AraC+LME-treated astrocyte cultures secreted IL-6 under basal condition, could happen either due to the minimal amount of microglia in the cultures or because of the LME-provoked astrocyte activation.

9.6. IL-1ß-production in astrocyte cultures purified by shaking

Our observations are in line with previous findings, that activated human astrocytes are able to secrete IL-1ß (108), however interestingly, IL-1ß mRNA expression could not be measured in mouse astrocytes after LPS-treatment (186).

LPS could provoke IL-1ß release in astrocyte cultures, however it should be noted that after the LME-treatment, the IL-1ß secretion was abolished, indicating the potential role of microglia in the IL-1ß production (187). As far as we know, there are no direct evidences that ammonia or Mn could increase the IL-1ß protein expression, thus ours is the first finding, suggesting that some factors involved in HE are able to induce IL-1ß secretion of astrocytes.

9.7. IL-10-production in highly purified astrocyte cultures

We also measured also the anti-inflammatory cytokine, IL-10 in the medium of primary astrocyte cultures after both kinds of purification procedures. The untreated groups of both cultures were able to secrete IL-10, but no change was detected in cultures purified by shaking treated with any of the test compounds. Interestingly, LPS provoked IL-10 release in the cultures purified by chemical microglia elimination. In another report, it has been established that astrocytes produce IL-10 under physiological conditions and also after oxygen glucose deprivation (188), however in this study the purity of cultures was only 95%, suggesting the potential role of residual microglia.

9.8. Glutamate assay

After the measurement of cytokine production in astrocyte cultures, we also examined the function of glutaminase, which represents another neuroinflammation-related event, beside ROS generation. We hypothesised that some PDE inhibitors can block the glutaminase activity in the primary rat astrocyte cultures, which could be demonstrated by the increase in glutamine level concomitant with a decrease in glutamate concentration. During the measurements of IC and EC glutamate concentrations, BPTES (10 µM) and DON (50 µM, 100 µM, 1000 µM) were used as a positive control. Unexpectedly, BPTES as well as 50 µM and 100 µM DON increased the EC glutamate level, moreover the same concentrations of DON caused elevated IC glutamate level as well.

These findings are not aligned with previous results in the field, since DON inhibits both isoforms of glutaminase (60), thereby reduces glutamate synthesis from glutamine (189) and BPTES, an allosteric glutaminase inhibitor (190) also causes decrease in glutamate synthesis (62).

After ammonia-exposure, EC glutamate was undetectable, moreover the IC glutamate reduction was not significant, however, it has been shown that acute ammonia treatment at the same concentration as we applied, should lead to glutamate release from astrocytes (191).

We found that both zaprinast and theophyilline increased the EC glutamate content in all the applied concentrations. This result might be linked to the zaprinast- and theophyilline-evoked elevation of cGMP, however this explanation was not supported by others, since reduced total content of GLAST (glutamate transporter, also called EAAT1) glutamate transporter in cerebellar slices of hyperammonemic rats could be normalized by the treatment with extracellular cGMP (192). Thus, in our experiments the increased level of cGMP after the treatment with PDE inhibitors should have reduced the glutamate content in the medium by increasing the expression of GLAST, which are mainly expressed by astrocytes and responsible for the glutamate uptake from the synaptic cleft (193). The connection between cGMP and glutamate is bidirectional, since not only cGMP affects the EC glutamate content, but also the changes of glutamate level contribute to the cGMP content. Glutamate activates the N-methyl-D-aspartate (NMDA) receptor, which causes increasing of calcium ion concentration in the post-synaptic neuron, followed by activation of nitric oxide (NO) synthase. It will lead to NO generation subsequently followed by an increase of cGMP (194). Thus, in our experiment, the zaprinast- and theophylline-evoked elevation of EC glutamate level might cause the increase of cGMP level, however there were no neurons in our cultures. Interestingly, it has been shown that NMDA receptors are not expressed exclusively in neurones, but also in astrocytes (195), thus we suppose that our observation has some relevances in highly purified astrocyte cultures as well.

After the treatment with either 100μM DON+5mM ammonia or 1000μM DON+5mM ammonia or 150 μM theophyilline, we have found that glutamate concentrations changed in the opposite direction in the EC and IC compartment. While, the combination of DON and ammonia increased the EC glutamate level, these treatments caused decrease in IC glutamate level. Similarly, 150 μM theophylline reduced EC glutamate concentration and raised IC level of glutamate. These findings are not in line with a previous report, which revealed that elevation of EC glutamate concentration leads to elevation of IC glutamate level in astrocytes, which itself causes the downregulation of GLAST, thereby EC glutamate is not able to enter astrocytes, subsequently leading to the increase of EC concentration of glutamate (196).

9.9. Glutamine assay

We have demonstrated that ammonia-exposure leads to decreased EC glutamine concentration, which is supported by a previous study in which ammonia inhibited glutamine transport from mouse cortical astrocytes (197). Interestingly, while in our study we used acute ammonia-treatment, in the abovementioned research, ammonia was applied for 24 h. Nevertheless, it remains unclear why the ammonia-induced reduction of EC glutamine level was not followed by an increase of IC glutamine level, as already described by others (47). One possible cause could be the existence of other alternative pathways of ammonia/glutamine metabolism. One of them, which has attracted little attention to date, is the so-called ‘glutaminase II pathway’.

The first step of this pathway represented by the transamination of glutamine to α-ketoglutaramate (α-KGM) by the enzymes *glutamine transaminases* (isozymes of *glutamine transaminases*: *Glutamine transaminase K*: GTK, *Glutamine transaminase L*: GTL). The activity of both GTK and GTL has been detected in the brain, in addition, it has been shown that both activities and expression of GTK are higher in the astrocytes than in the neurons (198). It is wo

th mentioning that it has been shown that GTK is identical to *kynurenine aminotransferase I*: KAT I (198)) and GTL is identical to KAT III (199).

Another alternative pathway of glutamate/glutamine cycle is suggested to be alanine formation, thereby glutamine synthesis is not the only route for ammonia metabolism, since ammonia might be metabolized also into alanine by the function of glutamate dehydrogenase (GDH) and alanine aminotransferase (ALAT) (56).

We have found that BPTES, DON, zaprinast and theophyilline caused significantly lower levels of IC glutamine when compared with untreated control (except 150 μM theophyilline). These results do not correspond with our hypothesis, since the inhibition of glutaminase should result in an increased IC concentration of glutamine. In fibrosarcoma cell line, both zaprinast and BPTES increased the glutamine level (62), moreover DON also increased the ammonia-evoked elevation of IC glutamine level in astrocyte culture (47).

10. Conclusion

In my work I have found that both shaking and AraC+LME-treatment are appropriate methods for the microglia-elimination, however only shaking resulted in a significantly higher purity of the astrocyte cultures. Furthermore, my experiments have revealed that both LDH release and NR uptake assays are suitable methods to detect the cytotoxicity of different compounds involved in the pathogenesis of HE, however NR uptake assay is more appropriate method for assessing H_2O_2- and ammonia- evoked cell death than LSH assay. Furthermore, we have demonstrated that Mn causes ROS formation in highly purified astrocyte cultures as well as in mixed glial cultures. Interestingly, H_2O_2 provoked oxidative stress exclusively in the mixed glial cultures, which suggests that using of primary rat astrocyte cultures without microglia elimination is not suitable for the examination of the H_2O_2-evoked ROS formation in astrocytes. During the measurements of cytokine production, we have found that TNF-α, IL-6 and IL-10 secretion differs between cultures, which also highlights the importance of the culture composition regarding the pro-and anti-inflammatory cytokine production in astrocyte cultures. Our investigations have shown that astrocyte cultures purified by shaking produce IL-1ß, moreover LPS, ammonia and Mn increase the protein level of this cytokine. Finally, we have studied the possible glutaminase-inhibitor effect of some PDE inhibitors, however already the examination of positive controls provided controversial data, thus further *in vivo*/clinical investigations are needed to clarify the mechanism of the unexpected results.

11. New scientific results

11.1. My research revealed that the method of shaking and AraC+LME-treatment of primary rat astrocyte cultures are not equivalent with each other, since shaking results in higher purity of astrocyte cultures than chemical treatment, verified by immunocytochemistry

11.2. During the cell viability measurements, similar results were obtained from LDH release and NR uptake assay, however, we have demonstrated that NR uptake assay provides more reliable results regarding the assessing of cytotoxicity of both H_2O_2 and ammonia due to the followings:

- in primary rat astrocyte cultures purified by shaking, the highest concentrations of H_2O_2 caused reduction of LDH release in the culture medium, albeit the reduction of NR uptake provided evidence for the significant increase of cell death.
- in primary rat mixed astrocyte-microglia cultures, the NR uptake assay showed significant increase of cell death after the incubation with ammonia for 24 h, and this result could not be detected by the elevation of extracellular LDH activity. Moreover, in contrast to LDH, NR uptake assay verified the H_2O_2-evoked cell death also at lower concentrations after 24 h.

11.3. My results are the first, to our knowledge, to demonstrate differences in regard to the intracellular ROS production in primary rat astrocyte cultures purified by either shaking or Arac+LME-treatment or in mixed astrocyte-microglia cultures.

- H_2O_2 did not cause oxidative stress in primary rat astrocyte cultures purified by AraC+LME-treatment, conversely provoked oxidative stress in primary rat astrocyte cultures purified by shaking at only one concentration and led to ROS production in the mixed primary rat astrocyte-microglia cultures at all of the applied concentrations.

11.4. Cytokine production

- The pro-inflammatory cytokine IL-6 was below the detection level in astrocyte cultures purified by shaking in the untreated group and in the treated groups as well, in contrast with this, both control and treated groups of cultures purified by AraC+LME-treatment demonstrated IL-6 secretion but no significant increases appeared after time- and dose-dependent LPS, Mn, or ammonia administration, while all the H_2O_2 levels totally eliminated the IL-6 production.

- Astrocyte cultures purified by shaking produced lesser amount of TNF-α than cultures purified by AraC+LME-treatment, moreover, in the latter case, LPS could not increase the TNF-α production in contrast to cultures purified by shaking
- LPS caused increased IL-10 secretion in the cultures purified by AraC+LME-treatment, in contrast, LPS did not have this effect in cultures purified by shaking
- Both Mn and ammonia could increase the IL-1ß production in astrocyte cultures purified by shaking

11.5.Glutamate/glutamine metabolism

- We have shown that both PDE inhibitors, zaprinast and theophyilline caused increase in the EC glutamate levels, which may have clinical significance with regard to the glutamate-NO-cGMP pathway and the treatment of HE.

12. References:

1. Barany Z, Sterczer A, Jocsak G, Frenyo L V., Kiss DS. The aetiology and some new pathophysiological aspects of hepatic encephalopathy. Literature review. Magy Állatorvosok Lapja. 2017;139(3):157–68.

2. Vilstrup H, Amodio P, Bajaj J, Cordoba J, Ferenci P, Mullen KD, et al. Hepatic encephalopathy in chronic liver disease: 2014 Practice Guideline by the American Association for the Study Of Liver Diseases and the European Association for the Study of the Liver. Hepatology. 2014;60(2).

3. Hassanein TI, Hilsabeck RC, Perry W. Introduction to the hepatic encephalopathy scoring algorithm (HESA). Dig Dis Sci. 2008;53(2).

4. Ridola L, Cardinale V, Riggio O. The burden of minimal hepatic encephalopathy: From diagnosis to therapeutic strategies. Vol. 31, Annals of Gastroenterology. 2018.

5. Bajaj JS, Saeian K, Christensen KM, Hafeezullah M, Varma RR, Franco J, et al. Probiotic yogurt for the treatment of minimal hepatic encephalopathy. Am J Gastroenterol. 2008;103(7).

6. Ridola L, Nardelli S, Gioia S, Riggio O. Quality of life in patients with minimal hepatic encephalopathy. Vol. 24, World Journal of Gastroenterology. 2018.

7. Bajaj JS, Saeian K, Schubert CM, Hafeezullah M, Franco J, Varma RR, et al. Minimal hepatic encephalopathy is associated with motor vehicle crashes: The reality beyond the driving test. Hepatology. 2009;50(4).

8. Chen QF, Zou TX, Yang ZT, Chen HJ. Identification of patients with and without minimal hepatic encephalopathy based on gray matter volumetry using a support vector machine learning algorithm. Sci Rep. 2020;10(1).

9. Fichet J, Mercier E, Genée O, Garot D, Legras A, Dequin PF, et al. Prognosis and 1-year mortality of intensive care unit patients with severe hepatic encephalopathy. J Crit Care. 2009 Sep 1;24(3):364–70.

10. Nakao M, Nakayama N, Uchida Y, Tomiya T, Ido A, Sakaida I, et al. Nationwide survey for acute liver failure and late-onset hepatic failure in Japan. J Gastroenterol. 2018;53(6).

11. Singh SK, Sarma M Sen, Yachha SK, Srivastava A, Poddar U. Late-Onset Hepatic Failure in Children: Risk Factors that Determine the Outcome. Dig Dis. 2020;38(5).

12. Prakash R, Mullen KD. Mechanisms, diagnosis and management of hepatic

encephalopathy. Vol. 7, Nature Reviews Gastroenterology and Hepatology. 2010.

13. Mokdad AA, Lopez AD, Shahraz S, Lozano R, Mokdad AH, Stanaway J, et al. Liver cirrhosis mortality in 187 countries between 1980 and 2010: A systematic analysis. BMC Med. 2014;12(1).

14. Poveda MJ, Bernabeu Á, Concepción L, Roa E, de Madaria E, Zapater P, et al. Brain edema dynamics in patients with overt hepatic encephalopathy. A magnetic resonance imaging study. Neuroimage. 2010;52(2).

15. Bajaj JS, O'Leary JG, Tandon P, Wong F, Garcia-Tsao G, Kamath PS, et al. Hepatic Encephalopathy Is Associated With Mortality in Patients With Cirrhosis Independent of Other Extrahepatic Organ Failures. Clin Gastroenterol Hepatol. 2017;15(4).

16. Dong V, Nanchal R, Karvellas CJ. Pathophysiology of Acute Liver Failure. Vol. 35, Nutrition in Clinical Practice. 2020.

17. EASL Clinical Practical Guidelines on the management of acute (fulminant) liver failure. J Hepatol. 2017;66(5).

18. Rech R, Barros C. Neurologic Diseases in Horses. Vol. 31, Veterinary Clinics of North America - Equine Practice. 2015.

19. Buczinski S, Duval J, d'Anjou MA, Francoz D, Fecteau G. Portacaval shunt in a calf: Clinical, pathologic, and ultrasonographic findings. Can Vet J. 2007;48(4).

20. Marçal VC, Oevermann A, Bley T, Pfister P, Miclard J. Hepatic encephalomyelopathy in a calf with congenital portosystemic shunt (CPSS). J Vet Sci. 2008;9(1).

21. Humann-Ziehank E, Bruegmann M, Ganter M. Hepatoencephalopathy in a goat: Clinical manifestation of an intrahepatic porto-systemic shunt. Small Rumin Res. 2001;42(2).

22. Kinde H, Pesavento PA, Loretti AP, Adaska JM, Barr BC, Moore JD, et al. Congenital portosystemic shunts and hepatic encephalopathy in goat kids in California: 11 cases (1999-2012). J Vet Diagnostic Investig. 2014;26(1).

23. Torisu S, Washizu M, Hasegawa D, Orima H. Brain magnetic resonance imaging characteristics in dogs and cats with congenital portosystemic shunts. Vet Radiol Ultrasound. 2005;46(6).

24. Barnes HL, Chrisman CL, Mariani CL, Sims M, Alleman AR. Clinical signs, underlying cause, and outcome in cats with seizures: 17 Cases (1997-2002). J Am Vet Med Assoc. 2004;225(11).

25. Da Fonseca Ferreira M, Mellanby RJ, Gow AG. Serum melatonin in dogs with congenital portosystemic shunting, with and without hepatic encephalopathy. In: Veterinary Record. 2020.

26. Shawcross DL, Olde Damink SWM, Butterworth RF, Jalan R. Ammonia and hepatic encephalopathy: The more things change, the more they remain the same. Vol. 20, Metabolic Brain Disease. 2005.

27. Lidbury JA, Cook AK, Steiner JM. Hepatic encephalopathy in dogs and cats. Vol. 26, Journal of veterinary emergency and critical care (San Antonio, Tex. : 2001). 2016.

28. Lidbury JA, Ivanek R, Suchodolski JS, Steiner JM. Putative precipitating factors for hepatic encephalopathy in dogs: 118 cases (1991–2014). J Am Vet Med Assoc. 2015;247(2).

29. Tobias KM, Rohrbach BW. Association of breed with the diagnosis of congenital portosystemic shunts in dogs: 2,400 Cases (1980-2002). J Am Vet Med Assoc. 2003;223(11).

30. Carrera I, Kircher PR, Meier D, Richter H, Beckman K, Dennler M. In vivo proton magnetic resonance spectroscopy for the evaluation of hepatic encephalopathy in dogs. Am J Vet Res. 2014;75(9).

31. Gow AG. Hepatic Encephalopathy. Vet Clin North Am Small Anim Pract. 2017 May;47(3).

32. Holecek M. Evidence of a vicious cycle in glutamine synthesis and breakdown in pathogenesis of hepatic encephalopathy-therapeutic perspectives. Vol. 29, Metabolic Brain Disease. 2014.

33. Clemmesen JO, Larsen FS, Kondrup J, Hansen BA, Ott P. Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. Hepatology. 1999;29(3).

34. Luo M, Li L, Yang EN, Dai CY, Liang SR, Cao WK. Correlation between interleukin-6 and ammonia in patients with overt hepatic encephalopathy due to cirrhosis. Clin Res Hepatol Gastroenterol. 2013;37(4).

35. Tivers MS, Handel I, Gow AG, Lipscomb VJ, Jalan R, Mellanby RJ. Hyperammonemia and systemic inflammatory response syndrome predicts presence of hepatic encephalopathy in dogs with congenital portosystemic shunts. PLoS One. 2014;9(1).

36. Oliveira MM, Tremblay M, Rose CF. A195 GLUTAMINE SYNTHETASE IN

ENDOTHELIAL CELLS OF THE BLOOD-BRAIN BARRIER: NEW TARGET FOR THE TREATMENT OF HEPATIC ENCEPHALOPATHY? J Can Assoc Gastroenterol. 2018 Mar 1;1(suppl_1).

37. Rose C, Ytrebø LM, Davies NA, Sen S, Nedredal GI, Belanger M, et al. Association of reduced extracellular brain ammonia, lactate, and intracranial pressure in pigs with acute liver failure. Hepatology. 2007;46(6).

38. Weissenborn K, Ahl B, Fischer-Wasels D, Van Den Hoff J, Hecker H, Burchert W, et al. Correlations between magnetic resonance spectroscopy alterations and cerebral ammonia and glucose metabolism in cirrhotic patients with and without hepatic encephalopathy. Gut. 2007;56(12).

39. Nath K, Saraswat VA, Krishna YR, Thomas MA, Rathore RKS, Pandey CM, et al. Quantification of cerebral edema on diffusion tensor imaging in acute-on-chronic liver failure. NMR Biomed. 2008;21(7).

40. Chavarria L, Alonso J, Rovira A, Córdoba J. Neuroimaging in acute liver failure. Vol. 59, Neurochemistry International. 2011.

41. Chastre A, Bélanger M, Nguyen BN, Butterworth RF. Lipopolysaccharide precipitates hepatic encephalopathy and increases blood-brain barrier permeability in mice with acute liver failure. Liver Int. 2014 Mar;34(3).

42. McMillin MA, Frampton GA, Seiwell AP, Patel NS, Jacobs AN, DeMorrow S. TGFβ1 exacerbates blood–brain barrier permeability in a mouse model of hepatic encephalopathy via upregulation of MMP9 and downregulation of claudin-5. Lab Investig. 2015 Aug 1;95(8).

43. Colombo E, Farina C. Astrocytes: Key Regulators of Neuroinflammation. Vol. 37, Trends in Immunology. Elsevier Ltd; 2016. p. 608–20.

44. Norenberg MD, Martinez-Hernandez A. Fine structural localization of glutamine synthetase in astrocytes of rat brain. Brain Res. 1979;161(2).

45. Tofteng F, Hauerberg J, Hansen BA, Pedersen CB, Jørgensen L, Larsen FS. Persistent arterial hyperammonemia increases the concentration of glutamine and alanine in the brain and correlates with intracranial pressure in patients with fulminant hepatic failure. J Cereb Blood Flow Metab. 2006;26(1).

46. Chavarria L, Alonso J, García-Martínez R, Simón-Talero M, Ventura-Cots M, Ramírez C, et al. Brain magnetic resonance spectroscopy in episodic hepatic encephalopathy. J Cereb Blood Flow Metab. 2013;33(2).

47. Jayakumar AR, Rao KVR, Murthy CRK, Norenberg MD. Glutamine in the mechanism of ammonia-induced astrocyte swelling. Neurochem Int. 2006;48(6–7).

48. Pichili VBR, Rama Rao K V., Jayakumar AR, Norenberg MD. Inhibition of glutamine transport into mitochondria protects astrocytes from ammonia toxicity. Glia. 2007;55(8).

49. Albrecht J, Zielińska M, Norenberg MD. Glutamine as a mediator of ammonia neurotoxicity: A critical appraisal. Vol. 80, Biochemical Pharmacology. 2010.

50. Rama Rao K V., Norenberg MD. Glutamine in the pathogenesis of hepatic encephalopathy: The Trojan horse hypothesis revisited. Vol. 39, Neurochemical Research. 2014.

51. Sepehrinezhad A, Zarifkar A, Namvar G, Shahbazi A, Williams R. Astrocyte swelling in hepatic encephalopathy: molecular perspective of cytotoxic edema. Metab Brain Dis. 2020 Apr 7;35(4).

52. Jayakumar AR, Panickar KS, Murthy CRK, Norenberg MD. Oxidative stress and mitogen-activated protein kinase phosphorylation mediate ammonia-induced cell swelling and glutamate uptake inhibition in cultured astrocytes. J Neurosci. 2006;26(18).

53. Rama Rao K V., Chen M, Simard JM, Norenberg MD. Increased aquaporin-4 expression in ammonia-treated cultured astrocytes. Neuroreport. 2003 Dec;14(18).

54. Ismail FS, Faustmann TJ, Corvace F, Tsvetanova A, Moinfar Z, Faustmann PM. Ammonia induced microglia activation was associated with limited effects on connexin 43 and aquaporin 4 expression in an astrocyte-microglia co-culture model. BMC Neurosci. 2021 Dec 25;22(1).

55. Drews L, Zimmermann M, Westhoff P, Brilhaus D, Poss RE, Bergmann L, et al. Ammonia inhibits energy metabolism in astrocytes in a rapid and glutamate dehydrogenase 2-dependent manner. Dis Model Mech. 2020 Jan 1;

56. Dadsetan S, Kukolj E, Bak LK, Sørensen M, Ott P, Vilstrup H, et al. Brain alanine formation as an ammonia-scavenging pathway during hyperammonemia: Effects of glutamine synthetase inhibition in rats and astrocyte-neuron co-cultures. J Cereb Blood Flow Metab. 2013;33(8).

57. Butterworth RF. Pathophysiology of hepatic encephalopathy: a new look at ammonia . Metab Brain Dis. 2002;17(4).

58. Martín-Rufián M, Tosina M, Campos-Sandoval JA, Manzanares E, Lobo C, Segura JA, et al. Mammalian Glutaminase Gls2 Gene Encodes Two Functional Alternative Transcripts by a Surrogate Promoter Usage Mechanism. PLoS One. 2012 Jun 5;7(6).

59. Cardona C, Sánchez-Mejías E, Dávila JC, Martín-Rufián M, Campos-Sandoval JA, Vitorica J, et al. Expression of Gls and Gls2 glutaminase isoforms in astrocytes. Glia. 2015 Mar;63(3).

60. Zimmermann SC, Wolf EF, Luu A, Thomas AG, Stathis M, Poore B, et al. Allosteric Glutaminase Inhibitors Based on a 1,4-Di(5-amino-1,3,4-thiadiazol-2-yl)butane Scaffold. ACS Med Chem Lett. 2016 May 12;7(5).

61. Thomas AG, Rojas C, Tanega C, Shen M, Simeonov A, Boxer MB, et al. Kinetic characterization of ebselen, chelerythrine and apomorphine as glutaminase inhibitors. Biochem Biophys Res Commun. 2013 Aug 23;438(2):243–8.

62. Elhammali A, Ippolito JE, Collins L, Crowley J, Marasa J, Piwnica-Worms D. A high-throughput fluorimetric assay for 2-Hydroxyglutarate Identifies Zaprinast as a Glutaminase Inhibitor. Cancer Discov. 2014;4(7).

63. Díaz-Herrero MM, Del Campo JA, Carbonero-Aguilar P, Vega-Pérez JM, Iglesias-Guerra F, Periñá N I, et al. THDP17 decreases ammonia production through glutaminase inhibition. A new drug for hepatic encephalopathy therapy. PLoS One. 2014;9(10).

64. Ampuero J, Ranchal I, Nuñez D, Díaz-Herrero M del M, Maraver M, Campo JA del, et al. Metformin Inhibits Glutaminase Activity and Protects against Hepatic Encephalopathy. PLoS One. 2012;7(11).

65. Bhatia V, Singh R, Acharya SK. Predictive value of arterial ammonia for complications and outcome in acute liver failure. Gut. 2006;55(1).

66. Sies H. On the history of oxidative stress: Concept and some aspects of current development. Vol. 7, Current Opinion in Toxicology. 2018.

67. Hayes JD, Dinkova-Kostova AT, Tew KD. Oxidative Stress in Cancer. Vol. 38, Cancer Cell. 2020.

68. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Vol. 39, International Journal of Biochemistry and Cell Biology. 2007.

69. Singh S, Mondal P, Trigun SK. Acute liver failure in rats activates glutamine-glutamate

cycle but declines antioxidant enzymes to induce oxidative stress in cerebral cortex and cerebellum. PLoS One. 2014;9(4).

70. Mansour DF, Nada SA, Eldenshary EES, Elmahmoudy BM, AbdElgayed SS. Antioxidant and hypo-ammonemic activities of alpha-lactalbumin and vitamin c in thioacetamide-induced liver and brain damage in rats. J Appl Pharm Sci. 2015;5(9).

71. Görg B, Qvartskhava N, Bidmon HJ, Palomero-Gallagher N, Kircheis G, Zilles K, et al. Oxidative stress markers in the brain of patients with cirrhosis and hepatic encephalopathy. Hepatology. 2010;52(1).

72. Montes-Cortes DH, Olivares-Corichi IM, Rosas-Barrientos JV, Manuel-Apolinar L, Martìnez-Godinez MDLA, Hernández-López JC, et al. Characterization of Oxidative Stress and Ammonia According to the Different Grades of Hepatic Encephalopathy. Dig Dis. 2020;38(3).

73. Lachmann V, Görg B, Bidmon HJ, Keitel V, Häussinger D. Precipitants of hepatic encephalopathy induce rapid astrocyte swelling in an oxidative stress dependent manner. Arch Biochem Biophys. 2013;536(2).

74. Panickar KS, Jayakumar AR, Rao KVR, Norenberg MD. Ammonia-induced activation of p53 in cultured astrocytes: Role in cell swelling and glutamate uptake. Neurochem Int. 2009;55(1–3).

75. Görg B, Karababa A, Häussinger D. Hepatic Encephalopathy and Astrocyte Senescence. Vol. 8, Journal of Clinical and Experimental Hepatology. 2018.

76. Tranah TH, Vijay GKM, Ryan JM, Shawcross DL. Systemic inflammation and ammonia in hepatic encephalopathy. Metab Brain Dis. 2013 Mar 7;28(1).

77. Leithead JA, Ferguson JW, Bates CM, Davidson JS, Lee A, Bathgate AJ, et al. The systemic inflammatory response syndrome is predictive of renal dysfunction in patients with non-paracetamol-induced acute liver failure. Gut. 2009;58(3).

78. Jeong JH, Park IS, Kim DH, Kim SC, Kang C, Lee SH, et al. CLIF-SOFA score and SIRS are independent prognostic factors in patients with hepatic encephalopathy due to alcoholic liver cirrhosis. Med (United States). 2016;95(26).

79. Iebba V, Guerrieri F, Di Gregorio V, Levrero M, Gagliardi A, Santangelo F, et al. Combining amplicon sequencing and metabolomics in cirrhotic patients highlights distinctive microbiota features involved in bacterial translocation, systemic inflammation and hepatic encephalopathy. Sci Rep. 2018;8(1).

80. Bernardi M, Moreau R, Angeli P, Schnabl B, Arroyo V. Mechanisms of decompensation and organ failure in cirrhosis: From peripheral arterial vasodilation to systemic inflammation hypothesis. Vol. 63, Journal of Hepatology. 2015.

81. Fouts DE, Torralba M, Nelson KE, Brenner DA, Schnabl B. Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease. J Hepatol. 2012;56(6).

82. Alexopoulou A, Agiasotelli D, Vasilieva LE, Dourakis SP. Bacterial translocation markers in liver cirrhosis. Ann Gastroenterol. 2017;30(5).

83. Kasravi FB, Wang L, Wang XD, Molin G, Bengmark S, Jeppsson B. Bacterial translocation in acute liver injury induced by D-galactosamine. Hepatology. 1996;23(1).

84. Yang R, Zou X, Tenhunen J, Tønnessen TI. HMGB1 and Extracellular Histones Significantly Contribute to Systemic Inflammation and Multiple Organ Failure in Acute Liver Failure. Vol. 2017, Mediators of Inflammation. 2017.

85. Jenkitkasemwong S, Akinyode A, Paulus E, Weiskirchen R, Hojyo S, Fukada T, et al. SLC39A14 deficiency alters manganese homeostasis and excretion resulting in brain manganese accumulation and motor deficits in mice. Proc Natl Acad Sci. 2018 Feb 20;115(8).

86. Gow AG, Frowde PE, Elwood CM, Burton CA, Powell RM, Tappin SW, et al. Surgical attenuation of spontaneous congenital portosystemic shunts in dogs resolves hepatic encephalopathy but not hypermanganesemia. Metab Brain Dis. 2015 Oct 5;30(5).

87. Kobtan AA, El-Kalla FS, Soliman HH, Zakaria SS, Goda MA. Higher Grades and Repeated Recurrence of Hepatic Encephalopathy May Be Related to High Serum Manganese Levels. Biol Trace Elem Res. 2016;169(2).

88. Mendieta Zerón H, Rodríguez MR, Montes S, Castañeda CR. Blood manganese levels in patients with hepatic encephalopathy. J Trace Elem Med Biol. 2011 Dec 1;25(4):225–9.

89. Rose C, Butterworth RF, Zayed J, Normandin L, Todd K, Michalak A, et al. Manganese deposition in basal ganglia structures results from both portal-systemic shunting and liver dysfunction. Gastroenterology. 1999;117(3).

90. Rama Rao K V., Reddy PVB, Hazell AS, Norenberg MD. Manganese induces cell swelling in cultured astrocytes. Neurotoxicology. 2007;28(4).

91. Stuntz E, Gong Y, Sood D, Liaudanskaya V, Pouli D, Quinn KP, et al. Endogenous Two-Photon Excited Fluorescence Imaging Characterizes Neuron and Astrocyte Metabolic Responses to Manganese Toxicity. Sci Rep. 2017 Dec 21;7(1).

92. Barany Z, Toth I, Jocsak G, Frenyo L V., Bartha T, Sterczer A, et al. Differential production of interleukin-6 and tumor necrosis factor-α in primary rat astrocyte cultures using two distinct methods of microglia elimination. Clin Exp Neuroimmunol. 2021 May 22;

93. Rama Rao KV, Reddy PVB, Hazell AS, Norenberg MD. Manganese induces cell swelling in cultured astrocytes. Neurotoxicology. 2007 Jul;28(4).

94. Jha MK, Jo M, Kim J-H, Suk K. Microglia-Astrocyte Crosstalk: An Intimate Molecular Conversation. Neurosci. 2019 Jun 22;25(3).

95. Brown GC, Vilalta A. How microglia kill neurons. Brain Res. 2015;1628.

96. Bahniwal M, Little JP, Klegeris A. High Glucose Enhances Neurotoxicity and Inflammatory Cytokine Secretion by Stimulated Human Astrocytes. Curr Alzheimer Res. 2017;14(7).

97. Lau LT, Yu ACH. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. J Neurotrauma. 2001;18(3).

98. Brenneman DE, Phillips TM, Hauser J, Hill JM, Spong CY, Gozes I. Complex array of cytokines released by vasoactive intestinal peptide. Neuropeptides. 2003 Apr 1;37(2):111–9.

99. Gowrisankar Y V., Clark MA. Angiotensin II induces interleukin-6 expression in astrocytes: Role of reactive oxygen species and NF-κB. Mol Cell Endocrinol. 2016 Dec 5;437:130–41.

100. Soto-Díaz K, Juda MB, Blackmore S, Walsh C, Steelman AJ. TAK1 inhibition in mouse astrocyte cultures ameliorates cytokine-induced chemokine production and neutrophil migration. J Neurochem. 2020 Mar 15;152(6).

101. van Neerven S, Nemes A, Imholz P, Regen T, Denecke B, Johann S, et al. Inflammatory cytokine release of astrocytes in vitro is reduced by all-trans retinoic acid. J Neuroimmunol. 2010;229(1–2).

102. Forshammar J, Block L, Lundborg C, Biber B, Hansson E. Naloxone and ouabain in ultralow concentrations restore Na +/K +-ATPase and cytoskeleton in

lipopolysaccharide-treated astrocytes. J Biol Chem. 2011;286(36).

103. Souza DG, Bellaver B, Souza DO, Quincozes-Santos A. Characterization of Adult Rat Astrocyte Cultures. PLoS One. 2013;8(3).

104. Tantarungsee N, Yisarakun W, Thongtan T, Lalert L, Srikam S, Reuangwechvorachai P, et al. Upregulation of Pro-inflammatory Cytokine Expression Following Chronic Paracetamol Treatment in Astrocyte. Neurotox Res. 2018 Jul 14;34(1).

105. Liu L, Chan C. IPAF inflammasome is involved in interleukin-1β production from astrocytes, induced by palmitate; implications for Alzheimer's Disease. Neurobiol Aging. 2014;35(2).

106. Samy ZA, Al-Abdullah L, Turcani M, Craik J, Redzic Z. Rat astrocytes during anoxia: Secretome profile of cytokines and chemokines. Brain Behav. 2018;8(7).

107. Kirkley KS, Popichak KA, Afzali MF, Legare ME, Tjalkens RB. Microglia amplify inflammatory activation of astrocytes in manganese neurotoxicity. J Neuroinflammation. 2017;14(1).

108. Choi SS, Lee HJ, Lim I, Satoh JI, Kim SU. Human astrocytes: Secretome profiles of cytokines and chemokines. PLoS One. 2014;9(4).

109. Walz W, Lang MK. Immunocytochemical evidence for a distinct GFAP-negative subpopulation of astrocytes in the adult rat hippocampus. Neurosci Lett. 1998 Dec 4;257(3):127–30.

110. Tatsumi K, Isonishi A, Yamasaki M, Kawabe Y, Morita-Takemura S, Nakahara K, et al. Olig2-Lineage Astrocytes: A Distinct Subtype of Astrocytes That Differs from GFAP Astrocytes. Front Neuroanat. 2018 Feb 14;12.

111. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature. 2017;541(7638).

112. Burda JE, Sofroniew M V. Seducing astrocytes to the dark side. Cell Res. 2017 Jun 17;27(6).

113. Welser-Alves J V., Milner R. Microglia are the major source of TNF-α and TGF-β1 in postnatal glial cultures; Regulation by cytokines, lipopolysaccharide, and vitronectin. Neurochem Int. 2013 Jul 1;63(1):47–53.

114. Chistyakov D V., Azbukina N V., Astakhova AA, Goriainov S V., Chistyakov V V., Sergeeva MG. Sex-mediated differences in lps induced alterations of TNFα, IL-10

expression, and prostaglandin synthesis in primary astrocytes. Int J Mol Sci. 2018;19(9).

115. Jiang W, Desjardins P, Butterworth RF. Direct Evidence for Central Proinflammatory Mechanisms in Rats with Experimental Acute Liver Failure: Protective Effect of Hypothermia. J Cereb Blood Flow Metab. 2009 May 4;29(5).

116. Saleh DO, Mansour DF, Fayez AM. Thioacetamide-induced acute hepatic encephalopathy: central vs peripheral effect of Allicin. Metab Brain Dis. 2021 Mar 25;

117. Bémeur C, Qu H, Desjardins P, Butterworth RF. IL-1 or TNF receptor gene deletion delays onset of encephalopathy and attenuates brain edema in experimental acute liver failure. Neurochem Int. 2010;56(2).

118. Rodrigo R, Cauli O, Gomez-Pinedo U, Agusti A, Hernandez-Rabaza V, Garcia-Verdugo JM, et al. Hyperammonemia induces neuroinflammation that contributes to cognitive impairment in rats with hepatic encephalopathy. Gastroenterology. 2010 Aug 1;139(2):675–84.

119. Hernandez-Rabaza V, Cabrera-Pastor A, Taoro-Gonzalez L, Gonzalez-Usano A, Agusti A, Balzano T, et al. Neuroinflammation increases GABAergic tone and impairs cognitive and motor function in hyperammonemia by increasing GAT-3 membrane expression. Reversal by sulforaphane by promoting M2 polarization of microglia. J Neuroinflammation. 2016;13(1).

120. Hellewell SC, Yan EB, Alwis DS, Bye N, Morganti-Kossmann MC. Erythropoietin improves motor and cognitive deficit, axonal pathology, and neuroinflammation in a combined model of diffuse traumatic brain injury and hypoxia, in association with upregulation of the erythropoietin receptor. J Neuroinflammation. 2013 Dec 18;10(1).

121. Zhang J, Guo J, Zhao X, Chen Z, Wang G, Liu A, et al. Phosphodiesterase-5 inhibitor sildenafil prevents neuroinflammation, lowers beta-amyloid levels and improves cognitive performance in APP/PS1 transgenic mice. Behav Brain Res. 2013 Aug 1;250:230–7.

122. Hernandez-Rabaza V, Agusti A, Cabrera-Pastor A, Fustero S, Delgado O, Taoro-Gonzalez L, et al. Sildenafil reduces neuroinflammation and restores spatial learning in rats with hepatic encephalopathy: underlying mechanisms. J Neuroinflammation. 2015 Dec 29;12(1).

123. Schwenkgrub J, Zaremba M, Joniec-Maciejak I, Cudna A, Mirowska-Guzel D, Kurkowska-Jastrzębska I. The phosphodiesterase inhibitor, ibudilast, attenuates

neuroinflammation in the MPTP model of Parkinson's disease. PLoS One. 2017 Jul 28;12(7).

124. Wang Y, Li Y, Zhao R, Wu B, Lanoha B, Tong Z, et al. Glutaminase C overexpression in the brain induces learning deficits, synaptic dysfunctions, and neuroinflammation in mice. Brain Behav Immun. 2017 Nov 1;66:135–45.

125. Wang K, Ye L, Lu H, Chen H, Zhang Y, Huang Y, et al. TNF-α promotes extracellular vesicle release in mouse astrocytes through glutaminase. J Neuroinflammation. 2017 Dec 20;14(1).

126. Gao G, Li C, Zhu J, Wang Y, Huang Y, Zhao S, et al. Glutaminase 1 Regulates Neuroinflammation After Cerebral Ischemia Through Enhancing Microglial Activation and Pro-Inflammatory Exosome Release. Front Immunol. 2020 Feb 7;11.

127. Tkach M, Théry C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Vol. 164, Cell. Cell Press; 2016. p. 1226–32.

128. Holm MM, Kaiser J, Schwab ME. Extracellular Vesicles: Multimodal Envoys in Neural Maintenance and Repair. Vol. 41, Trends in Neurosciences. Elsevier Ltd; 2018. p. 360–72.

129. Park KW, Baik HH, Jin BK. IL-13-Induced Oxidative Stress via Microglial NADPH Oxidase Contributes to Death of Hippocampal Neurons In Vivo. J Immunol. 2009 Oct 1;183(7).

130. Liu J, Du L. PERK pathway is involved in oxygen-glucose-serum deprivation-induced NF-kB activation via ROS generation in spinal cord astrocytes. Biochem Biophys Res Commun. 2015 Nov 13;467(2):197–203.

131. Simmons LJ, Surles-Zeigler MC, Li Y, Ford GD, Newman GD, Ford BD. Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells in vitro involves the NF-kappa B pathway. J Neuroinflammation. 2016 Dec 6;13(1).

132. Santa-Cecília F V., Socias B, Ouidja MO, Sepulveda-Diaz JE, Acuña L, Silva RL, et al. Doxycycline Suppresses Microglial Activation by Inhibiting the p38 MAPK and NF-kB Signaling Pathways. Neurotox Res. 2016 May 8;29(4).

133. Jocsak G, Ioja E, Kiss DS, Toth I, Barany Z, Bartha T, et al. Endocrine Disruptors Induced Distinct Expression of Thyroid and Estrogen Receptors in Rat versus Mouse Primary Cerebellar Cell Cultures. Brain Sci. 2019 Dec 5;9(12).

134. Gupta S, Knight AG, Gupta S, Keller JN, Bruce-Keller AJ. Saturated long-chain fatty

acids activate inflammatory signaling in astrocytes. J Neurochem. 2012;120(6).

135. Hung C-Y, Hsu T-I, Chuang J-Y, Su T-P, Chang W-C, Hung J-J. Sp1 in Astrocyte Is Important for Neurite Outgrowth and Synaptogenesis. Mol Neurobiol. 2020 Jan 18;57(1).

136. Facci L, Barbierato M, Marinelli C, Argentini C, Skaper SD, Giusti P. Toll-Like Receptors 2, -3 and -4 Prime Microglia but not Astrocytes Across Central Nervous System Regions for ATP-Dependent Interleukin-1β Release. Sci Rep. 2015 May 29;4(1).

137. Pascual O, Ben Achour S, Rostaing P, Triller A, Bessis A. Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proc Natl Acad Sci. 2012 Jan 24;109(4).

138. Uliasz TF, Hamby ME, Jackman NA, Hewett JA, Hewett SJ. Generation of primary astrocyte cultures devoid of contaminating microglia. Methods Mol Biol. 2012;814.

139. Hewett JA, Hewett SJ. Induction of nitric oxide synthase-2 expression and measurement of nitric oxide production in enriched primary cortical astrocyte cultures. Methods Mol Biol. 2012;814.

140. Pont-Lezica L, Colasse S, Bessis A. Depletion of microglia from primary cellular cultures. Methods Mol Biol. 2013;1041.

141. Holm TH, Draeby D, Owens T. Microglia are required for astroglial toll-like receptor 4 response and for optimal TLR2 and TLR3 response. Glia. 2012 Apr;60(4).

142. Jebelli J, Piers T, Pocock J. Selective depletion of microglia from cerebellar granule cell cultures using L-leucine methyl ester. J Vis Exp. 2015;2015(101).

143. Xu X, Zhang A, Zhu Y, He W, Di W, Fang Y, et al. MFG-E8 reverses microglial-induced neurotoxic astrocyte (A1) via NF-κB and PI3K-Akt pathways. J Cell Physiol. 2019 Jan;234(1).

144. Qian D, Li L, Rong Y, Liu W, Wang Q, Zhou Z, et al. Blocking Notch signal pathway suppresses the activation of neurotoxic A1 astrocytes after spinal cord injury. Cell Cycle. 2019 Nov 2;18(21).

145. Zhu D. Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. J Cell Sci. 2005 Aug 15;118(16).

146. Du F, Qian ZM, Zhu L, Wu XM, Qian C, Chan R, et al. Purity, cell viability, expression of GFAP and bystin in astrocytes cultured by different procedures. J Cell Biochem.

2009;

147. Schildge S, Bohrer C, Beck K, Schachtrup C. Isolation and culture of mouse cortical astrocytes. J Vis Exp. 2013;(71).

148. da Silva AB, Cerqueira Coelho PL, das Neves Oliveira M, Oliveira JL, Oliveira Amparo JA, da Silva KC, et al. The flavonoid rutin and its aglycone quercetin modulate the microglia inflammatory profile improving antiglioma activity. Brain Behav Immun. 2020 Mar 1;85:170–85.

149. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. Nat Protoc. 2008;3(7).

150. Hamdi Y, Masmoudi-Kouki O, Kaddour H, Belhadj F, Gandolfo P, Vaudry D, et al. Protective effect of the octadecaneuropeptide on hydrogen peroxide-induced oxidative stress and cell death in cultured rat astrocytes. J Neurochem. 2011 Aug;118(3).

151. Zhang J, Johnston G, Stebler B, Keller ET. Hydrogen Peroxide Activates NF *κ* B and the Interleukin-6 Promoter Through NF *κ* B-Inducing Kinase. Antioxid Redox Signal. 2001 Jun;3(3).

152. Du L, Chang H, Xu W, Wei Y, Wang Y, Yin L, et al. Effect of NMO-IgG on the interleukin-6 cascade in astrocytes via activation of the JAK/STAT3 signaling pathway. Life Sci. 2020 Oct;258.

153. Prajeeth CK, Kronisch J, Khorooshi R, Knier B, Toft-Hansen H, Gudi V, et al. Effectors of Th1 and Th17 cells act on astrocytes and augment their neuroinflammatory properties. J Neuroinflammation. 2017;14(1).

154. De Almeida LMV, Piñeiro CC, Leite MC, Brolese G, Leal RB, Gottfried C, et al. Protective effects of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures. Neurochem Res. 2008;33(1).

155. Wakida NM, Cruz GMS, Ro CC, Moncada EG, Khatibzadeh N, Flanagan LA, et al. Phagocytic response of astrocytes to damaged neighboring cells. PLoS One. 2018 Apr 30;13(4).

156. Bondarenko A, Chesler M. Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts. Glia. 2001 Apr 15;34(2).

157. Rieger AM, Hall BE, Luong LT, Schang LM, Barreda DR. Conventional apoptosis assays using propidium iodide generate a significant number of false positives that prevent accurate assessment of cell death. J Immunol Methods. 2010 Jun 30;358(1–

2):81–92.

158. Pathipati P, Ferriero DM. The Differential Effects of Erythropoietin Exposure to Oxidative Stress on Microglia and Astrocytes in vitro. Dev Neurosci. 2017;39(1–4).

159. Kim J, Min K-J, Seol W, Jou I, Joe E. Astrocytes in injury states rapidly produce anti-inflammatory factors and attenuate microglial inflammatory responses. J Neurochem. 2010 Dec;115(5).

160. Fauconneau B, Petegnief V, Sanfeliu C, Piriou A, Planas AM. Induction of heat shock proteins (HSPs) by sodium arsenite in cultured astrocytes and reduction of hydrogen peroxide-induced cell death. J Neurochem. 2002 Dec 11;83(6).

161. Oh SJ, Kim H, Liu Y, Han HK, Kwon K, Chang KH, et al. Incompatibility of silver nanoparticles with lactate dehydrogenase leakage assay for cellular viability test is attributed to protein binding and reactive oxygen species generation. Toxicol Lett. 2014;225(3).

162. Kendig DM, Tarloff JB. Inactivation of lactate dehydrogenase by several chemicals: Implications for in vitro toxicology studies. Toxicol Vitr. 2007;21(1).

163. Eysseric H, Gonthier B, Soubeyran A, Richard M., Daveloose D, Barret L. Effects of chronic ethanol exposure on acetaldehyde and free radical production by astrocytes in culture. Alcohol. 2000 Jun;21(2).

164. Hohnholt MC, Blumrich EM, Dringen R. Multiassay analysis of the toxic potential of hydrogen peroxide on cultured neurons. J Neurosci Res. 2015 Jul;93(7).

165. Schmuck G, Röhrdanz E, Tran-Thi Q-H, Kahl R, Schlüter G. Oxidative stress in rat cortical neurons and astrocytes induced by paraquatin vitro. Neurotox Res. 2002 Jan;4(1).

166. Guerra M, Tortorelli LS, Galland F, Da Ré C, Negri E, Engelke DS, et al. Lipopolysaccharide modulates astrocytic S100B secretion: a study in cerebrospinal fluid and astrocyte cultures from rats. J Neuroinflammation. 2011;8(1).

167. Görg B, Qvartskhava N, Keitel V, Bidmon HJ, Selbach O, Schliess F, et al. Ammonia induces RNA oxidation in cultured astrocytes and brain in vivo. Hepatology. 2008;48(2).

168. Milatovic D, Yin Z, Gupta RC, Sidoryk M, Albrecht J, Aschner JL, et al. Manganese induces oxidative impairment in cultured rat astrocytes. Toxicol Sci. 2007;98(1).

169. Chen CJ, Liao SL. Oxidative stress involves in astrocytic alterations induced by

manganese. Exp Neurol. 2002;175(1).

170. Bi J, Jiang B, Liu JH, Lei C, Zhang XL, An LJ. Protective effects of catalpol against H2O2-induced oxidative stress in astrocytes primary cultures. Neurosci Lett. 2008;442(3).

171. Ni M, Li X, Yin Z, Sidoryk-Węgrzynowicz M, Jiang H, Farina M, et al. Comparative study on the response of rat primary astrocytes and microglia to methylmercury toxicity. Glia. 2011 May;59(5).

172. Röhl C, Armbrust E, Kolbe K, Lucius R, Maser E, Venz S, et al. Activated microglia modulate astroglial enzymes involved in oxidative and inflammatory stress and increase the resistance of astrocytes to oxidative stress in vitro. Glia. 2008;56(10).

173. Matejuk A, Ransohoff RM. Crosstalk Between Astrocytes and Microglia: An Overview. Front Immunol. 2020 Jul 16;11.

174. Moriyama M, Kurebayashi R, Kawabe K, Takano K, Nakamura Y. Acetate Attenuates Lipopolysaccharide-Induced Nitric Oxide Production Through an Anti-Oxidative Mechanism in Cultured Primary Rat Astrocytes. Neurochem Res. 2016 Nov 20;41(11).

175. Song JH, Marszalec W, Kai L, Yeh JZ, Narahashi T. Antidepressants inhibit proton currents and tumor necrosis factor-α production in BV2 microglial cells. Brain Res. 2012;1435.

176. Wang B, Chen T, Wang J, Jia Y, Ren H, Wu F, et al. Methamphetamine modulates the production of interleukin-6 and tumor necrosis factor-alpha via the cAMP/PKA/CREB signaling pathway in lipopolysaccharide-activated microglia. Int Immunopharmacol. 2018;56.

177. Wang MJ, Huang HY, Chen WF, Chang HF, Kuo JS. Glycogen synthase kinase-3β inactivation inhibits tumor necrosis factor-α production in microglia by modulating nuclear factor κB and MLK3/JNK signaling cascades. J Neuroinflammation. 2010;7.

178. Leist M, Jäättelä M. Triggering of apoptosis by cathepsins. Cell Death Differ. 2001 Apr 1;8(4).

179. Pišlar A, Božić B, Zidar N, Kos J. Inhibition of cathepsin X reduces the strength of microglial-mediated neuroinflammation. Neuropharmacology. 2017 Mar 1;114:88–100.

180. Kavčič N, Butinar M, Sobotič B, Hafner Česen M, Petelin A, Bojić L, et al. Intracellular cathepsin C levels determine sensitivity of cells to leucyl-leucine methyl ester-triggered apoptosis. FEBS J. 2020 Dec;287(23).

181. Souza DG, Bellaver B, Souza DO, Quincozes-Santos A. Characterization of Adult Rat Astrocyte Cultures. PLoS One. 2013 Mar 28;8(3).

182. Seyedzadeh MH, Safari Z, Zare A, Gholizadeh Navashenaq J, Razavi SA, Kardar GA, et al. Study of curcumin immunomodulatory effects on reactive astrocyte cell function. Int Immunopharmacol. 2014;22(1).

183. Keene SD, Greco TM, Parastatidis I, Lee S-H, Hughes EG, Balice-Gordon RJ, et al. Mass spectrometric and computational analysis of cytokine-induced alterations in the astrocyte secretome. Proteomics. 2009 Feb;9(3).

184. Zhang Y, He X, Wu X, Lei M, Wei Z, Zhang X, et al. Rapamycin upregulates glutamate transporter and IL-6 expression in astrocytes in a mouse model of Parkinson's disease. Cell Death Dis. 2017 Feb 9;8(2).

185. Ahlemeyer B, Kölker S, Zhu Y, Hoffmann GF, Krieglstein J. Cytosine arabinofuranoside-induced activation of astrocytes increases the susceptibility of neurons to glutamate due to the release of soluble factors. Neurochem Int. 2003 Jun 1;42(7):567–81.

186. Pontecorvi P, Banki MA, Zampieri C, Zalfa C, Azmoon P, Kounnas MZ, et al. Fibrinolysis protease receptors promote activation of astrocytes to express pro-inflammatory cytokines. J Neuroinflammation. 2019 Dec 6;16(1).

187. Barbierato M, Facci L, Argentini C, Marinelli C, Skaper S, Giusti P. Astrocyte-Microglia Cooperation in the Expression of a Pro-Inflammatory Phenotype. CNS Neurol Disord - Drug Targets. 2013 Jul 1;12(5).

188. He ML, Lv ZY, Shi X, Yang T, Zhang Y, Li T-Y, et al. Interleukin-10 release from astrocytes suppresses neuronal apoptosis via the TLR2/NFκB pathway in a neonatal rat model of hypoxic-ischemic brain damage. J Neurochem. 2017 Sep;142(6).

189. Hyzinski-García MC, Vincent MY, Haskew-Layton RE, Dohare P, Keller Jr. RW, Mongin AA. Hypo-osmotic swelling modifies glutamate-glutamine cycle in the cerebral cortex and in astrocyte cultures. J Neurochem. 2011 Jul;118(1).

190. Amin SA, Adhikari N, Gayen S, Jha T. First report on the structural exploration and prediction of new BPTES analogs as glutaminase inhibitors. J Mol Struct. 2017 Sep 5;1143:49–64.

191. Rose C, Kresse W, Kettenmann H. Acute Insult of Ammonia Leads to Calcium-dependent Glutamate Release from Cultured Astrocytes, an Effect of pH. J Biol Chem. 2005 Jun;280(22).

192. Cabrera-Pastor A, Arenas YM, Taoro-Gonzalez L, Montoliu C, Felipo V. Chronic hyperammonemia alters extracellular glutamate, glutamine and GABA and membrane expression of their transporters in rat cerebellum. Modulation by extracellular cGMP. Vol. 161, Neuropharmacology. Elsevier Ltd; 2019. p. 107496.

193. Huang S, Tong H, Lei M, Zhou M, Guo W, Li G, et al. Astrocytic glutamatergic transporters are involved in Aβ-induced synaptic dysfunction. Brain Res. 2018 Jan 1;1678:129–37.

194. Piedrafita B, Cauli O, Montoliu C, Felipo V. The function of the glutamate-nitric oxide-cGMP pathway in brain in vivo and learning ability decrease in parallel in mature compared with young rats. Learn Mem. 2007 Apr 1;14(4).

195. Verkhratsky A, Chvátal A. NMDA Receptors in Astrocytes. Neurochem Res. 2020 Jan 14;45(1).

196. Takaki J, Fujimori K, Miura M, Suzuki T, Sekino Y, Sato K. L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in neuroinflammation: the 'collusion' hypothesis for increased extracellular L-glutamate concentration in neuroinflammation. J Neuroinflammation. 2012 Dec 23;9(1).

197. Dąbrowska K, Albrecht J, Zielińska M. Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes. Neurochem Int. 2018 Sep 1;118:225–32.

198. Cooper AJL. The role of glutamine transaminase K (GTK) in sulfur and α-keto acid metabolism in the brain, and in the possible bioactivation of neurotoxicants. Vol. 44, Neurochemistry International. Elsevier Ltd; 2004. p. 557–77.

199. Pinto JT, Krasnikov BF, Alcutt S, Jones ME, Dorai T, Villar MT, et al. Kynurenine aminotransferase III and glutamine transaminase L are identical enzymes that have cysteine S-conjugate β-lyase activity and can transaminate L-selenomethionine. J Biol Chem. 2014 Nov 7;289(45):30950–61.

13. Publications in peer-reviewed journals related to the thesis

Jocsak G, Ioja E, Kiss DS, Toth I, Barany Z, Bartha T, et al. Endocrine Disruptors Induced Distinct Expression of Thyroid and Estrogen Receptors in Rat versus Mouse Primary Cerebellar Cell Cultures. Brain Sci. 2019 Dec 5;9(12).

Bárány, Z. B., Sterczer, Á., Jócsák, G., Frenyo, V. L., & Kiss, D. S. (2017). The aetiology and some new pathophysiological aspects of hepatic encephalopathy. Literature review. Magyar Állatorvosok Lapja, 139(3), 157-168.

Barany, Z., Toth, I., Jocsak, G., Frenyo, L. V., Bartha, T., Sterczer, A., & Kiss, D. S. (2021). Differential production of interleukin-6 and tumor necrosis factor-α in primary rat astrocyte cultures using two distinct methods of microglia elimination. Clinical and Experimental Neuroimmunology.

14. Publications in peer-reviewed journals not related to the thesis

Kiss, D. S., Ioja, E., Toth, I., Barany, Z., Jocsak, G., Bartha, T., ... & Zsarnovszky, A. (2018). Comparative analysis of zearalenone effects on thyroid receptor alpha (TRα) and beta (TRβ) expression in rat primary cerebellar cell cultures. International journal of molecular sciences, 19(5), 1440.

Kiss, D. S., Toth, I., Jocsak, G., Barany, Z., Bartha, T., Frenyo, L. V., ... & Zsarnovszky, A. (2020). Functional Aspects of Hypothalamic Asymmetry. Brain Sciences, 10(6), 389.

15. Scientific presentations, posters

Bárány ZB., Kiss DS, Tóth I., Jócsák G., Bartha T., Frenyó VL., Sterczer Á.: Primer patkány asztroglia sejtek citokin termelése oxidatív stressz hatására, P1.1.3, In: Joint Conference of the Hungarian Physiological Society and the Hungarian Pharmacology, Microcirculation and Physiological Societies/ A Magyar Élettani Társaság, a Magyar Kísérletes és klinikai Farmakológiai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság közös Vándorgyűlése, Debrecen, Hungary, 2017.

Bárány Z., Kiss DS., Tóth I., Jócsák G., Bartha T., Frenyó VL., Sterczer Á.: Cytokine production induced by oxidative stress in primary rat astrocytes, P1-338, In: Meeting of the Hungarian Neuroscience Society & Federation of European Neuroscience Societies Regional Meeting, Pécs, Hungary, 2017.

Bárány Z, Kiss DS, Sterczer A: Az asztrogliák neuroinflammációs szerepének vizsgálata hepaticus encephalopathiaban. Akadémiai Beszámoló. 2018. január 22-25.

Bárány Z, Kiss DS, Tóth I, Jócsák G, Frenyó VL, Bartha T, Zsarnovszky A, Sterczer A: Az oxidatív stressz és a tumor nekrózis faktor alfa (TNF-α)-termelés vizsgálata hepaticus encephalopathiaban. Akadémiai Beszámoló. 2019. január 21-24.

Barany Z., Kiss DS., Toth I., Jocsak G., Bartha T., Frenyo VL., Zsarnovszky A., Sterczer A.: Examination of the oxidative stress and the tumor necrosis factor (tnf)-α-production in hepatic encephalopathy, T14-045A In: XIV European Meeting on Glial Cells in Health and Disease, *Lisboa,* Portugal, 2019.

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