Development of neuron-astrocyte co-culture system for mechanistic and pharmacological studies in neurodegeneration

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Liudmila Efremova

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Universität Konstanz

Mathematisch-Naturwissenschaftliche Sektion
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Gutachter/in:
1. Prof. Dr. Marcel Leist
2. Prof. Dr. Christof Hauck

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Summary

One of the main obstacles for development of the neuroprotective therapies for Parkinson’s disease and other neurodegenerative diseases is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. There is an urgent need to get a better insight into molecular mechanisms of the diseases. One major hindrance for development of novel neuroprotective strategies and discovery of early disease biomarkers is the limited availability of appropriate in vitro models. Moreover, most of the available ones are lacking active neuron-glia interactions. However, it is known that astrocytes play a crucial role in a healthy brain as well as in pathology, and therefore their absence has an impact on relevance of these models.

In a first step we introduce the new immortalized mouse astrocytes cell line IMA 2.1 as an alternative to currently used primary astrocytes cultures. IMA 2.1 were directly compared with primary mouse astrocytes with respect to their response to proinflammatory stimuli, expression of typical astrocytes markers, and to the cell line’s capacity to metabolize the parkinsonian toxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP) to its toxic metabolite MPP⁺.

MPTP causes Parkinsonism in humans which is almost indistinguishable from PD of conventional ethiology. Therefore in a second step we generated a human in vitro counterpart of the widely used MPTP mouse model. We took advantage of human neuronal cell line (LUHMES), the conditionally-immortalized mesencephalic neuronal precursors that can be differentiated to fully postmitotic DA neurons, combined with IMA 2.1. This new co-culture system allowed us to model MPTP metabolism in vitro and to reveal differences in the pharmacological activities of mechanistically diverse neuroprotective experimental compounds in comparison with neuronal monocultures exposed to MPP⁺. Our data demonstrate that neurons grown in co-culture with glia show a radically different pharmacological behaviour than neuronal monocultures.

Decades of studies on pathology of neurodegenerative disease have been focused preferentially on neuronal abnormalities, but it is becoming increasingly obvious that other neural cells may also be the important players. Indeed, glial cells undergoing dramatic changes in pathology seem to be one of the additional culprits of the diseases. In a final step, we studied the effects of astrocytes activated by inflammatory stimuli on human neurons. This IMA-LUHMES neuroinflammation model was suitable for separate profiling of both glial-
directed and directly neuroprotective strategies. Moreover, direct evaluation in co-cultures of the same cells allowed testing of therapeutic effectiveness in more complex settings, in which astrocytes affect pharmacological properties of neurons.

We showed that the presence of glial cells *in vitro* can drastically affect the pharmacology of neuroprotection. The novel model systems are closer representing the situation in human brain tissue than conventional monocultures, and its use for screening of candidate neuroprotectants may increase the predictiveness of a test battery.
Zusammenfassung


Wir haben gezeigt, dass die Anwesenheit von Gliazellen die pharmakologische Wirkung neuroprotektiver Therapien in vitro stark beeinflussen kann. Diese neuen Modellsysteme sind näher an der realen Situation humanen Hirngewebes als herkömmliche Kultursysteme und ihre Anwendung, um potentielle neuroprotektive Wirkstoffe zu untersuchen, könnte die Voraussagekraft von Testbatterien erhöhen.
Chapter A.

Introduction

1. Clinical picture and diagnosis of PD

The main PD features were originally described by English physician James Parkinson in monograph “An Essay on the Shaking Palsy” in 1817 (Parkinson, 1817). One hundred years later it was discovered that pathological feature of PD is death of neurons in SNpc. Since then lot of efforts were made to investigate deeper pathological mechanisms and develop a curative treatment for PD. Approximately 1-2% of the population over 65 years is affected by this disorder, and the number of cases could be increased twice by 2030 due to lifespan increase (Schule et al., 2009). Although clinically heterogeneous, PD is characterized by the six cardinal motor symptoms:

- tremor-at-rest, a rhythmic muscle contraction or shaking hands which decreases with voluntary movement;
- bradykinesia, a slowness of movement;
- rigidity, an increase in muscle tone of limbs;
- flexed posture of neck, trunk and limbs;
- loss of postural reflexes;
- freezing of gait.

Besides the motor disabilities PD patients suffer from non-motor symptoms, mainly at the late stages of the disease. Slowness of mental function, decreased motivation and apathy, dementia, fatigue, depression, anxiety and sleep disorders, and also sensory complaints that also decrease the quality of life. Patients diagnosed with PD can live more than 20 years. Death is usually caused due to the concurrent unrelated illness or due to decreased mobility.

The average disease onset age is 55, and number of disease cases increases considerably over time (Dauer et al., 2003). PD affects from 1% in those over 60 years of age to 4% of the population over 80. Some studies have proposed that it is more common in men than women, but others failed to detect any differences between the two sexes (de Lau et al., 2006).

First parkinsonian motor symptoms start to appear by the time when approximately 60% of DA neurons of the SNpc degenerated corresponding to 80% decrease of dopamine level in the striatum. Therefore, the diagnosis is made quite late to rescue lost neurons. In spite of different organizations tried to standardize the diagnostic process in the early stages of the
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disease, one unified single test is still not widely used. Usually for diagnostics the physicians perform a neurological test for motor symptoms. Not all of six major motor disabilities should present in patients, but at least two of them are sufficient for diagnosis of the disease (Jankovic, 2008). Sometimes following relief after Levodopa consumption or identification of Lewy bodies (LB) inclusions in postmortem midbrain autopsy can prove that the correct diagnosis of PD was made. Five stages of disease were first described by Hoehn and Yahr (Hoehn et al., 1967). Later in 1987 the Unified Parkinsons’s disease Rating Scale (UPDRS) was developed as a gold standard for the disease progression measurement (Meara et al., 1999; Movement Disorder Society Task Force on Rating Scales for Parkinson’s, 2003). In spite of existing diagnostic approaches incorrect diagnosis can still reach in up to 25% (Meara et al., 1999). Therefore, development of early and correct biomarkers of the disease is also a pressing issue.

Although a number of different biomarkers have already been identified for healthy state as well as for early identification of pathological process and therapeutic response, they still need to be validated and remain expensive for the routine analysis (Morgan et al., 2010).

2. Etiology of PD

The factors triggering PD onset long time were considered to be unknown. Upgraded knowledge in the last decades improved insight on disease causes. Around 95% of the disease cases are sporadic, the rest are caused by genetic component. Although aging is considered to be a main risk factor, in certain cases the environmental toxins (Paraquat, Rotenone) appear to be the cause (Betarbet et al., 2000; Brooks et al., 1999). Serendipitous finding of neurotoxic effect of MPTP provided useful model of the PD (Burns, Chiueh et al. 1983, Langston, Ballard et al. 1983). Identification of correlation of mutations in α-synuclein (α-syn) gene with onset of the disease changed the point of view that PD has no genetic background (Polymeropoulos et al., 1997).

3. Therapy in PD

Much has been learned about new target sites of PD that led to the development of different treatment strategies. Since the disease is multifactorial, the treatment also must be complex due to existence of many target sites for neuroprotection. In addition, different stages of the disease also require specific approaches. One can categorize current methods of the PD treatment into three major components: medications, surgery and physical therapy. They will be described below.
3.1. Medications

The classic PD therapy is based on dopamine replacement. L-dopa (L-3,4-Dihydroxyphenylalanine), a dopamine precursor, is able to cross the BBB and being converted to dopamine increase the it concentration in the brain. L-dopa is used in the combination with COMT (catechol-O-methyl transferase) inhibitors, Entacapone and Tolcapone, which prolong L-dopa half-life and allow the larger amount of L-dopa to reach the brain (Olanow et al., 2004). It is still considered to be one of the most effective and powerful symptomatic medication. In spite of great improvement after around 3 years up to 80% of patients develop “wearing-off” effects such as dyskinesia, diminished voluntary movements (Bezard et al., 2001; Swope, 2004). Certain metabolites of the drug could be toxic for DA neurons and therefore eventually lead to disease aggravation (LeWitt et al., 2004). This therapy also leads to acute side effects due to hyper activation of DA system in other regions of CNS and PNS resulting in vomiting, hypotension and nausea. Moreover, L-dopa is not able to remove the non-motor symptoms such as dementia, constipation and excessive sweating. MAO-B inhibitors (Rasagiline and Selegiline) are able to prolong the half-life of dopamine and postpone the L-dopa use in the early stages. They also possess neuroprotective properties rendering antiapoptotic and antioxidative effects and regulate a number of DA neuron-related genes (Jenner, 2004). Dopamine agonists (e.g. Rotigotine and Cabergoline) also used as monotherapy to delay the use of L-dopa or in combination with it to reduce the risk of dyskinesia acquirement (Im et al., 2003). Some neurotrophic factors (glial-derived neurotrophic factor (GDNF), brain-derived neurotropic factor (BDNF), and insulin-like growth factor (IGF)) showed neuroprotective potential and also could be used in the disease treatment in future when the suitable delivery method will be developed (Erickson et al., 2001; Guan et al., 2000; Roussa et al., 2004). Targeting of genes essential for survival and development of DA neurons (Nurr1, HSP70, Parkin) could be a useful therapeutic tool in future (Chen et al., 2006). Antioxidant compound CoQ10 showed promising effects in clinical trials (Shults et al., 2002).

3.2. Surgery

Although the deep brain stimulation is also used for the patient in the late stages of disease or in case if the medications were ineffective. The method is costly, risky and may trigger neurological deficit (Bronstein et al., 2011), however can lead to a significant improvement in side effects such as dyskinesias (involuntary movements caused by long-term use of levodopa). Researchers are continuing to study DBS and to develop ways of improving it.
Application of different stem cell types for replacement therapy showed beneficial effects in clinical trials. The risk of tumor formation or dyskinesia development requires further investigation in this field (Politis et al., 2012).

Currently, drugs which are able to stop or slow down the disease progression do not exist. Therapy remains to be not only symptomatic, but also does little to treat non-motor symptoms. Partially this might be explained by delayed diagnosis of the disease which leads to the treatments at the irreversible stage. Current trend in PD treatment is to change the strategy of symptomatic treatment to disease-modifying therapies. Deeper understanding of molecular pathogenesis of the disease will allow us in future define new targets for neuroprotection as well as to develop new tools for early disease diagnosis.

4. Potential pathogenic mechanisms

Although the great efforts were made to identify main pathological events which contribute to cell death of DA neurons in SNpc, knowledge on extent of their contribution for disease progression as well as sequence in which they occur is still incomplete. Here I provide the list and a brief summary of several most frequent and well-studied factors contributing to PD. A short resume of known pathological events and cellular and molecular players is depicted in Figure 1.

![Figure 1. The complexity of pathological events involved in PD.](image)

Oxidative stress occurs in PD and plays a remarkable role in degeneration of DA neurons. Oxidative modifications of DNA and RNA (e.g. formation of 8-hydroxyguanine), lipids and proteins were identified in postmortem material of PD patients (Alam et al., 1997; Dexter et
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al., 1989; Saggu et al., 1989). The major triggers of such devastating changes are reactive oxygen species (ROS) and reactive nitrogen species (RNS). However, the exact source of oxidative damage in PD is unknown and is still under the debate. Mitochondrial Complex I deficiency in SNpc of PD patients was reported (Schapira et al., 1989). This complex is a part of cell respiration system, which function is conversion of molecular oxygen by mitochondrial respiratory chain. Herewith $O_2^{-}$ is byproduct of this process, which yield in normal state is minimal. Decreased activity of Complex I can lead to dramatic increase of $O_2^{-}$ production which can be further converted to peroxynitrite causing damage of lipids, proteins and nucleic acids (Siddique et al., 2010). The possible endogenous inhibitors of Complex I is hypothesized (Dauer et al., 2003). In spite of a general agreement that PD is associated with a modest loss of Complex I activity, there are studies showing that it could also be a systemic effect (Haas et al., 1995), in contrast with fact that a specific group of neurons degenerating on PD. Moreover, DA neurons permanently undergo OS due specific dopamine metabolism, since dopamine could be auto-oxidized to dopamine-quinone species, superoxide radicals and hydrogen peroxide (Graham, 1978). Decreasing activity of detoxification system in the neuron resulting in insufficient scavenging due to, e.g. aging process can also contribute to OS level (Mecocci et al., 1993; Sohal et al., 1996). It is still not clear yet whether the OS triggers the disease onset or is the consequence of other initiating events in PD. Since clinical trials based on antioxidants failed, it indicates that OS is not the single process contributing to disease progression (Halliday et al., 2011).

4.2. Mutations

As it was already briefly mentioned before a number of mutations are known to cause PD (Hardy et al., 2009; Klein et al., 2012). Here I provide more detailed information about the first discovered and well-known ones. The first found familial forms of PD are linked to mutations in three genes, e.g. PARK1, PARK2 and PARK5 encoding α-synuclein, parkin and UCHL1 (Ubiquitin carboxy-terminal hydrolase L1) respectively (Lansbury et al., 2002). Mutation in PARK1 gene results in modification in α-synuclein protein, which acquires the tendency to form toxic species, protofibrils (Conway et al., 2000a; Conway et al., 2000b; Spira et al., 2001). Mutation in PARK1 was not found in sporadic PD cases (Lynch et al., 1997). Mutation in PARK2 gene coding parkin protein is also associated with familial form of PD. The gene product is an E3 ubiquitin ligase which is involved in protein degradation (Kitada et al., 1998). This familial form is not associated with LB formation (Mizuno et al., 2001). It is not well known yet how loss of parkin functions leads to degeneration, but
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probably the accumulation of misfolded parkin is responsible (Siddique et al., 2010). Two patients with mutation in PARK5 encoding UCHL1, enzyme involved in Ubiquitin proteasome system (UPS) were so far identified (Leroy et al., 1998). The last two mutations indicate that UPS is also involved in PD pathogenesis. Mutations in gene encoding for leucine-rich repeat serine/threonine kinase (LRRK2) the most widespread cause for autosomal-dominant PD. Six point mutations in this gene leading to the amino acid substitutions (R1441G, R1441C, N1437H, Y1699C, G2019S, I2020T) are known to trigger the disease onset. Mutations in the gene for PTEN-induced putative kinase1 (PINK1) cause an autosomal-recessive form of PD and result in the loss of protein functions. Two mutations, L166P and D149A, in the gene for DJ-1 mediate early onset of PD (Blandini et al., 2012).

4.3. Inflammation: Role of glial cells in pathogenesis of PD

Neuroinflammation was shown to contribute to neuron demise in several neurodegenerative diseases, such as AD, HD and ALS (McGeer et al., 2004). The body of evidences exist that neuroinflammatory processes also play a role in PD. The special role in the neuroinflammation processes are played by glial cells: therefore I will begin with the overview of their functions. A hypothetical intermodulation between cell types in neuroinflammation is summarized in Figure 2.

![Figure 2. Interconnection of cell types in pathological state.](image)

The state of all three cell type is influenced in pathology. Neurons secrete disease proteins influencing microglia and astrocytes. Microglia become activated and secrete neurotoxic ROS, RNS as well as inflammatory cytokines, which trigger astrocytes reaction. Activated astrocytes reduce their neuronal support; secrete cytokines resulting in further microglia activation and secrete soluble factors which are toxic for neurons.
4.3.1. Role of glial cells in pathogenesis of PD

Although many factors initiating and propagating degeneration of DA neurons are already defined in PD, failure of current treatment to halt the disease progression indicates that additional processes participate in neurodegeneration. Therefore, revealing putative pathogenic events is crucial for developing of effective therapeutic strategies. In accordance to this goal, current state of PD research was reviewed to identify additional putative culprits.

One of the potential important processes identified recently which was proposed to contribute to PD pathogenesis is so-called gliosis. Gliosis is a nonspecific reactive change of glial cells in response to damage to CNS. In most cases, gliosis involves the proliferation or hypertrophy of several different types of glial cells, including astrocytes, microglia, and oligodendrocytes (Buffo et al., 2010). As mentioned before multiple functions are performed by glial cells in the undamaged healthy brain, although their activities are not confined to “housekeeping” ones. However, the role of these cells in neurodegeneration was long time neglected. Indeed, dramatic changes occurring in glia phenotype during the pathological process result in their contribution to neuronal demise. Activated microglia was identified by using following markers human leucocyte antigen DR (HLA-DR), cell surface MHC class II receptor and ferritin in all autopsies of PD patients in the SNpc (Banati et al., 1998; Langston et al., 1999; McGeer et al., 1988) as well as in animal models (Czlonkowska et al., 1996; Liberatore et al., 1999). Activated astrocytes were identified in many but not in all cases of PD relying on increasing expression of GFAP protein (Forno et al., 1992; Langston et al., 1999; Mirza et al., 2000). The role of oligodendrocytes in PD initiation was not yet reported (Wilkin et al., 1999).

Moreover, SNpc is relatively rich in microglia in comparison with other brain regions, meaning that nigrostriatal neurons are more susceptible to deleterious effects of activated microglia (Kim et al., 2000). This is consistent with idea that gliosis indeed plays relevant role in PD. Moreover, the severe damage of neurons was observed in striatum, where the astrocytic density is low. In turns it makes neurons more vulnerable since they lack essential support (Damier et al., 1993).

Animal model of PD showed that MPTP injection in mice, killed at different time points, led to simultaneous appearance of reactive astrocytes and features of DA neurons degeneration. However, activation of microglia occurs much early than that of astrocytes and reaches it’s maximum before the neuronal distress (Liberatore et al., 1999). Although, the role of astrocytes in PD is poorly understood, it is likely that they do not initiate a cell death.
A body of evidence indicate that gliosis comprises a number of different effects which depending on a situation may mediate beneficial or harmful events.

4.3.2. Deleterious effects of glial cells in PD

Studies of human brain post-mortem material of PD patients indicated that neuroinflammatory process indeed affects the brain. However, it is still not clear whether activation of glial cells is able to initiate a pathological process or is subsequent event. There are data indicating that microglia activation is sufficient to initiation of DA neurons degeneration. LPS injection in SNpc of rats resulted in microglia activation which was followed by DA degeneration, in turns pharmacological inhibition of activation in turn prevented neuronal demise (Liu et al., 2000). The source of microglia stimulation could be neuronal death or toxins. Activated microglia is characterized by macrophages markers; it can produce a number of detrimental compounds as ROS, RNS, pro-inflammatory prostaglandins and cytokines. Increased expression of inducible NO-synthase (iNOS) in SNpc was found in activated microglia but not on DA neurons of PD patients as well as PD mice models (Dehmer et al., 2000; Hunot et al., 1996). Inducible NOS up-regulation leads to prolonged production of NO which is deleterious for neurons (Xia et al., 1997). ROS have been involved in PD pathogenesis (Przedborski et al., 2003). One of the major sources of ROS is NADPH-oxidase, presented on microglia, which main subunit was upregulated in the SNpc of PD patients and MPTP-treated mice (Wu et al., 2003). Cyclooxygenase 2 (COX2) is an important enzyme in inflammation process. Prostaglandin E2 (PGE2) synthesis by COX2 is accomplished with ROS production as a by-product. Increased expression of COX2 along with elevation of PGE2, which is considered to be cytotoxic, was shown to be present in glial cells of SNpc in PD patients as well as MPTP mice model (Mattammal et al., 1995; Teismann et al., 2001). However, some groups did not observe COX2 upregulation on glial cells, but on neurons in post-mortem PD samples (Teismann et al., 2003). Activated microglia is also able to produce pro-inflammatory tumor necrosis factor α (TNF-α) as well as interleukin-1β (IL-1β) which level was also increased in SNpc of PD patients (Mogi et al., 1996; Mogi et al., 1994). These cytokines may stimulate astrocytes to produce even more of them which lead to amplifications of the inflammatory response (Giulian et al., 1988). Moreover, TNF-α may bind to TNF-α-receptor 1 which expression is increased on DA neurons in PD. This binding leads to activation of cell death signaling pathways including a translocation of transcriptional factor NF-κB from cytoplasm to the nucleus and activation of genes inducing apoptosis (Boka et al., 1994; Mogi et al., 2000). Indeed, nuclear localization of NF-κB was increased in PD
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19 patients (Hunot et al., 1997). The pathological role of astrocytes is still not well defined. Other deleterious effects of reactive astrocytes are likely to be confined to their debilitating of their neurotrophic functions. Although, it was mentioned that microglial activation is a rather subsequent event in PD pathogenesis which is triggered by neuronal damage. However, recent studies indicate that inflammation could be a risk factor to PD development (Hirsch et al., 2009).

5. Protective effects of glial cells in PD

Some studies report beneficial effect of glial cells in PD. IL-1β injection in SNpc of 6-hydroxydopamine (6-OHDA) treated mice led to astrocytes activation and DA neurons protection (Saura et al., 2003). Astrocytes also render a trophic factor support to mesencephalic neurons, e.g. by secretion of mesencephalic astrocytes-derived neurotrophic factor (MANF) which was shown to improving their survival in vitro (Petrova et al., 2003; Sortwell et al., 2000).

Putative protective compound is also GDNF which can be released by activated microglia as well as by astrocytes and improve viability of neurons (Batchelor et al., 1999; Burke et al., 1998; Lin et al., 1993). Its neuroprotective properties were shown in vitro as well as in vivo to rescue DA neurons (Kordower et al., 2000). BDNF also secreted by activated microglia and astrocytes was shown to render neuroprotective effect on DA neurons in PD models (Knott et al., 2002; Kordower et al., 1999; Spina et al., 1992). Another protective mechanism could be based on scavenging and detoxification of ROS by glutathione (Przedborski et al., 2000). Cytotoxic dopamine released from degenerating neurons could be metabolized by astrocytes expressing monoamine oxidase B (MAO-B) enzyme (Hirsch et al., 1999).

According to aforementioned data it is clearly demonstrates that neuroinflammation plays a significant role in PD. Moreover, it is seems that microglia render rather deleterious effect then neuroprotective in contrast to astrocytes. It also unlikely that glial cells initiate death of DA neurons but rather propagate degenerative process. Many gaps in understanding of precise pathological pathways and their key players participating in PD neuroinflammation should be fulfilled.

6. Properties of glial cells in normal state

The term “glia”, originated from Greek word what means “glue”, was first introduced by Rudolf Virchow in 1846 to describe a connective tissue holding neurons together in the brain. Until recently, it was assumed that these non-neuronal cells perform only passive structural
functions in the brain. In recent years it became obvious that glial cells serve multitude of different tasks for neuronal development, functioning and survival in the healthy state. In the CNS glial cells consist of macroglia, including oligodendrocytes and astrocytes, and microglia.

Recent studies showed that astrocytes not only hold neurons together but also provide a critical support for neuronal function (Barres, 2003; Seth et al., 2008). They are important for homeostasis of extracellular environment by scavenging neuronal waste products or neurotransmitters. For instance, participation on glutamine-glutamate cycle is one of the key functions of astrocytes in synaptic modulation. They uptake residual glutamate, a neurotransmitter released from axonal terminal, from the synaptic cleft and convert it to glutamine which is again recycled available for neuronal signaling (Allen et al., 2009).

They also provide neurons with necessary metabolic substrates. Neurons need lactic acid subsequently will be converted to pyruvate needed for energy metabolism. Astrocytes supply neurons with lactic acid converted from glucose (Danbolt, 2001). Moreover, astrocytes are also crucial for control of ions concentration in the brain. Accumulation of potassium ions is buffered by potassium channels located on astrocytes (Kofuji et al., 2004). The link between neuronal activity and cerebral blood flow is also mediated by astrocytes (Takano et al., 2006). Astrocytes supply neurons with trophic factors such as GDNF, NGF and BDNF which are crucial for survival, maturation and differentiation of neurons during the development. Emerging evidence suggests that astrocytes are involved in neurogenesis (Heins et al., 2002; Pillai et al., 2006).

Microglia serves as an innate immune system for CNS and represented by represents resident macrophages in the brain. It is main functions are scavenging and phagocytosis. It is able to find and recognize damaged and apoptotic cells and clean cellular debris. These processes occur mostly during the development when removing of neuronal excess is necessary. Microglial cells can also engulf bacteria and viruses in inflamed state and act as the antigen-representing cells recruiting T-cells. Microglia sensitivity is significantly higher than that of the rest of the immune system; they could react to minimal pathological signs in the brain (McGeer et al., 2008). If infectious agent crosses the BBB, microglia reacts quickly to neutralize it before its wide spreading (Gehrmann et al., 1995).

Oligodendrocytes participate mainly in myelination process and supply neurons with neurotrophic factors. They insulate axons by wrapping them with produced myelin sheaths
consisting of lipids and proteins. This in turns increase the impulse speed of neuronal impulse transmission, protect from ion leakage and maintain neuronal membrane capacitance (Baumann et al., 2001).

7. Models of PD

Although current therapeutic strategies greatly improved the quality of life and survival of PD patients, nowadays there is no treatment available to stop the progression or modify the disease.

Modeling of PD is essential for understanding precise mechanisms as well as for testing putative drug candidates. There are several key players and pathological events in PD already known, which is challenging to recapitulate in one single system. Models neither animal nor cellular reflecting all prominent feature of PD do not exist yet. Therefore, a variety of models having their benefits and limitations currently are used. Studying on each level of disease pathogenesis by using a number of different models at the same time can help to bring all parts of this puzzle together. There are various models currently available for PD research. At present this field is of great interest and is developing impetuously.

7.1. Toxin-based models

Pathogenesis and pathophysiology of PD has been widely studied by using a different number of animal models. Classically these models were based on neurotoxins administrations, but later after revealing of genetic background, transgenic animals had appeared to reflect familial PD forms. Toxicant-based models are the oldest ones in PD research and recapitulate both pathological and behavioral alterations in rodents and primates. MPTP model is a classic and systemic one which exerts a selective toxicity on DA neurons. MPTP was first discovered in 1983 when it caused a parkinsonism in young drug consumers after intravenous injection. MPTP is able to cross the BBB and within the brain, it is metabolized to toxic 1-Methyl-4-phenyl-pyridin (MPP⁺) by MAO-B localized in astrocytes (Figure 3). This process is followed by its uptake by dopaminergic neurons via the dopamine transporter (DAT) where it blocks Complex I of mitochondrial respiratory chain (Langston et al., 1983). Subject’s animals, mice or primates, from this experimental model represent all main symptoms of PD and respond to L-dopa treatment. MPTP animal model greatly contributed to development of novel therapeutic strategies. The main model imperfection is considered to be lacking of LBs formation followed by MPTP injection. In spite of wide
usage model is considered to be not robust and reproducible as it discussed in the literature. Additionally, inability of MPTP to trigger degeneration in rat is still not clear.

Figure 3. Schematic representation of MPTP metabolism.

After systemic administration, MPTP crosses the blood brain barrier and is converted to MPDP⁺ by MAO-B in glial cells. The subsequent transformation into MPP⁺ and the release into the extracellular space are not yet fully understood. MPP⁺ is then actively taken up and concentrated into DA neurons via DAT.

Revealing of putative role of other environmental toxins in disease pathogenesis led to their wide application in PD modeling. Sequential epidemiological studies showed that chronic exposure to pesticides and rural life style increase the risk of PD development (Blandini et al., 2012). Thereby intravenous administration of Rotenone, known pesticide and insecticide, was first described as disease model in rats (Betarbet et al., 2000). Both behavioral phenotype and selective degeneration of dopaminergic neurons was observed and more important for the first time in PD modeling the LB inclusions were present. Rotenone also crosses the BBB, but its penetration into DA neurons in contrast to MPP⁺ does not require DAT. Inside the cell rotenone inhibits both Complex I and proteasome activity resulting in ROS formation and proteolytic stress correspondently. Several shortcomings such as irreproducibility, high unspecific toxicity and unspecificity restrict the model application.

Paraquat, non-selective herbicide, represents another systemic model of PD. Since paraquat is not able to cross the BBB it requires neural amino acid transporter (e.g. system L carrier LAT-1). Paraquat does not rely on DAT to get into neurons but use a sodium-
dependent transport mechanism. Within the neuron it raises an OS and also initiates intrinsic mitochondrial apoptotic pathway. It is selective for DA neurons and leads to decreased motor activity and aggregation of α-synuclein. Both toxins rotenone and paraquat though induced models are quite attractive, but their general toxicity restricts the application.

Local injection of 6-OHDA, which is not able to cross BBB, is another PD animal model. Injection leads to dramatic decrease of DA neurons in the striatum followed by the cell loss in SNpc. Neurotoxic 6-OHDA is hydroxylated analogue of dopamine. Due to the DAT affinity it is easily taken up by DA neurons. Inside the cell 6-OHDA undergoes auto-oxidation exerting later to hydrogen peroxide formation. Moreover, it may inhibit Complex I of mitochondrial respiratory chain (Blandini et al., 2012). It is widely used for antiparkinsonian drugs screening. This model allows mimicking “wearing off” effect.

As was already mentioned above that LPS, which are not toxic to neurons themselves, could be injected directly into rat SNpc leading to microglia activation followed by DA neuron degeneration. Animals exhibited behavioral changes as well as α-synuclein und ubiquitin accumulation in SNpc neurons (Hunter et al., 2009).

7.2. Transgenic models

Although aforementioned toxin-based models are extremely useful and widely used, they lack several important aspects of disease pathogenesis as age-dependency and slow progressive mode of degeneration, LBs absence. Mutations of α-synuclein gene have been shown to cause familial form of the disease but transgenic mice carrying those mutations did not show remarkable degeneration. Expression of wild-type α-synuclein leads to decrease of the number of DA neurons and slowness of movement. Some of these models represent also non-motor symptoms of PD (Lam et al., 2011). Six mutations in gene for LRRK2 are known to cause the disease onset. Transgenic mice with over expression of R1441G mutant showed age-dependent and progressive motor activity deficiency (Kumar et al., 2011). Mouse with mutated PINK1 gene showed decreased motor activity, although LBs were not present (Gispert et al., 2009). Around 100 mutations causing PD have been defined in the gene for parkin. Parkin KO mice are sensitive to neurotoxins but did show neither dopaminergic nor behavioral abnormalities. Transgenic mice over expressing wild-type orf mutant of DJ-1 have not been reported yet. Moreover, multiple mutant mice were also developed, although no consistent data exist. New models started to appear in PD research, e.g. MitoPark mice with disrupted mitochondrial function. Transgenic rat with monogenic PD mutations are now
commercially available. Several drawbacks of above-mentioned models as an absence of nigrostriatal degeneration led to generation of alternative more selective models as *Drosophila melanogaster*, *C. elegans* and Zebrafish, which are simpler, less expensive and allow performing high throughput screenings (Blandini et al., 2012). Unexpectedly, transgenic models appeared to be unavailing to gain a proper disease model. Probably combined effect of toxins and transgenes mutation could model pathogenic process more precisely. Nowadays, one single “ideal” *in vivo* model reflecting full PD pathology does not exist. Moreover, the multiple causes and characteristics of disease as well as profound differences between human and animal neuronal systems make it almost impossible to develop. The choice of the model should depend on its strong sides and must be defined by exact research tasks.

7.3. Cell culture models

For a long time one of the most common sources of cells for *in vitro* studies were primary cells from human or rodent’s postmortem material with limited life-spans. Therefore, they could not be successfully cultivated. Early studies were done on rat embryonic midbrain cultures, where the amount of DA neurons did not exceed 2%. Moreover, the species discrepancies are especially pronounced in the neuronal tissues, causing the differences in main molecular players of key pathways. All this drives an interest for establishing of cultivatable human-based cellular models. The summary of the most commonly used neuronal and non-neuronal cell lines for PD and their main characteristics are listed in Table 1. Each of these monoculture models has their strong and weak points, but none of them could be considered as a fully appropriate model the disease. All above-mentioned cellular models contribute greatly to PD research, although they lack a necessary neuron-glia interaction aspect. In Table 2 several astrocytes cultures from different sources representing benefits for neurodegenerative disease studies are listed.

Finally, various arts of co-cultures are currently available for PD research. Since the pathological picture of the disease is rather complicated, different co-culture systems are tailored to address the specific issues (Table 3).

*Direct cultures (cell co-cultures, organotypic, 3D cultures)*

The direct co-cultures represent a powerful tool to model Parkinsonism *in vitro* and are widely used for testing of potential neuroprotective compounds. In this approach, neurotoxic compounds have traditionally been applied to the media to induce a uniform dopaminergic
A. General introduction

cell death or activation of glial cells was triggered to affect neuronal viability. These models possess an advantage – a direct neuron-glia interaction, which might be one of the key factors for the neuronal survival modulation. Organotypic cultures such as brain slices and tissue material can be in culture for several weeks to months, however there is a huge variation among the slices (Gahwiler et al., 1997; Stahl et al., 2009). 3D cultures received an enormous interest in PD research. These culture systems differ from organotypic ones by a number of features: they have additional artificial biocompatible scaffold/framework, e.g. hydrogel, which helps culture to be correctly formed/differentiated and reflect an in vivo architecture; they represent spheres containing neurons and astrocytes or only neurons (neurospheres). Indeed, 3D cultures allow an exploration of new aspects and expanding the existing tools for PD, however, the establishing procedure still remains complicated and laborious.

Separate cultures (transwell systems, medium transfer, microfluidic, IdMOC approaches)

An indirect co-culturing of neurons and astrocytes offers the great advantage of separate analysis of alterations occurring in both astrocytes and neurons after the particular interventions such as intracellular metabolites, viability testing, helping to reveal key players of pathological molecular pathways involved. Additionally, indirect cultures are used to study α-synuclein aggregation and cell-to-cell transfer between different neural cell types. The various indirect co-cultures used in PD, their characteristics and types of studies one may perform are summarized in Table 3.

In spite of a vast of models existing and appearing in the field of PD in vitro research, the direct contact of astrocytes and neurons still remains to be the most frequently used classic system that combines simplicity and reproducibility and simultaneously reflects important neuron-glia interactions.
<table>
<thead>
<tr>
<th>Cell line/source</th>
<th>Advantages for PD</th>
<th>Drawbacks for PD</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human neuroblastoma cell lines (SH-SY5Y, SK-N-MC, SK-N-BE)</td>
<td>Cell line with human origin expressing the TH, dopamine D2 and D3 receptors, DAT, and VMAT2 upon differentiation with RA and TPA, respond to Parkinson's neurotoxin (MPP+), protein overexpression is possible.</td>
<td>Cells are not authentic DA neurons. No unified differentiation protocol is available. Cells are not authentic DA neurons.</td>
<td>1,9,16</td>
</tr>
<tr>
<td>Embryonic teratocarcinomas (NT2, hNT)</td>
<td>Neuronal progenitors with human origin which can be differentiated to DA neurons with high outcome of TH-positive cells (&gt; 80%) and other DA markers such as AADC, DAT, Nurr1, TrkB and TrkC, GFRA1; respond to neurotransmitters, and exhibit electrophysiological excitability, protein overexpression is possible.</td>
<td>Tumor background, failed in neurotransplantation studies</td>
<td>4,14,17</td>
</tr>
<tr>
<td>Immortalized human embryonic mesenchymal cells (LUHMES, NGC-407, ReNcell IVM NCS)</td>
<td>LUGHMES: rapid in vitro differentiation process towards human mature neurons with a high degree of dopaminergic phenotype (expression of TH, DAT, VMAT2, respond to MPP+, including release of dopamine and neuronal electric properties). Cells can be efficiently transfected with lentiviral approach.</td>
<td>LUHMES: Cells do not maintain phenotype in vivo.</td>
<td>12,13,15</td>
</tr>
<tr>
<td>Neuron-like rat pheochromocytoma cell line (PC12)</td>
<td>Differentiate to neuronal phenotype by NGF, respond to 6-OHDA</td>
<td>Do not acquire DA phenotype, rat origin</td>
<td>2,10</td>
</tr>
<tr>
<td>Human embryonic kidney (HEK293)</td>
<td>High transfection efficiency allows to study kinetics of asyn aggregation</td>
<td>Do not acquire neuronal phenotype</td>
<td>8,14,18</td>
</tr>
<tr>
<td>Stem cell models</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human embryonic and adult neural stem cells</td>
<td>Cellular ability to expand and differentiate, upon optimized differentiation, expression of most midbrain DA markers, DA release and electrophysiological characteristics in a subset of cells, TH positive cells outcome 18-56%</td>
<td>Cells are largely heterogeneous, studies are needed to optimize culture conditions and differentiation approaches</td>
<td>7</td>
</tr>
<tr>
<td>Human mesenchymal stem cells</td>
<td>Can be easily obtained (e.g., from bone marrow or umbilical cord tissue), success rate for TH-positive neurons 12.7-67%, expression of En1, En2, Nurr1</td>
<td>Low yield of TH-positive neurons, questions regarding the authenticity of neuronal cells differentiated from cell types whose potency is generally limited to mesodermal derivatives.</td>
<td>20</td>
</tr>
<tr>
<td>-Human iPSCs</td>
<td>TH expression in iPSCs from patients with idiopathic PD</td>
<td>Optimised protocols should still be developed</td>
<td>3,19</td>
</tr>
<tr>
<td>Patient specific cell line</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cybrid cell lines (e.g., cytoplasmic fusion of cells that lack mitochondrial DNA (mtDNA) fused with platelet mtDNA from PD patients)</td>
<td>Reduced complex I activity, enhanced susceptibility for H2O2 and MPP+, fibrillar and vesicular inclusions after prolonged culture conditions</td>
<td>Technical difficulties in developing these cell lines, non neuronal phenotype</td>
<td>5</td>
</tr>
<tr>
<td>Primary human fibroblasts from patients (e.g., PINK1-deficient fibroblasts)</td>
<td>Upregulation of alpha-synuclein and synaptic protein genes, lower respiratory activity, increased ROS production, and decrease in cytochrome C</td>
<td>Do not have neuronal phenotype; cells become senescent in a long-term culture; may also transform and/or undergo clonal selection.</td>
<td>6,11</td>
</tr>
</tbody>
</table>

Table 1. Summary of neuronal cell culture models for PD research.
Table 1. Summary of neuronal cell culture models for PD research.

<table>
<thead>
<tr>
<th>Literature</th>
<th>Description</th>
</tr>
</thead>
</table>
Table 2. Astrocytes sources and studies performed.

<table>
<thead>
<tr>
<th>Cell line/source</th>
<th>Basic characteristics available</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human astrocytes (LONZA)</td>
<td>Primary-derived culture, limited doublings number with guaranteed properties, express GFAP</td>
<td>3, 9, 10</td>
</tr>
<tr>
<td>Human Astrocytes (Sciencell)</td>
<td>Primary astrocytes isolated from human brain tissue (cerebral cortex), limited number of passages, GFAP-positive</td>
<td>1, 4, 5</td>
</tr>
<tr>
<td>Human astrocyte cell line (A735)</td>
<td>Immortalised human brain-derived cell line with morphological, growth, and functional properties of astrocytes</td>
<td>7</td>
</tr>
<tr>
<td>iCell @Astrocytes (Cellular dynamics)</td>
<td>Heterogeneous and highly pure human astrocytes population derived from iPS cells, express GFAP and s100b. Upon stimulation with cytokine mix demonstrate changes in cell morphology and upregulation of the IL-6 secretion.</td>
<td>11</td>
</tr>
<tr>
<td>Astrocytoma cell line (1321N1) (Sigma)</td>
<td>Human Astrocytoma cell line derived from malignant glioma</td>
<td>2</td>
</tr>
<tr>
<td>Mouse astrocytes generated from embryonic stem cells (mAGES)</td>
<td>Pure postmitotic astrocyte cell culture with phenotypic heterogeneity, expressing GFAP, aquaporin-4 or glutamine synthetase, undergo inflammatory activation with cytokine mix, render neuronal support</td>
<td>6</td>
</tr>
<tr>
<td>Immortalized mouse astrocytes (IMA 2.1)</td>
<td>Expression of typical astrocyte markers, able to metabolize the parkinsonian toxin MPTP to its toxic metabolite MPP+, under stimulation express iNOS and COX-2, and the release of various inflammatory mediators.</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3. Various co-culture models involved in PD research.

<table>
<thead>
<tr>
<th>Model system</th>
<th>Characteristics/Types of studies</th>
<th>Example references</th>
</tr>
</thead>
</table>
| Direct contact cultures                     | 1. Primary dopamine-enriched midbrain neuron-astrocyte(microglia) co-cultures, or co-culturing of established cell lines where MPP+, 6-OHDA, rotenone or H₂O₂ trigger neurotoxicity  
  2. The accumulation, aggregation and distribution of alpha-synuclein among the neurons and astrocytes can be studied  
  3. Primary microglia-neuronal co-culture as well as established cell lines exposed to inflammatory stimuli such as LPS, MPP+/dopamine | 5, 8, 11, 14, 19 |
| - Neuron glia co-cultures                  | 4. Tissues are isolated from genetically-transformed rodent or animals exposed to MPTP/6-OHDA/MPP+.  
  5. Slices isolated from rodents exposed to Parkinsonian drugs or neuroinflammatory stimuli; human post mortem material from PD and healthy patients | 2, 7, 4, 15, 23 |
| - Organotypic cultures (Brain slices/tissue samples) | 6. These systems used as cell-based drug delivery system (deliver dopamine)  
  7. Neurospheres containing neurons with astrocytes or microglia which are exposed to neurotoxins or genetically-manipulated via viral transduction | 3, 9 |
| - 3D cultures                              | Neurons and astrocytes (primary isolated cells or cell lines) underwent either to genetic interventions (e.g., DJ-1 mutation) or exposed to Parkinsonian toxins (MPP+, 6-OHDA, rotenone); evaluation of asynuclein aggregates transfer among the cells can be studied | 10, 16, 20 |
| Indirect contact cultures                  | One cell type is grown in the well of the plate, another cell type in inserts with pores allowing diffusion factors penetration | 12, 22 |
| Transwell plate                            | Conditioned medium from one cell type partially or completely transferred to another one | 6, 17, 18, 21 |
| Medium transfer                            | Different cell types are localised in separate compartments connected by a number of microchannels | 13 |
| Microfluidic platform                      | Non-neural cells (dental pulp stem cells, olfactory ensheathing cells) are as well used for co-culturing with neurons in order to evaluate their support against neurotoxins. |             |
| IdMOC system Li                            | Wells-within-a-well concept for the co-culturing of cells or tissue slices from different organs as physically separated (discrete) entities in the small inner wells |             |
| In silico models                           | Computational model to study impact of astrocytes in neural network function | 1 |

8. Aims of the thesis:

The *in vitro* models available for fundamental studies as well as for screening of therapeutic candidates often lack important neuron-glial interactions in the system. For co-culture systems, the major focus has been on microglia as the second cell type besides neurons (McGeer *et al.*, 2008). However, it is becoming more evident that astrocytes are also important players in PD and other neurological disorders (Forno *et al.*, 1992; Maragakis *et al.*, 2006). Appropriate *in vitro* models are needed in order to gain insights on possible pharmacological approaches to target specific pathways and influence the clinical treatment of neurodegenerative disorders.

The aims of this thesis were:

1. A phenotypic and functional characterization of IMA 2.1 as astrocytes model system, including direct comparison with primary mouse astrocytes with respect to their response proinflammatory stimuli, expression of typical astrocytes markers, and to the cell line’s capacity to metabolize the parkinsonian toxin MPTP to its toxic metabolite MPP⁺, compatibility with neuronal cell line LUHMES. The evaluation of the suitability of IMA cells for disease modeling and neuropharmacological studies in the field of PD (Chapter B).

2. The generation of a human *in vitro* counterpart of the widely-used MPTP mouse model for PD to test new experimental therapies. The system should comprise a neuron-glia interaction and allow us to model MPTP metabolism (Chapter C).

3. The generation of *in vitro* co-culture model allowing study of the neuropharmacological relevance of astrocytes, their involvement in progress of neuronal death and therapeutic potential (Chapter D).
Chapter B

Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocyte functions.

Stefan Schildknecht¹, Susanne Kirner¹, Anja Henn¹, Karlo Gasparic¹, Regina Pape¹, Liudmila Efremova¹, Olaf Maier², Roman Fischer², Marcel Leist¹

Affiliations:
¹Doerenkamp-Zbinden Chair for In vitro Toxicology and Biomedicine, University of Konstanz, 78457 Konstanz, Germany
²Institute for Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

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B. Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocytes functions

1. Abstract

Astrocytes are activated in most chronic neurodegenerative diseases associated with inflammatory events such as Parkinson’s Disease or Alzheimer’s Disease, but also in stroke. Due to an aging population worldwide, research efforts in these areas are likely to expand in the future. This will entail an increased demand for appropriate experimental models.

We introduce here the new immortalized mouse astrocyte cell line IMA 2.1 as alternative to the currently used primary astrocyte cultures. IMA 2.1 were directly compared with primary mouse astrocytes with respect to their response to proinflammatory stimuli, the expression of typical astrocyte markers, as well as to the cell line’s capacity to metabolize the experimental parkinsonian drug MPTP to its toxic metabolite MPP⁺. Under inflammatory conditions, mimicked by the addition of a cytokine mix, IMA 2.1 responded similar to primary astrocytes by mRNA upregulation, expression of iNOS and COX-2, as well as by the release of various inflammatory mediators. Analysis of astrocytic markers indicated that IMA 2.1 represent a relatively early, GFAP-negative, stage of astrocyte development. Moreover, conversion of MPTP by monoamine oxidase-B proceeded in IMA at least as fast as in primary cells. For all endpoints investigated, the cell line IMA 2.1, derived from a single clone, delivered reproducible results over a period of several years and allowed upscaling of experiments due to its easy handling compared with primary cells.
2. Introduction

Astrocytes are highly differentiated cells of the central nervous system that serve numerous functions, such as nutrient supply of neurons, regulation of cerebral blood flow, orchestration of neuronal growth and differentiation, maintenance of extracellular glutamate levels, and ion and liquid balance (Kettenmann, 2005). They are furthermore involved in inflammatory processes in the central nervous system that are not only observed following the invasion of pathogens, but also in association with chronic neurodegenerative diseases such as Alzheimer’s Disease (AD) or Parkinson’s Disease (PD) (Falsig et al., 2008). The primary effector cells of the brain in response to bacteria are microglia (Lund et al., 2006; Lund et al., 2005). They release proinflammatory cytokines that trigger the subsequent activation of astrocytes as second line of defence (Falsig et al., 2006; Henn et al., 2011; Lee et al., 1993). Following stimulation, both cell types become rich sources of cytokines and prostanoids (Meeuwsen et al., 2003; Murphy et al., 1988). Moreover, they produce the free radicals nitric oxide (\(^{\text{3}}\text{NO}\)) and superoxide (\(^{\text{3}}\text{O}_2\)) that finally can lead to the formation of several cytotoxic species such as peroxynitrite (\(^{\text{3}}\text{ONO}_2\)) or hydroxyl radicals (\(^{\text{3}}\text{OH}\)) (Beckman et al., 1996; Brown et al., 2010). These molecules can attack and modify cellular proteins, lipids, and DNA in neighbouring neurons, as illustrated in numerous reports, particularly in association with chronic brain diseases (Schildknecht et al., 2011).

In a widely used \textit{in vivo} model of PD, astrocytes are responsible for the conversion of the parkinsonian toxin 1-methyl-\textit{1-methyl-1-phenyl-1,2,3,6-tetrahydropyridine (MPTP)} into its active metabolite \textit{1-methyl-4-phenylpyridinium (MPP\textsuperscript{+})} (Di Monte et al., 1991; Ransom et al., 1987). The responsible enzyme has been identified as monoamine oxidase-B (MAO-B).
Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocyte functions

After release from astrocytes, MPP$^+$ is selectively taken up by dopaminergic neurons and leads to their death (Poltl et al., 2012; Schildknecht et al., 2009).

Primary rodent astrocytes have been used for many in vitro disease models and to obtain biochemical insight into astrocyte function (Christiansen et al., 2011; Falsig et al., 2004b; Kim et al., 2011). Although highly sophisticated isolation procedures have been developed, batch-to-batch variations and contamination with microglia represent considerable shortcomings in the use of primary cultures (Hansson, 1986). As primary astrocytes hardly proliferate, the high demand for animals puts an additional burden on this type of cellular model. For example, highly purified primary astrocyte preparations, as used for gene expression profiling (Falsig et al., 2004a; Falsig et al., 2006) may yield less than 1 million cells per mouse, and a single study may require the sacrifice of several hundreds of mice. In order to find alternative approaches, numerous attempts have been undertaken to generate astrocyte cell lines by retroviral transduction of immortalizing oncogenes. The first approach dates back to 1990, when progenitor cells isolated from mouse brains were transduced with the myc-oncogene that allowed the generation of cell lines with neuronal and glial phenotypes (Ryder et al., 1990). In a more sophisticated approach, mouse cortical astrocytes of varying differentiation stages have been immortalized by SV40 T antigen, and this allowed the generation of mature and immature mouse astrocyte lines (Frisa et al., 1994). In an attempt to generate an immortalized human astrocyte cell line, astrocytes obtained from human fetal brain have been purified and transfected with the SV40 T antigen. However, it was noted that the high proliferation capacity of this newly generated A735 line was associated with downregulation of GFAP expression and subsequent loss of other astrocyte specific markers (Price et al., 1999), thus representing no adequate substitute for primary cells. In a new attempt to provide an alternative to primary astrocyte cultures, we immortalized cortical astrocytes of wildtype BALB/c mice with the SV40 large T antigen, created single cell clones, and selected the clone IMA 2.1 with particular emphasis on its ability to respond to proinflammatory cytokines and with respect to its expression of functional monoamine oxidase-B (MAO-B).

During more than three years in use in our laboratory, its phenotype and response pattern towards cytokines remained stable. This makes the IMA 2.1 cells a reliable new model for certain astrocyte studies (Leist et al., 2010). In the present manuscript, we provide data on the basic characterization of this new cell line and suggest the IMA 2.1 cell line as a useful tool
for a variety of *in vitro* studies of astrocyte functions including astrocyte–neuron crosstalk. It is intended as an overview for interested researchers to enable a first judgement on whether IMA 2.1 may be useful for their planned investigations.
3. Materials and Methods

Immortalization of primary astrocytes from BALB/c mice

Cerebral cortices were isolated from one or two day old mice. After removal of the meninges, cortices were chopped into small pieces with a razor blade and further homogenized by enzymatic digestion in PBS/0.5 % trypsin/EDTA (Gibco/Invitrogen, Karlsruhe, Germany), 0.5 % DNase I (Sigma-Aldrich, Steinheim, Germany) for 15 min at 37 °C. Trypsin was inactivated by addition of fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany) and tissue was triturated using a Pasteur pipette. The homogenates were seeded on poly-D-lysine hydrobromide (PDL; 10 µg/ml; Sigma-Aldrich, MW 30-70 kDA) coated culture flasks at a density of approximately 40,000 cells/cm2 in DMEM (Gibco/Invitrogen) supplemented with 10 % FCS, 2 mM L-Glutamin (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen). After eight days in culture, an astrocyte layer, overgrown by numerous microglial cells, oligodendrocyte precursor cells and oligodendrocytes, can be observed. To prepare purified astrocyte cultures, oligodendrocytes and surface microglia were flushed away from the astrocyte layer. The remaining microglia were removed by trypsinization of the primary cultures and differential adhesion for 30 min on uncoated culture dishes. The astrocytes remaining in the supernatant were collected, and approximately 0.5 x 10^6 cells were plated in PDL-coated 6-well plates. Cells were grown to confluency in culture medium without antibiotics. On the day of transfection, medium was changed to Opti-MEM I (Gibco). Lipofectamine 2000 (Invitrogen) was diluted 1:50 in Opti-MEM I and incubated for 5 min at room temperature. The plasmid psV3neo (Chang et al., 1986), coding for the large T antigen from SV40 (500 ng) was diluted in Opti-MEM I and mixed in a ratio of 1:1 with the lipofectamine dilution. The resulting transfection complexes were preincubated at room temperature for additional 20 minutes, then the transfection mixture (500 µl/well) was added carefully to the cells, and incubated at 37 °C. After 6 h, the transfection mixture was replaced by standard cell culture medium and the cells were incubated for further 24 hours. Stably transfected cells were selected with 800 µg/ml genetin (G418; Invitrogen). The resultant immortalized mouse astrocytes (IMA) were cultivated at 37 °C (5 % CO2) in DMEM Glutamax (high glucose) (GIBCO) with 5 % FCS (fetal calf serum, from PAA) without antibiotics. They were trypsinized for 2 min with 0.5% trypsin/DMEM every 2-3 days and reseeded at a ratio of 1:5 or 1:10. Experiments were performed in DMEM containing 2 % FCS.
B. Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocytes functions

Primary astrocyte cultures

Primary cortical astrocytes were generated from BALB/c mice, bred at the animal facility of the University of Konstanz, Germany. All mice were housed at 22 °C and 55 % relative humidity in a 12 h day/night rhythm with free access to food and water according to national regulations and EU guideline 86/609/EEC. Primary cortical astrocytes were prepared from mouse pups at 24 - 48 h post partum as described earlier (Henn et al., 2011). In brief, brains were removed, the cortices were dissected out, and hippocampi and meninges were carefully removed before digestion with trypsin and DNase. After trituration, the cell suspension was carefully layered over a 30 % Percoll solution and centrifuged at 150 x g for 10 min. The astrocyte fraction was recovered, washed, and resuspended in DMEM (high glucose), 20 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were seeded at a density of 2 million cells (corresponding to 2 brains) per T75 flask, and cultured at 37 °C in a 5 % CO₂ atmosphere. The medium was changed after 4 days, and subsequently every second day. After 14 days in primary culture, cells were trypsinized and incubated in DMEM for 45 min at room temperature in a T75 flask for differential adhesion of any residual microglia. Non-adherent cells (astrocytes) were reseeded in DMEM plus 10 % FCS, containing 4.5 g/l D-glucose and 2 mM Glutamax, and cultured in multiwell dishes for 7-9 days before use in experiments. This protocol was shown to result in microglia or oligodendrocyte contaminations below our detection limit (< 1 %) by FACS, PCR, or immunostaining.

Co-culture model

Primary astrocytes or IMA were seeded into 24-well plates (Nunclon, Nunc, Roskilde, Denmark), when confluency was reached, the cells were maintained for additional 7 days. Then, pre-differentiated (2 days) human mesencephalic cells (LUHMES) were seeded on top of the glial cell layer at a density of 180,000/cm² in Advanced DMEM (Gibco), containing 2 mM L-glutamine, 1 x N2 supplement, and 1 µg/ml tetracycline for additional 4 days. LUHMES cells were previously characterized with respect to their applicability as in vitro model of terminally differentiated dopaminergic neurons (Lotharius et al., 2005; Schildknecht et al., 2009; Scholz et al., 2011). To selectively visualize the neurons in these cocultures, cells were fixed with 4 % paraformaldehyde (PFA) for 15 min and permeabilized with 0.2 % Triton X-100 in PBS for 10 min. Following blocking with 1 % bovine serum albumin (BSA) in PBS for 1 h, LUHMES were stained with an antibody against the neuronal class of β-III-tubulin (TUJ-1; Convance, mouse, 1:1000) and fluorescence-labeled secondary antibodies.
Translocation of NF-κB

Cells were plated in 96-well plates at a density of 15,000 cells/well and cultured in DMEM plus 2 % FCS. The cells were stimulated as indicated, then fixed with 4 % PFA for 15 min, permeabilized with 0.1 % Triton X-100 for 10 min, blocked with 10 % FCS for 1 h, immunostained with purified monoclonal mouse anti-NF-κB p65 (clone 20/NF-κB/p65; final dilution 1:300) antibody (BD Transduction Laboratories) over night, and detected with anti-mouse IgG Alexa-488 secondary antibody. Images were recorded with a high-resolution CCD camera on a Cellomics ArrayScan™, which is based on an automated Zeiss Axiovert-100 microscope equipped with a 20x objective and a FITC/H-33342 filter set (λ_ex-1 = 365 nm, λ_ex-2 = 475 nm, λ_em = 535 nm). Nuclei, stained with H-33342, were imaged first (channel 1) for automated focusing and identification of valid objects. Subsequently, the stained antigens were imaged in the corresponding fields (channel 2). Based on these data sets, the nuclear translocation of NF-κB was quantified for each cell with the predefined algorithm ‘nuclear translocation’, essentially as described earlier (Henn et al., 2011; Henn et al., 2009).

Flow cytometry

Cells from 6-well plates were trypsinized and washed twice with PBS, containing 10 % FCS. They were centrifuged for 5 min at 500 x g. FITC-conjugated anti-TLR2 antibody (clone 6C2; eBioscience; Alasdair Stewart, U.K) diluted in 2 % FCS/PBS with a concentration of 1 µg per million cells (in 200 µl) was added for 50 min at 4 °C. An appropriate isotype control was used in a similar way. Cells were washed twice and fixed with 0.5 % PFA. For each sample, a minimum of 10,000 cells within the gated cell population was analysed using a flow cytometer (Accuri Cytometers; Cambs, U.K.) and the corresponding software CFlow. The percentage of positively stained cells relatively to isotype control (maximum 1 % positive staining) was determined.

RNA extraction, RT-PCR and quantitative PCR

Total RNA was extracted with TRIzol from Invitrogen (Darmstadt, Germany) and 1 µg of total RNA was reverse transcribed with Superscript™ II Reverse Transcriptase (Invitrogen, Darmstadt, Germany) using random hexamers and Oligo-dT-Primer in a 20 µl reaction according to the manufacturer’s protocol. PCR amplification of the cDNA was quantified using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen, Darmstadt, Germany). The threshold cycle (C_T) was determined for each sample using the iCycler MyiQ
qPCR detection system (BioRad Laboratories, Hercules, CA, USA) and corresponding data analysis software. The housekeeping gene control was *gapdh*. The relative cDNA levels were compared among different groups using the delta-delta C_t method. Absolute levels were calculated by the delta C_t method. The following primer sequences were used in the study:

**AQP 4:** sense 5´-GCT CAG AAA ACC CCT TAC CTG TGG-3´, antisense 5´-TTC CAT GAA CCG TGG TGA CTC C-3´; **Cnx 43:** sense 5´-CCA CTC TCA CCT ATG TCT CCT CC-3´, antisense 5´-CGT CAG GGA AAT CAA ACG GCT GG-3´; **COX-2:** sense 5´-CCA TTG AAC CTG GAC TGC AGA AGG C-3´, antisense: 5´-CGG AAC TAA GAG GAG CAG CAA TGC-3´; **GAPDH:** sense 5´-CAG GCA GCT CAG GTT GAC TCT G3´, antisense 5´-GGG AAT TTG AGG TCT TTT C-3´; **GFAP:** sense 5´-CGA GAT CGC CAC CTA CAG GAA GC-3´, antisense: 5´-CTT TAA TGA CCT CTC CAT CCC GC-3´; **GLAST:** sense 5´-CTC TAC GAG GCT TTG GCT GC-3´, antisense: 5´-GAG GCG GTC CAG AAA CCA GTC-3´; **GLT-1:** sense: 5´-GCA GCC ATC TTC ATA GCC CAA ATG-3´, antisense: 5´-CTC ATT CTA TCC AGC AGC CAG TCC-3´; **GS:** sense: 5´-GTA GAC ACA GCC TCA GAC AGC C-3´, antisense: 5´-GGA GAG GAT GC AAT CAA ACG GCT GG-3´; **IL-1β:** sense: 5´-TTT TTG TTG TTC ATC TCG GAG CCT GTA G-3´, antisense: 5´-GAG CAC CTT CTT TTC CAT CTT TG-3´; **IL-6:** sense: 5´-CCT CTG GTC TTT TCG AGG ATC ACC ATA GC-3´, antisense: 5´-GGA GAG CAT TGG AAA TTG GGG TAG G-3´; **IL-23:** sense: 5´-CAT GCA CCA CGG GGA CAT ATG-3´, antisense: 5´-CAG ACC TTG GCG GAT CCT TTG-3´; **Kir4.1:** sense: 5´-GTA GAC ACA GCC TCA GAC AGC C-3´, antisense: 5´-GGT AGG AAG TGC GAA CTT GCC-3´; **MAO-B:** sense: 5´-AAC AGT ATT CTG GGG CTT GCT GCT ACA-3´, antisense: 5´-AGG GAC ATC CAA AGA TTC TGG TTC TG-3´; **MCP-1:** sense: 5´-CAT AGC AGA ACA GAA GAA CAG-3´, antisense: 5´-GTC TTT GAG GGT TTT CTT TG-3´; **Nestin:** sense: 5´-CTG GAA GGT GGG TAG CAG CAA CT-3´, antisense: 5´-ATT AGG CAA GGG GGA AGA GGT G-3´; **NOD-2:** sense: 5´-CAG GCA GCT CAG GTT GAC TCT GCT G-3´, antisense: 5´-TAG AAA GCG GCA AAA AAG CAC TGG AAG-3´; **NOS-2:** sense: 5´-TTG CCA CGG ACG AGA CGG ATA GG-3´, antisense: 5´-GGG GTT GCT GAA CTT CCA GTC-3´; **S100β:** sense: 5´-GGA TGC CCT CAT TGA TGT CTT CCA C-3´, antisense: 5´-GAG TCT CCT GCT CCT TTA TTT CCA G-3´; **TLR-2:** sense: 5´-CCT CCG TCT TGG AAT GTC ACC AGG-3´, antisense: 5´-GAG CCA CGC CCA CAT CAT TC-3´; **TLR-3:** sense: 5´-GCC CCC TTT GAA CTC CTC TTC TC-3´, antisense: 5´-AGA TCC TCC AGC CCT CGA TG-3´; **TNF-α:** sense: 5´-TAG CAA ACC ACC AAG TGG AGG AG-3´, antisense: 5´-GCA GCC TTG TCC CTT GAA GAG AA-3´;
TYKi: sense: 5´- GGA TGC GCT GCA CAC CAA TT-3´, antisense : 5´- TAG CTC CTT GGC CTC CGT TTG-3´.

Measurements of prostanoids and nitrite in cell culture supernatants

Prostaglandin E$_2$ (PGE$_2$) was determined by using commercially available EIA-kits (Assay Designs, MI, USA) according to the manufacturer’s instructions. Nitrite (NO$_2$), the stable autoxidation product of *NO, was measured by the Griess assay. Briefly, 30 µl 12.5 µM sulfanilamide (Sigma) and 30 µl 6 M HCl were mixed with 200 µl cell culture supernatant at room temperature and incubated for 5 min. Absorbance was measured before and after the addition of 25 µl N-(1-naphthyl)ethylenediamine (12.5 µM) (Sigma) at 560 nm using a microtiter plate reader. Nitrite concentrations were calculated from a NaNO$_2$ standard curve in the range of 0.5-10 µM.

HPLC analysis

Detection of MPTP, MPP$^+$ and MPDP$^+$ was performed on a Kontron system (Goebel Analytic, Au/Hallertau, Germany) composed of a model 520 pump, model 560 autosampler, models 535 and 430 diode array detectors, set at 245 nm for MPTP, 295 nm for MPP$^+$ and 345 nm for MPDP$^+$. Samples were acidified with 9 µl perchloric acid (70%) per ml volume of sample and centrifuged at 10.000 x g for 15 min. The supernatant was filtered through a Chromaphil PET-20/15MS-filter with 0.2 µm pore size from Macherey Nagel (Düren, Germany). Separation was carried out on a C18 nucleosil column (250 x 4.6 mm; 5 µm particle size) from Macherey Nagel (Düren, Germany) at room temperature. The mobile phase consisted of acetonitrile : distilled water : triethylamine : sulphuric acid (12.50 : 86.18 : 1.04 : 0.28, v/v, pH 2.3) The mobile phase was degassed with an online vacuum degasser and delivered isocratically at a flow rate of 1 ml/min at an average pressure of 145 bar. Data analysis was performed with Geminyx II software (Goebel Analytic).

Monoamine oxidase (MAO) activity assay

The assay is based on the oxidation of kynuramine to 4-hydroxyquinoline by MAO. Cells were homogenized in PBS and total protein content was adjusted to 1 mg/ml. Then, 180 µl of cell homogenate were supplemented with 20 µl substrate kynuramine (Sigma) in PBS to yield a final concentration of 100 µM. In parallel, a standard curve of 4-hydroxyquinoline (0-100 µM) was prepared. After 2 h of incubation, the reaction was terminated by the addition of 80 µl perchloric acid (400 mM). Samples were centrifuged at 12.000 x g for 1 min, 200 µl
supernatant was transferred into a new tube and mixed with 400 µl of NaOH (1 M). Samples of the standard curve were treated accordingly and fluorescence was detected at $\lambda_{\text{ex}}$ 315 nm / $\lambda_{\text{em}}$ 380 nm.

**Cyclooxygenase (COX) activity**

COX activity was determined by the conversion of the $^{14}$C-labeled substrate arachidonic acid ($^{14}$C-AA) to prostanoids (Schildknecht et al., 2008). Cells were washed twice, collected in cold PBS and centrifuged at 1000 x g for 3 min. The pellet was dissolved in lysis buffer (20 mM HEPES, 1 % Triton X-100, 1 % aprotinin, 10 % glycerol, pH 7.5) for 30 min on ice. Following centrifugation at 12,000 x g for 1 min, the supernatant was incubated with reaction buffer (80 mM Tris-HCl, 0.1 mM phenol, 5 µg/ml hematin, 5 µM $^{14}$C-AA, pH 8.0) for 20 min. The reaction was terminated by the addition of ethyl acetate/ 2 M citric acid (30:1). After vortexing for at least 1 min, the organic phase was collected, evaporated and used for analysis on silica TLC plates (Silica 60, Merck, Darmstadt, Germany) by chromatography. The mobile phase consisted of ethyl acetate : 2,2,4-trimethylpentane : acetic acid : water (110:50:20:100). Plates were dried and used to expose a PhosphorImager™ screen overnight. For reading the screen, a PhosphorImager™ system from Molecular Dynamics, USA was used. Quantification was performed by the detection of total prostanoid formation utilizing ImageQuant™ software.

**Western-blot analysis**

Cells were lysed in RIPA buffer (50 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1 % NP40, 1 mM Na$_2$VO$_4$, 50 mM NaF, pH 7.5). Protein determination was performed by using a BCA protein assay kit (Pierce). After loading of 25 µg protein to a 8 % polyacrylamide SDS gel, proteins were transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK). Loading and transfer were controlled by brief Ponceau staining. Washed membranes were then blocked with 5 % milk in PBS-Tween (0.1 %) for 2 h. Primary antibodies were added at 4 °C over night (COX-2: Cayman, polyclonal, 1:1000; NOS-2: BD Transduction, polyclonal rabbit 1:5000; β-actin: Sigma, monoclonal mouse 1:2000). Following washing steps with PBS-Tween, horseradish peroxidase-conjugated secondary antibodies were added for 1 h at room temperature. For visualization, ECL western blotting substrate (Pierce) was used. As secondary antibodies, anti-mouse HRP 1:5000, Jackson Immuno Research and anti-rabbit HRP 1:5000, GE Healthcare were used.
Inflammatory stimulation

IMA or primary astrocytes were stimulated with cytokines or the TLR-2 ligand FSL-1 (Invitrogen, San Diego, CA). Murine TNF-α, IL-1β, and IFN-γ were obtained from R+D Systems (Wiesbaden, Germany). The combination of IL-1β (10ng/ml), TNF-α (10ng/ml), and IFN-γ (20ng/ml) was termed complete cytokine mix (CCM).

Immunocytochemistry

Cells were grown on 10 mm glass cover slips (Menzel, Braunschweig, Germany) in 24-well plastic cell culture plates (Nunclon™). Following treatment, cells were fixed with 4 % PFA for 20 min at 37 °C and washed with PBS. After blocking with 1 % BSA (Calbiochem, San Diego, CA) for 1 h, primary antibodies (anti-CD44, 1:500, rat monoclonal, Caltag Laboratories; anti-nestin, 1:500, rat monoclonal, Millipore) were added in PBS-Tween (0.1 %) at 4 °C over night. Secondary antibodies (anti rat IgG-Alexa 488, Invitrogen, Darmstadt, Germany) were added for 45 min at RT. For visualization, an Olympus IX 81 microscope (Hamburg, Germany) equipped with a F-view CCD camera was used. Nuclei were stained with H-33342 (1µg/ml) for 15 min prior to the final washing step. For image processing, Cell P software (Olympus) was used.

Statistics

NF-κB translocation data are based on at least 200 valid cells per well and are indicated as the means ± SEM of at least three wells from independent experiments. Flow-cytometry analysis was done in duplicates. Nitrite and PGE₂ detection, enzyme activity assays and mRNA experiments were all performed in triplicates using independent cell preparations. Statistical analysis was performed with GraphPad Prism software (Version 4.03), using t-test or one-way ANOVA with Bonferroni’s post-hoc test, as appropriate.
4. Results

4.1. Characterization of inflammatory responses

Initially, we used a pool of IMA, obtained by selection of the transfected cells by antibiotics, and then expanded the cells by passaging in normal culture medium. After stable growth conditions were reached, such cells were tested for their capacity to react to inflammatory cytokines. Altogether, six cell pools from different transfections were used (IMA 1-6) to assess NF-κB translocation from the cytosol to the nucleus. This signaling response is usually observed after stimulation of astrocytes with various cytokines as well as toll-like receptor (TLR) ligands. Here, it was quantitatively assessed 30 min after stimulation with IL-1β, TNF-α, IFN-γ, or a combination of all three cytokines (complete cytokine mix = CCM). TNF-α or IL-1β stimulation alone was sufficient to evoke maximal NF-κB translocation (Figure 1A). In these experiments, IMA 2 showed the best responses and were used in the future. In order to obtain a homogeneous cell population, IMA 2 were diluted to the single cell level and different clones originating from this procedure were generated and compared. Five IMA 2 subclones (designated IMA 2.1 - IMA 2.5) were investigated with respect to their response to different cytokines. In all subclones, IL-1β, TNF-α or CCM were observed to trigger strong NF-κB translocation to the nucleus. Clone 2.1 showed slightly higher growth rates, and was therefore selected for all further experiments and characterizations, if not otherwise indicated.

Figure 1. Inflammatory response to different cytokines.

(A) IMA 2 were stimulated with IL-1β (10 ng/ml), TNF-α (10 ng/ml), or IFN-γ (20 ng/ml), respectively by a Complete Cytokine Mix (CCM) composed of all three cytokines for 30 min. Cells were then fixed and stained with an anti-NF-κB p65 antibody. Quantitative assessment of NF-κB translocation from the cytosol to the nucleus was performed with an automated microscope (Cellomics array Scan). This method allows detection of NF-κB translocation on the single cell level. Data are displayed as percentage of cells positive for NF-κB translocation compared to all cells detected. (B) IMA 2.1 were stimulated with cytokines as indicated for 16 h; nitric oxide synthase-2 (NOS-2) mRNA was detected as representative for an early immediate gene. Data are expressed as means ± SD of quadruplicate samples. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05.
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The inducible isoform of nitric oxide synthase (NOS-2) was chosen as a representative example for pathophysiologically-relevant astrocyte enzymes. NOS-2 mRNA expression was slightly increased after 16 h of stimulation by TNF-α or IL-1β (Figure 1B). Combination of the two cytokines did not further increase NOS-2 mRNA, and IFN-γ had no effect at all, when given alone (not shown). Combination of the three cytokines (stimulation with CCM) resulted in a two orders of magnitude higher mRNA expression that exceeded by far the sum of TNF-α and IL-1β signal intensities (Figure 1B). A similar pattern was observed for the induction of NOS-2 and the release of nitrite from primary astrocytes (Falsig et al., 2004a; Falsig et al., 2006). This indicates that IMA behaved similarly to murine astrocytes with respect to the cytokine response and in particular to the synergism of TNF-α and IL-1β with IFN-γ.

In a CCM-stimulation time course, NOS-2 mRNA displayed a rapid rise after 4 h, followed by a subsequent decline (Figure 2A). Induction of the mRNA coding for toll-like receptor-2 (TLR-2) was qualitatively similar to the situation observed with NOS-2. However, when compared quantitatively, the induction was far less pronounced compared with NOS-2 (Figure 2B). In contrast to TLR-2, TLR-4 expression was not regulated by CCM stimulation (not shown).

For a more comprehensive comparison of the IMA subclone IMA 2.1 with primary mouse astrocytes, both cell populations were activated with CCM for 4 h and mRNA expression levels of a set of astrocyte–relevant marker genes was measured. The results indicated that the IMA 2.1 response resembles the response observed in primary astrocytes (Figure 2C).

Following the analysis of a set of inflammation-related target genes on the mRNA level, we selected NOS-2 and COX-2 for a more detailed analysis on the protein and activity level. IMA 2.1 were stimulated with CCM for various time intervals, and both NOS-2 and COX-2 protein became detectable after 4 h of CCM stimulation (Figure 3A). Interestingly, NOS-2 mRNA peaked at 4 h, while NOS-2 protein expression displayed a peak after 16 h. Similar observations have been made in other inflammatory cells, such as lipopolysaccharide-stimulated primary rat alveolar macrophages (unpublished Schildknecht).
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Figure 2. Time course and spectrum of inflammatory responses.

IMA were stimulated by CCM for the time intervals indicated. Total mRNA was isolated and served as template for cDNA synthesis and PCR amplification. (A+B) The inducible proinflammatory enzyme nitric oxide synthase-2 (NOS-2) as well as toll-like receptor 2 were determined by real-time PCR. Data are expressed relative to GAPDH expression. (C) Cells were activated by CCM for 4 h, data were normalized to GAPDH and displayed as ΔΔct values relative to the respective mRNA expression in unstimulated cells. COX-2 = cyclooxygenase-2; NOS-2 = nitric oxide synthase-2; TYK1 = inducible thymidylate kinase; TNF-α = tumor necrosis factor α; IL-1β = interleukin-1β; IL-6 = interleukin 6; IFN-β = interferon-β; IL-23 = interleukin-23; MCP-1 = monocyte chemotactic protein 1; TLR 3 = toll-like receptor 3; NOD 2 = nucleotide binding oligomerization domain 2; TLR 2 = toll-like receptor 2. Data are expressed as means ± SD of triplicate samples. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05.

Accumulation of prostaglandin E2 (PGE2) in the supernatant served as indirect indicator of COX activity. Unlike the gradual and continuing increase of nitrite as marker of NOS activity, PGE2 demonstrated an increase up to 16 h after CCM stimulation. From then on, no further elevation was observed, despite a pronounced COX-2 protein expression (Figure 3 A,B). To test whether inducible NOS-2 was alone responsible for the nitrite accumulation, selective inhibitors of NOS-2, AMT and L-NMMA, were applied in different concentrations together with CCM for 16 h. The complete block of nitric oxide synthesis by these compounds suggests that no other NOS-isoform contributed to the observed NO synthesis (Figure 3 C,D).

For a further investigation on the lack of PGE2 synthesis after 16 h of CCM activation, COX activity in homogenates of CCM-treated IMA was directly assessed by the addition of the 14C-labeled substrate arachidonic acid, followed by separation and quantification of the prostanoids formed. In close correlation to the Western-blot data in Figure 3A, a constant
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increase in activity up to 12 h of CCM stimulation was observed, that was followed by a rapid decline in COX activity to almost baseline values (Figure 4A).

Figure 3. Induction of cyclooxygenase-2 and nitric oxide synthase-2.
IMA were activated by CCM (IL-1β, TNF-α, IFN-γ) for the time periods investigated. (A) The amount of cyclooxygenase-2 (COX-2) and nitric oxide synthase-2 (NOS-2) was evaluated on the protein level by Western blot analysis. (B) Prostaglandin E2 (PGE2) as indicator for COX activity and nitrite as indicator for NOS activity were assessed in the respective cell culture supernatants. (C+D) To discriminate between NOS-2-dependent NO formation and NO derived from constitutively expressed NOS proteins, the NOS-2 selective inhibitors AMT or L-NMMA were applied in different concentrations together with a cytokine mix for 16 h, nitrite served as indicator for NOS activity. Data are expressed as means ± SD of quadruplicate samples. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05

Similar observations were made in RAW 264.7 (Schildknecht et al., 2006) and primary rat alveolar macrophages (unpublished), in which a nitration and inactivation of COX-2 was observed. Although not investigated in detail in IMA 2.1 so far, it is likely that a similar mechanism is responsible for the observed effects, since we found that inactivation of COX-2 was prevented by the application of NOS-2 inhibitors (not shown).

We examined TLR-2 protein expression on the surface of CCM-stimulated IMA 2.1 by FACS analysis (Figure 4B) in addition to the mRNA profiling (Figure 2B). The surface expression continuously increased for up to 12 h. The delay between the time course of mRNA and protein surface expression likely originated from a time period required for the
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synthesis and transport of newly formed TLR-2 from the Golgi- to the plasma-membrane. To test the importance of translocation, the secretory vesicle assembly blocker brefeldin A (BFA) was used. Under these conditions, a significant reduction in TLR-2 surface expression was detected. This inhibitory effect was also seen for the basic TLR-2 surface expression of unstimulated cells (Figure 4C). Together, these data indicate that IMA showed inflammatory responses, similar to the ones of primary astrocytes or other inflammatory cells. Moreover, the data suggest the importance of activity measurements and protein quantification, in addition to the mRNA profiling.

Figure 4. Regulation of inflammation-relevant proteins.

(A) IMA were stimulated with CCM for the time intervals indicated, enzymatic activity of COX was directly assessed in cell lysates by the addition of 14C-labeled substrate arachidonic acid. (B) IMA were activated with a cytokine mix as indicated, cells were stained with an anti-TLR-2 antibody and detected by flow cytometry. (C) IMA were stimulated with CCM, brefeldin A (BFA), or a combination of CCM and BFA for 16 h, TLR-2 surface expression was analyzed by flow cytometry. Data are expressed as means ± SD of quadruplicate samples. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05.
4.2. Expression of immature astrocyte markers

For an initial comparison of the marker expression and morphology of primary mouse astrocytes and IMA 2.1, cells were stained with antibodies selective for nestin and CD 44 (Figure 5A). These two proteins with cytoskeletal (nestin) and membraneous (CD44) localization allow the characterization of different morphological features. According to this,

IMA2.1 took the typical star-shape of astrocytes. Both markers were expressed in primary astrocytes and IMA 2.1, but the astrocytic extensions of IMA 2.1 were slightly smaller

Figure 5. Phenotypic and functional characteristics of IMA.

(A) IMA or primary murine astrocytes were fixed, permeabilized, and stained with antibodies against the cell surface glycoprotein CD 44 and the intermediate filament protein nestin (green). Nuclei were labelled with H-33342 (red). (B) Alternatively, fluorescence-conjugated phalloidin, selectively binding to F-actin, was used for visualization of general cell morphology of IMA 2.1 and primary astrocytes. Additionally, living IMA 2.1 were labelled with calcein-AM (1 µM; 30 min), the same region was also photographed in the phase contrast mode. (C) IMA were seeded at a density of 10,000 cells/cm² (2 % FCS), respectively 5,000 cells/cm² (5 % and 10 % FCS) in the presence of the various serum concentrations (2 %, 5 %, 10 %). For each serum concentration, variable glutamine/glutamate levels were added, cells were allowed to grow for a total of 10 days and every 12 h, the number of cells was determined manually. The doubling time was calculated from the growth curves. Doubling time for cells grown with 2 % serum and 0.1 mM glutamine could not be determined as cells did not tolerate these growth conditions. The value was set to 270 h for graphical representation. Data are expressed as means ± SD of quadruplicate samples. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05.
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compared with primary astrocytes. Both, nestin and CD44 are not considered typical astrocyte markers (Kuegler et al., 2012; Kuegler et al., 2010), but are rather typical for immature precursors. However, they are often found in primary neonatal astrocyte cultures (which are commonly used for many experiments). The strong expression in IMA suggests their rather early-stage astrocyte precursor phenotype. For the assessment of general cell morphology, IMA 2.1, respectively primary astrocytes, were stained with fluorescent phalloidin that selectively binds to F-actin. Confluent IMA 2.1 were additionally stained by calcein-AM (1 µM; 30 min), the same region was photographed in the phase contrast mode (Figure 5 B). In order to test the functionality of the mature astrocyte marker glutamine synthase in IMA 2.1, cells were maintained under different culture conditions. In the presence of 2 mM glutamine, astrocytes had optimal growth conditions and they proliferated largely independent of the fetal calf serum (FCS) concentrations in the culture. At lower glutamine concentrations, IMA increased their doubling time and became more dependent on high concentrations of FCS. Addition of glutamate compensated for the lack of glutamine supply, at least at high FCS concentrations. These data indicate that IMA were capable of enzymatic conversion of glutamate to glutamine is a typical function of astrocytes in the brain.

For further characterization of the enzymes involved in the glutamine/glutamate uptake and synthesis as well as other glial-related targets, mRNA expression was directly compared between primary mouse astrocytes, IMA 2.1, and the astrocyte-unrelated, murine control cell types 3T3 or BV-2. In the set of markers selected, expression in primary astrocytes was in general much higher, compared with IMA 2.1 (Figure 6A,B). In most cases, the expression in IMA was similar to the one of a non-astrocytic cell (3T3-fibroblasts or BV-2 microglia) (Henn et al., 2009). The RNA data were confirmed also immunocytochemically, e.g. by an absence of staining for glial fibrillary acidic protein (GFAP). Four notable exceptions were found: aquaporin 4, a typical astrocyte marker was expressed 200-fold higher in IMA than in 3T3. Nestin was expressed in IMA to a similar extent as in primary astrocytes, which is consistent with immunostaining results. S100β and Cnx43, typical markers expressed early in astrogenesis, had an elevated expression relative to non-astrocytic markers. In summary, these data suggest that IMA resemble the GFAP-negative astrocytes that can be derived from stem cells (Kuegler et al., 2012) and are at a relatively immature stage. We also examined other IMA 2 clones for mRNA expression. IMA 2.3 showed a slightly higher marker expression, but they were also negative for GFAP. Therefore, experiments were continued with IMA 2.1
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Figure 6. Comparison of mRNA expression in IMA, primary mouse astrocytes, and non-astrocytic cells.

Total mRNA was extracted as template for reverse transcription and PCR amplification. Data are shown relative to GAPDH expression in the same well. In (A), 3T3 cells served as non-astrocytic reference, in (B) BV-2 was used as reference. GS= glutamine synthetase; GLT-1 = glutamate transporter 1; GLAST = glutamate-aspartate transporter; Aqp 4 = aquaporin 4; MAO-B = monoamine oxidase B; GFAP = glial fibrillary acidic protein; Cnx43 = connexin 43; Kir 4.1 = inwardly rectifying K+ channel. Data are means ± SEM of three different cultures.

4.3. Metabolic capacity of IMA 2.1

In addition to the descriptive phenotype markers, we also examined the actual metabolic competence of IMA. MAO-B activity was chosen because of its prime importance in neurotoxicology. IMA 2.1 were compared with HEK293, HeLa, the neuronal cell line LUHMES and SH-SY5Y neuroblastoma cells with respect to MAO activity. Addition of the MAO-substrate kynuramine to homogenized cells indicated a significantly higher MAO activity in IMA compared with the other cell types investigated (Figure 7A). Thus, as in the case of NOS-2 and TLR-2, the protein data did not correlate with the mRNA data. For a potential application of IMA 2.1 in coculture models together with dopaminergic neurons in the field of Parkinson’s disease research, conversion of the parkinsonian toxin MPTP into the dopaminergic neurotoxin MPP+ (Figure 7B) was investigated in IMA 2.1.

Addition of MPTP to IMA 2.1 led to a time-dependent conversion into its active metabolite MPP+. The reaction intermediate MPDP+ was also detectable in the cell culture supernatant (Figure 7C).
B. Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocytes functions

Figure 7. Monoamine oxidase activity and MPTP metabolism.

(A) For a direct comparison of monoamine oxidase (MAO) activity, homogenates (1 mg/ml protein) of IMA 2.1, the neuronal cell lines LUHMES and SH-SY5Y, or HeLa and HEK 293 were incubated with the MAO substrate kynuramine (100 µM) for 2 h. The reaction product hydroxychinoline was measured fluorometrically and the resulting concentrations were plotted. (B) Overview on the structures of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the reaction intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) and the active toxin 1-methyl-4-phenylpyridinium (MPP⁺). (C +D) MPTP (20 µM) was added to IMA 2.1 or to primary mouse astrocyte cultures. At the time points indicated, supernatant was collected and MPTP, MPDP⁺, and MPP⁺ were measured by HPLC. (E) To investigate which MAO isoform accounts for the observed activity, MPTP (20 µM) was added together with the MAO-A selective inhibitor moclobemide, the MAO-B selective inhibitor (-)-deprenyl, or the isoform-unselective inhibitor pargyline. After 24 h, MPP⁺ was evaluated in the supernatant by HPLC. (F) For a comparison on the impact of inflammation on MAO-B activity, IMA were stimulated with CCM for 6 h. Then, MPTP (20 µM) was added, and MPP⁺ was detected by HPLC in the supernatant after the time periods indicated. Data are expressed as means ± SD of quadruplicate samples.

The same experiment was then run with primary mouse astrocytes that displayed a similar MPTP conversion rate compared with IMA 2.1 (Figure 7D). To identify the MAO-isoform responsible for the observed MPTP conversion by IMA 2.1, cells were pretreated with the MAO-A-selective inhibitor moclobemide, the MAO-B-selective inhibitor (-)-deprenyl, or the isoform-unselective inhibitor pargyline for 30 min before MPTP was added. These experiments indicated an almost exclusive contribution of MAO-B to the observed conversion of MPTP (Figure 7E). For a direct comparison of the influence of proinflammatory conditions
B. Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocytes functions

on MAO-B activity, IMA 2.1 were pre-treated with CCM for 6 h for full activation. Then, MPTP was added as MAO-B substrate; MPP⁺ was detected as readout in the supernatant and revealed no significant difference between the control and the CCM group (Figure 7F). These findings correlate well with a large body of literature, examining MPTP in the in vivo mouse model.

4.4. Compatibility with neuronal co-cultures

Finally, we tested whether IMA 2.1 can be used in a coculture model together with the human dopaminergic neuronal cell line LUHMES. For direct comparison, primary astrocytes or IMA 2.1 were grown until confluency was reached. Then, predifferentiated LUHMES were added and differentiated for additional four days. The morphology of LUHMES was analyzed by staining of the neuronal marker protein β-III-tubulin. As illustrated in Figure 8, differentiated LUHMES grown alone, or on top of either primary astrocytes or IMA 2.1 displayed similar neurite structures and cell morphology. These observations indicate that IMA 2.1 can be applied in coculture models either in direct contact with neurons as illustrated herein, or in a transwell coculture system, where both cell types are spatially separated.
Figure 8. Application of IMA in co-culture models.

Neuronal LUHMES cells were either differentiated alone (control), or on top of confluent layers of IMA 2.1, or primary rat glia cells. For a phenotypic assessment, LUHMES were stained for neuron-specific β-III-tubulin (green), nuclei were stained with Hoechst H-33342 (red).
5. Discussion

In the present manuscript, we characterized the mouse cell line IMA with respect to its potential as substitute for primary astrocyte cultures. Particular emphasis was put on IMA’s response to pro-inflammatory stimuli and their ability to serve as model for the conversion of the parkinsonian toxicant MPTP. In the field of PD research, *in vitro* models are desired that recapitulate these two events. The question arises, how a new astrocyte cell line can contribute to the promotion of the 3Rs concept (refinement, reduction, replacement) (Leist *et al.*, 2008). In an aging population, the incidence of chronic neurodegenerative diseases such as PD or AD is constantly increasing. This results in intensified basic and clinical research in this field, as evidenced by the higher use of experimental animals for basic research, and in particular, the widespread generation and use of new transgenic animal models (Leist *et al.*, 2008).

The standard method to obtain astrocytes is the establishment of primary cell cultures by isolation of cells from brain tissue of newborn animals. This costly and time-consuming procedure represents a clear logistic bottleneck in molecular mechanistic research on astrocyte function. Hence, replacement of primary astrocytes by an adequate cell line has a high potential to advance our knowledge about astrocyte biology and, at the same time, contributes to reduce the number of experimental animals. For successful replacement, new *in vitro* models however require the acceptance by the scientific community. Previous astrocyte cell lines failed to become widely distributed, and they failed to be adopted by the research community.

Primary cultures of astrocytes are undoubtedly closer as any immortalized cell line to an *in vivo* situation; however primary cells have, aside from limited availability, clear cut limitations, too. For example, contamination with microglia can significantly affect the outcome of an investigation (Saura, 2007). It is also important to realize that brain region-dependent differences in astrocytic phenotype exist that can have profound impact on the observations made. Moreover, it has been clearly demonstrated that the used cultures of neonatal astrocytes differ vastly from adult astrocytes in the brain (Zhang *et al.*, 2010b).

In the present study, we evaluated the mouse astrocyte cell line IMA with respect to its applicability in inflammation studies and for its suitability as parkinsonian MPTP conversion model. In direct comparison to primary astrocytes, IMA are easy to handle, do not require laborious isolation methods that usually lead to batch-to-batch variations, and were stable in
B. Characterization of the mouse cell line IMA 2.1 as potential model system to study astrocytes functions

their phenotype over years, which is a prerequisite for the reproducibility of results and inter-laboratory comparability. This aspect also opens the field for potential applications in validation assays. We also observed that in contrast to other cell types, genetic overexpression by transfection or electroporation, respectively the siRNA-mediated knockdown of targets of interest can be performed in IMA (not shown).

The suitability of IMA as inflammation model was assessed by the detection of NF-κB translocation into the nucleus, which represents one of the key events involved in the induction of so-called immediate early genes. The detection method by an automated microscope system on the single cell level showed that all IMA reacted simultaneously to different cytokines. Such a homogenous cell population is of great advantage for many biochemical and signal transduction studies. For a general overview of the inflammatory competence, we have chosen changes of mRNA levels as readout, as suggested previously (Kuegler et al., 2010). However, characterization based on transcript levels alone represented only a first overview. Further information on the actual expression, localization, and activity of a protein or enzyme was therefore presented here for NOS-2, MAO-B, or TLR-2, as examples.

The low GFAP mRNA expression, and the absence of GFAP staining (not shown) in IMA might raise concerns about the astrocytic origin of the cells. However, the original cell pool stained uniformly positive for GFAP directly after transduction. During the four passages thereafter, the expression of GFAP was lost, but the cells maintained the same morphologic features. It has previously been reported that GFAP expression depends highly on the proliferation status of cells. Accordingly, GFAP was reported to be downregulated in a proliferating astrocyte cell line. This might explain the low abundance observed in IMA. It is also becoming increasingly clear, that a large subpopulation of astrocytes does not express GFAP, also under in vivo conditions. Confluent cells kept for more than a week displayed elevated levels of GFAP that were however still significantly lower compared with the expression in primary astrocytes. Further support for genuine astrocyte functions of the established cell line IMA stems from functional expression of glutamine synthetase, as well as specific uptake of glutamate, both typical features of astrocytes. These observations suggest a potential application of IMA in coculture models in the field of glutamate / EAAT-toxicity research.
For potential applications in parkinsonian MPTP models, we compared the MAO-dependent conversion of MPTP by IMA and primary mouse glial cells that indicated a comparable MAO-B activity in IMA when adjusted to equal total protein content. The IMA cell line therefore represents an easily accessible platform for mechanistic studies on MPTP conversion.

In order to closer simulate the situation in the brain, we then tested whether IMA can form cocultures with the human dopaminergic cell line LUHMES. The LUHMES cell line that can be differentiated into post-mitotic neurons with a dopaminergic phenotype is very sensitive to culture conditions. Therefore, the successful cocultivation was initially surprising, and these data indicate that IMA 2.1 can most likely also be used in combination with other neuronal cell types (Stiegler et al., 2011; Zimmer et al., 2011b). The rationale for using a human neuronal cell line is based on the intended use of such co-cultures for human disease modelling. The relevant target cells for most diseases are neurons. To our current knowledge, LUHMES cells represent one of the most favourable in vitro models of dopaminergic neurons. We recently characterized this cell line (Schildknecht et al., 2009; Scholz et al., 2011) extensively, and we believe that no mouse cell line with similar features is available at present. Vice versa, the use of human astrocytes is much less established than the one of murine astrocytes, and the cells are less accessible. Notably, the combination of cells from different species does not result in histoincompatibility reactions, as long as no T cells are present. Moreover, most metabolites that are exchanged, in addition to most neurotransmitters, lipid mediators, growth factors and xenobiotic metabolites relevant to the co-culture, are not species-specific. Therefore, combining cell types of different species created no difficulties. We are aware of the disadvantages of the two-species system for the study of certain immune mediators (e.g. IFN-γ) that are species-specific. On the other hand, two species systems also have enormous advantages, as certain cytokines derived from one of the cell types may be blocked selectively, without influence on the same cytokine from the other cell type (e.g. human or murine CCL-5), and especially the regulation of transcripts can be followed for each cell type selectively by choosing appropriate PCR primers. Such advantages can be very important for mechanistic studies of reciprocal cell-type interactions. In preliminary results (not shown), we observed that MPTP addition to the coculture model of IMA and LUHMES leads to a time-dependent conversion into the active toxin MPP⁺ that subsequently is taken up by LUHMES cells and leads to a selective neurite degeneration and
ultimately cell death while viability of IMA, in which the conversion takes place, is not affected. These findings however require further investigations and confirmation.

The suitability of IMA for co-culture models in combination with neurons opens a promising perspective for a new *in vitro* system to study cellular interactions that may be relevant for disease. For instance, mechanistic and kinetic aspects of the toxicity of the Parkinsonism-inducing toxicant MPTP may be studied, which is not possible in pure neuronal cultures.

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Chapter C

Prevention of human dopaminergic neurodegeneration in an astrocytes co-culture system allowing endogenous drug metabolism

Liudmila Efremova¹,², Stefan Schildknecht¹, Martina Adam¹, Regina Pape¹, Simon Gutbier¹,², Benjamin Hanf²,³, Alexander Bürkle³ and Marcel Leist¹

Affiliations:
¹Doerenkamp-Zbinden Chair for In vitro Toxicology and Biomedicine, University of Konstanz, PO Box M657; D-78457 Konstanz, Germany;
²Research Training Group 1331 (RTG1331), University of Konstanz; ³Molecular Toxicology Group, University of Konstanz, Germany

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1. Abstract

BACKGROUND: Few neuropharmacological model systems are based on human neurons. Moreover, available test systems rarely reflect functional roles of co-cultured glial cells. The generation of a human in vitro counterpart of the widely-used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model has therefore remained a challenge in drug discovery technology.

EXPERIMENTAL APPROACH: We addressed this by successfully growing an intricate network of human dopaminergic (DA) neurons on top of a dense layer of astrocytes. In these co-cultures, MPTP was metabolized to 1-methyl-4-phenyl-pyridinium (MPP\(^+\)) by the glial cells, and the toxic metabolite was taken up through the dopamine transporter into neurons.

KEY RESULTS: For initial model characterization, we studied the activation of poly-(ADP-ribose)-polymerase (PARP). Similar to mouse models, MPTP exposure lead to (poly-ADP-ribose) synthesis, and neurodegeneration was blocked by PARP inhibitors. A panel of different putative neuroprotectants was then compared in monocultures and co-cultures. Rho kinase inhibitors worked in both models; CEP1347, ascorbic acid or a caspase inhibitor protected in monocultures from MPP\(^+\) toxicity, but did not show any protection in co-cultures, when used alone or in combinations. Application of oxidized glutathione (GSSG) prevented degeneration in co-cultures, but not in monocultures. The surprisingly different pharmacological profiles of the models suggest that the presence of glial cells, and the in vivo-like generation of the toxic metabolite MPP\(^+\) within the layered cultures played an important role for predictions of neuroprotection.

CONCLUSIONS: The novel model system is closer to the situation in human brain tissue than conventional cultures, and its use for screening of candidate neuroprotectants may increase the predictiveness of a test battery.

ABBREVIATIONS: CEP1347,(3,9-bis[(ethylthio)methyl]-K-252a); DA, dopaminergic; DAT, dopamine transporter; DHQ, 1,5-isoquinolinediol; DM, differentiation medium; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); GDNF, glial derived neurotrophic factor; GSSG, L-glutathione oxidized; IMA, immortalised mouse astrocytes; LDH, lactate dehydrogenase; MAO, monoamine oxidase; MPP\(^+\), 1-methyl-4-phenyl-pyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PARP, poly-(ADP-ribose)-polymerase; PD, Parkinson’s disease; PLO, poly-L-ornithine; ROCK, Rho kinase.
2. Introduction

Degeneration of dopaminergic (DA) neurons plays an important role in Parkinson’s disease (PD), and therefore, there is a great need for new models that allow testing of potential neuroprotective drugs (Schapira et al., 2005; Schule et al., 2009; Youdim, 2010). While some forms of PD have a genetic background, such as mutations of alpha-synuclein, about 90% of disease cases have an unclear aetiology. Some of these may be caused or promoted by toxicants such as manganese, rotenone or dieldrin (Caudle et al., 2012; Pan-Montojo et al., 2012). Several neuroprotective drugs have been developed so far, but none of the pharmacological agents has yet been shown convincingly to slow the progression of PD (Meissner et al., 2011). One major hindrance for the development of novel neuroprotective strategies and the discovery of early disease biomarkers is the limited availability of human cell-based neuronal models. Moreover, most of the available ones are lacking active neuron-glia interactions (Schule et al., 2009), although astrocytes are increasingly recognized for their effects on neurons. For instance, they may regulate neurite outgrowth not only during fetal development but also during regeneration from damage (Giordano et al., 2012).

The best studied chemical that reproducibly triggers DA neuron degeneration is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP). This compound triggers toxicity in the human nigrostriatal pathway with high specificity (Langston et al., 1984b). MPTP itself does not adversely affect neuronal cell cultures. The toxicity requires metabolic transformation to the ultimate toxicant MPP⁺. Conversion of MPTP to its toxic metabolite requires monoamine oxidase (MAO) activity, which is mainly found in astrocytes within the brain (Schulz et al., 1995). MPP⁺ then is taken up by DA neurons via their dopamine transporter.

Thus, the metabolism, distribution and neurotoxicity require complex neuron-glia interactions (Dauer et al., 2003). In most cases, MPTP toxicity is modeled in vitro by direct application of the active metabolite MPP⁺ to cells (Leist et al., 1998; Peng et al., 2013; Poltl et al., 2012; Sun et al., 2013; Zeng et al., 2006). This approach neglects the influence of glia cells, and necessarily alters the concentration-time profile of MPP⁺ exposure.

Many potentially neuroprotective drugs showed robust effects in preclinical studies but failed to be effective in clinical trials (Olanow et al., 2009). A typical example is the mixed lineage kinase inhibitor CEP-1347. The compound was protective in various rodent models and in vitro test systems, including human neurons exposed to MPP⁺ (Falsig et al., 2004b; Maroney et al., 1999; Schildknecht et al., 2009) but it failed in clinical studies due to a lack of
efficacy (Parkinson Study Group, 2007; Yacoubian et al., 2009). There have been several attempts to improve cell culture-based test systems for clinical predictivity by a transition from rodent to human cells, and by incorporation of glial cells to neuronal cultures (Liu et al., 2009; McNaught et al., 1999). For co-culture systems, the major focus has been on microglia as the second cell type besides neurons (McGeer et al., 2008), but astrocytes have been used, e.g. with motor neurons or in order to stabilize stem cell-derived neurons (Reinhardt et al., 2013; Zhang et al., 2013). Concerning the transition to human cells, often tumor cell lines have been used as surrogate for neurons (Chong et al., 2014; Zhao et al., 2013). Only recently, the broader availability of stem cells has facilitated the generation of human neuronal cultures for the study of neurodegenerative disease (Morizane et al., 2010; Perrier et al., 2004; Schulz et al., 2004). As the precise culture composition is difficult to control, when cells are directly generated from pluripotent stem cells, a start from intermediate stages (neural stem cells or committed precursor cells) is frequently used in drug screening and pharmacology (Reinhardt et al., 2013; Scholz et al., 2011).

In a related approach, we took advantage of LUHMES cells. These are conditionally-immortalized mesencephalic neuronal precursors that can be differentiated to fully postmitotic DA neurons (Lotharius et al., 2005; Scholz et al., 2011). These were combined with immortalized murine astrocytes that have been extensively characterized for their metabolic capacity and inflammatory competence (Schildknecht et al., 2012).

This new co-culture system allowed us to model MPTP metabolism in vitro and to reveal differences in pharmacological activities of mechanistically diverse neuroprotective experimental compounds in comparison with neuronal monocultures exposed to MPP+. Our data demonstrate that neurons grown in co-culture with glia show a radically different pharmacological behaviour than neuronal monocultures. Co-cultures could provide a valuable additional model in pharmacological and toxicological studies.
3. Materials and methods

Cell culture

LUHMES cells are ventral mesencephalic precursor cells from a human female fetus that have been conditionally immortalized by v-myc under the control (tet-off) of tetracycline. Inactivation of the transgene and addition of differentiation factors allows a rapid and synchronized conversion of fully post mitotic neurons with neurites of > 1000 µm length and expression of typical dopaminergic markers. Cells were cultured as described in detail earlier. (Krug et al., 2014; Schildknecht et al., 2013; Scholz et al., 2011).

Immortalised IMA 2.1 mouse astrocytes (IMA) were generated and used as previously described in detail (Schildknecht et al., 2012). Primary cortical astrocytes were generated from Wistar rats bred at the animal facility of the University of Konstanz, Germany. Primary glia cells were prepared from rat pups at 24 h post partum as described earlier (Falsig et al., 2004a).

For co-culture with LUHMES neurons, IMA or mixed primary glial cells were seeded in multi-well plates (BD Falcon, Bedford, MA) in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin at a density 15,000/cm². After 24 h, LUHMES differentiation medium was added for another 2 days. Then pre-differentiated (day 2) LUHMES cells were seeded on top of astrocytes at a density of 300,000 cells per well (24 well plates) and were further differentiated for another 4 days in LUHMES differentiation medium. The density of IMA on day 6 of the culture was 195,000/cm².

Cell viability assays

The resazurin assay was performed as described (Falsig et al., 2004a; Schildknecht et al., 2009; Zimmer et al., 2011a). Lactate dehydrogenase (LDH) activity was detected in cell culture medium (supernatant) and cell lysates separately. LDH release was expressed as 100 x LDH(supernatant)/LDH(supernatant) +LDH (lysate). For the detection of ATP levels, a commercially available ATP assay reaction mix (Sigma-Aldrich), containing luciferin und luciferase, was used (Volbracht et al., 1999). As main endpoint to quantify neuronal viability within co-cultures, we used an imaging-based quantification of the neuronal area, essentially as described earlier (Krug et al., 2013; Lotharius et al., 2005; Schildknecht et al., 2009). The method uses an automated microscope that automatically records images from many different fields, and uses an established algorithm to determine the respective neuronal areas of the
fields. An image-supported illustration of the method (see in Figure S3) and a cross-validation of the method with manual counting of cell somata (see in Figure S5) have been provided in the supplemental material.

**GSH assay**

Cell were washed with PBS and lysed in 250 µl of 1% sulfosalicylic acid (w/v) for 3 min. Following sonication and centrifugation at 12000 x g at 4°C for the removal of cell debris, supernatant was collected. Total glutathione content was determined by a DTNB (5,5’-Dithiobis(2-nitrobenzoic acid)) reduction assay. Supernatants were diluted 1:5 in H2O, 100 µl of sample was mixed with 100 µl assay mixture containing 300 µM DTNB, 1 U/ml glutathione-reductase, 400 µM NADPH, 1 mM EDTA in 100 mM sodium phosphate buffer, pH 7.5 (all Sigma). DTNB reduction was measured photometrically over time at 405 nm (Schildknecht et al., 2009).

**NAD⁺ measurement**

Cellular NAD⁺ content was determined as described before. In brief, cells were lysed with 0.5 M perchloric acid on ice. After 15 min, samples were centrifuged at 1500×g for 10 min at 4°C. The supernatant (500 µl) was combined with 350 µl of 1 M KOH, 0.33 M K2HPO4, 0.33 M KH2PO4 and NAD⁺ was determined by using an enzymatic cycling assay (Weidele et al., 2010).

**Quantitative image analysis**

Following treatment, cells were stained for the neuronal marker betaIII-tubulin (using the monoclonal antibody Tuj1) or for PAR. An Array-Scan VTI HCS Reader (Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera was used for image acquisition. Thirty fields per well (330 µm × 330 µm size each) were imaged in one channel using a 20 × objective. Image sets were analysed using the Thermo Scientific Cellomics Spot Detector Bioapplication V4 as described earlier (Krug et al., 2013). For each data point, 30 images were captured automatically and analysed by the software, as demonstrated in Figure S3.

**siRNA mediated knock-down of PARP1**

LUHMES were differentiated for 2 days according to the standard protocol. For transfection, solution A, consisting of 7.5 µl of Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) and 150 µl Opti-MEM I (Gibco) and solution B, consisting of 120 pmoles of the respective siRNA and 150 µl Opti-MEM I, were mixed and incubated for 5 min
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at room temperature. Following incubation, both solutions were combined, transferred into 6-well plates and incubated for 20 min. The amounts indicated refer to one well of a 6-well plate. Next, predifferentiated and trypsinised LUHMES cells were added in a volume of 1.7 ml differentiation medium to the siRNA containing solution at a density of 1.5×10^6 cell/well (6-well plate) and thoroughly mixed. After additional three days, cells were harvested for western blot analysis and for toxicological experiments. siRNA oligonucleotides were obtained from Sigma targeting the 3′UTR of PARP1: AACGTGTTAAAGGTTTTCTCTAA, or a randomized control sequence of the PARP1 target sequence, termed PARP1 scrambled AATCCGATGTTATTCGTGTATAA.

Statistics

All data were confirmed in at least two (usually three) different experiments. Values are expressed as the mean ± SD (n≥3) (unless otherwise stated). Data were analysed by one-way ANOVA, two-way ANOVA or Student’s t-test as appropriate. Differences between treatment groups in multiple comparisons were determined by Bonferroni’s or Dunnet’s post hoc test (Graph Pad software, San Diego, CA, USA).
4. Results

4.1. Establishment of the co-culture model

For monocultures of LUHMES or IMA cells (Figure 1A), usually different types of media are being used. In particular, the full differentiation of LUHMES into mature, post-mitotic DA neurons requires the presence of the differentiation factors dibutyryl-cAMP and glial derived neurotrophic factor (GDNF) in a strictly defined growth medium. Pilot experiments showed that astrocytes can be maintained under these medium conditions without significant influence on their function and morphology. For the construction of co-cultures with precisely defined cell ratios, LUHMES cells were pre-differentiated for two days to arrest their cell cycle, before they were plated onto a dense layer of IMA in LUHMES differentiation medium (DM). Then, the cells were allowed to differentiate further to neurons with an extended neurite network during the following four days. Co-cultures had an astrocytes:neuron ratio of 1.3 and were used at this stage for treatment with MPTP and pharmacological agents (Figure 1B). Cell type-specific immunostaining with anti-β-III-tubulin (LUHMES) or with anti-S100β (IMA) antibodies allowed a clear identification of both cell types, and showed that LUHMES cells were mainly layered on top of the astrocytes. Characterization of the culture morphology and composition across many separate experiments indicated that LUHMES can be reproducibly differentiated in the presence of IMA astrocytes to a phenotype similar to that obtained by differentiation of LUHMES monocultures (Figure 1C). The co-cultured neurons contained more tyrosine hydroxilase (TH) (Figure 1D), and had a higher dopamine content (Figure S1A). Immunostaining showed that they express TH, the dopamine transporter (DAT) and the vesicular monoamine transporter (VMAT) (Figure 1E, Figure S1B).

4.2. MPTP metabolism and toxicity in co-cultures

MPTP is a non-toxic compound and it leaves LUHMES cells unharmed (Schildknecht et al., 2009), while its metabolite MPP⁺ is very toxic for dopaminergic cells. We examined, whether the co-culture model recapitulated the in vivo conversion of MPTP and subsequent neurotoxicity. We observed a time-dependent decrease of MPTP levels in the supernatant that was accompanied by the formation of MPP⁺. The metabolism was completely blocked by the MAO-B inhibitor deprenyl (Figure 2A). In order to define which of the cells in the co-culture were responsible for the conversion, IMA and LUHMES monocultures were exposed to...
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Figure 1. Schematic representation and phenotypic characterisation of neuron-astrocyte co-cultures.

(A) Fully differentiated LUHMES (neurons) were stained after 6 days (d6) for the neuronal-specific marker Tuj1 (β-III-tubulin) (orange). IMA 2.1 (astrocytes) were stained for the astrocyte-specific marker S100β (green). (B) Experimental procedure for generation and application of co-cultures using neurons and astrocytes. IMA were seeded in culture dishes and grown until confluence was reached. Then, LUHMES differentiation medium was added to the IMA mono-culture for 2 days. In parallel, LUHMES in mono-culture were pre-differentiated for 2 days, trypsinized, seeded on top of the IMA cultures and differentiated for additional 4 days to yield a total LUHMES differentiation period of 6 days. (C) Co-cultures of neurons and astrocytes were generated as described in (B), and phenotypic assessment was performed on d6 of LUHMES differentiation, by immunostaining for Tuj1 (orange) and S100β (green). Nuclei were stained with H-33342 (blue). (D) Cell lysates from monoculture of fully differentiated LUHMES (mono), IMA monocultures (IMA) and co-cultures (Co) were analyzed by Western blot for TH protein. The detected band was 60 kDa. The neuronal marker Tuj was analyzed as loading control. (E) LUHMES cells differentiated in monocultures and co-cultures were stained on day 6 (d6) for tyrosine hydroxylase (TH) (green). Nuclei were stained with H-33342 (red). Scale bar = 20 μm.

MPTP (30 μM) and the amount of remaining MPTP or formed metabolites (MPDP⁺ and MPP⁺) was detected by HPLC. IMA showed similar MPTP conversion kinetics as co-cultures, while LUHMES had no metabolizing capacity. Data obtained with the IMA cell line were fully confirmed with primary astrocytes. Thus, we assumed that all metabolic conversion depended on the added astrocytes (Figure S2).
When we examined intracellular levels of MPTP and its metabolite MPP⁺ in co-cultures, it became evident, that the MPP⁺ concentration reached saturation levels after at most 24 h. MPTP concentrations of 60-100 µM did not produce higher intracellular MPP⁺ levels than 30 µM MPTP, but they resulted in a higher release of extracellular MPP⁺ and a higher passive diffusion of MPTP into the cells (Figure 2A, B). In this context, it is important to note that MPP⁺ did not accumulate at all in IMA monocultures, so that it is reasonable to assume that all cellular MPP⁺ measured in co-cultures was contained in LUHMES cells. This intracellular MPP⁺ amount was well within the range required to evoke neurotoxicity in MPP⁺ exposed LUHMES monocultures (Schildknecht et al., 2009). We concluded that conversion of MPTP by IMA astrocytes is sufficient to trigger degeneration of co-cultured LUHMES cells.

Figure 2. MPTP conversion in co-culture of LUHMES and IMA.

(A) Co-cultures were treated with 30 µM MPTP in the presence or absence of the MAO-B inhibitor deprenyl (10 µM). After the time periods indicated, extracellular and intracellular MPTP and MPP⁺ contents were measured by HPLC. Intracellular concentrations are expressed as the amount of compound per number of neurons. Parallel experiments showed that intracellular levels of MPP⁺ in IMA monocultures were always under the detection limit. (B) Different concentrations of MPTP were added to co-cultures. The amounts of extracellular and intracellular MPTP and MPP⁺ were measured by HPLC after two and four days. Data are expressed as means ± SD of quadruplicates.

For specific detection of neurodegeneration in the mixed co-culture model, cells were stained for the neuronal protein β-III-tubulin. Then, the overall neuronal area was recognized
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and quantified by an automated and unbiased imaging algorithm on a high content imaging system. Treatment of co-cultures with MPTP for 5 days led to a pronounced loss of neurons (Figure S3A). This loss was reproducibly quantifiable with a small relative error. Toxicity was concentration-dependent in the range of 0 - 50 µM MPTP and then reached a saturation level of about 50 % (Figure S3B, C). The data from IMA-LUHMES co-cultures were essentially confirmed in co-cultures of primary rat astrocytes and LUHMES (Figure S4). Moreover, we counted surviving cells after staining of the culture for the neuronal nuclear marker NeuN. As we observed that neurite-degeneration preceded the loss of cell somata, quantification of cell counts was done 24 h after the quantification of the neuronal area. Under these conditions, the overall neuronal area correlated well with a manual count of remaining neuronal cell bodies (Figure S5). Thus, the addition of astrocytes to human DA neurons allowed the examination of MPTP neurotoxicity.

As final control, we examined whether MPTP or its metabolites affected cells. They were characterized for different functional and viability parameters. Resazurin reduction, intracellular ATP and GSH levels remained at control levels. Moreover, no morphological changes were observed (Figure S6A,B). We also observed no inflammatory activation when IMA were directly challenged, but they upregulated e.g. IL-6, when exposed to debris from dead neurons (Figure S6 C,D).

An additional finding of these experiments was that the presence of astrocytes affected neurons not only by their xenobiotic metabolism. The toxicity of MPTP co-cultures was consistently less pronounced than MPP⁺ toxicity in monocultures, also when higher MPTP concentrations were applied. The difference was not due to obviously altered neuronal morphology or numbers, and the amount of betaIII tubulin per culture well was very similar in both models. Therefore, we expected some biochemical changes, and more detailed characterization experiments showed indeed that co-cultured neurons took up less MPP⁺ than the same cells in monoculture (Figure S7). In subsequent experiments we examined whether the differences in the culture systems also resulted in pharmacological differences when potentially neuroprotective agents were tested.
4.3. Involvement of PARP activation in the LUHMES/MPP⁺ model.

The damaging mechanism and pharmacological target process most consistently found in rodents exposed to MPTP, and also other Parkinson’s disease models, is the activation of poly-(ADP-ribose) polymerase (PARP) (Lee et al., 2014b; Lee et al., 2013; Mandir et al., 1999). The resulting formation of poly-(ADP ribose) (PAR) can be readily measured on a single cell level. We examined here first whether PARP played also a role in human LUHMES neurons exposed to MPP⁺. In monocultures exposed to the toxicant, we observed formation of PAR in the nucleus of individual neurons, and this was prevented by the PARP inhibitor DHQ (Figure 4A). The drug also prevented neuronal death, as assessed by the resazurin reduction assay (Figure 4B), and Western blot analysis showed that protein
PARylation was efficiently reduced (Figure 4C). The prevention of cell death by DHQ correlated well with preservation of cellular ATP and NAD$^+$ levels (Figure S8).

Figure 4. Protection from MPP$^+$ toxicity in LUHMES monoculture by PARP1 inhibition.

(A) LUHMES (d6) were treated with 5 µM MPP$^+$ alone or in combination with the PARP inhibitor 1,5-isoquinolinediol (DHQ; 50 µM). After 24 h, cells were fixed and immunostained for poly-ADP ribose (PAR), nuclei were counterstained with H-33342. (B) LUHMES were pre-differentiated for 2 days and re-plated in the presence of 120 pmoles siRNA for additional 3 days. A siRNA against PARP1 and a scrambled siRNA (control) were used. On day 5 of differentiation, cells were treated with 5 µM MPP$^+$ or solvent for 3 additional days. The PARP inhibitor 1,5-isoquinolinediol (DHQ; 50 µM) was used as a positive control. Viability was assessed by the resazurin reduction assay. (C) LUHMES (d6) were treated with 5 µM MPP$^+$ alone or in combination with 50 µM DHQ. After 24 h, cell lysates were analyzed by Western blot for proteins modified by PAR. Detection was in the molecular weight range of 250-400 kDa. (D) LUHMES cells were exposed to siRNA (as in B) on day 2 of differentiation and incubated for additional 72 h. Then, cell lysates were analyzed by Western blot on day 5 of LUHMES differentiation for the PARP1 protein content. Data are means ± SD of quadruplicates.

We probed the role of PARP also by using a siRNA selective for PARP-1. The reduction of cell death was also found for this intervention (Figure 4B), and these effects correlated with
a down-regulation of PARP-1 levels (Figure 4D). Thus, PARP played a role in the degeneration of human LUHMES neurons exposed to MPP⁺.

4.4. Involvement of PARP activation in the LUHMES/IMA co-culture MPTP model

We observed PAR formation within neurons upon MPTP treatment, and this was blocked by the PARP inhibitor DHQ. IMA alone were not affected (Figure 5A, B). Inhibition of PARP by DHQ (at 50-200 µM) resulted in a rescue from MPTP toxicity in co-cultures (Figure 5C, D), and ABT-888, another PARP inhibitor showed similar rescuing effects (at 10 µM). Taken together, these data show that the co-culture model shows degeneration mechanisms also known from *in vivo* models, and that it is able to identify potentially neuroprotective compounds.

*Figure 5. Neuroprotective effects of PARP inhibition in co-culture of neurons and astrocytes.*

(A) Co-cultures were treated with MPTP (30 µM) alone or in the presence of DHQ (200 µM) for 72 h. Then, cells were fixed and immunostained for PAR. (B) Images of co-cultures or IMA alone were additionally analyzed by an automated imaging acquisition system. The algorithm applied for quantification recognizes cell nuclei stained for PAR and quantifies the number of PAR-positive cells in 30 fields per well. (C) Representative images (β-III-tubulin staining) of co-cultures exposed to MPTP (30 µM) in the presence or absence of DHQ (200 µM) for 5 days. (D) Concentration-dependent effects of the PARP inhibitors DHQ and ABT-888 on neuronal area in co-cultures exposed to MPTP (30 µM) for 5 days. Cells were stained for β-III tubulin after treatment, and the neuronal area was analyzed by an automated imaging approach. Data are presented as means ± SD.
4.5. Pharmacological protection in the mono- vs co-culture models

To obtain a broader range of data on the effect of drugs and experimental treatments in the co-culture MPTP model, we assembled a set of tool compounds to be tested. These were compared in standard monocultures exposed to MPP⁺ (Figure 6A and 7A), and LUHMES/IMA co-cultures exposed to MPTP (Figure 6B and 7B). Viability of the neurons was assessed by morphological observation, and by high content imaging of neurons. LUHMES monocultures were protected from MPP⁺ by ascorbic acid (Vit C), by the mixed lineage kinase inhibitor CEP1347, or by the caspase/protease inhibitor zVAD.

Figure 6. Profiling of neuroprotective candidate compounds in LUHMES monocultures and co-cultures.

Monocultures (A) were exposed to MPP⁺ (2 µM, 4 days); co-cultures (B) were exposed to MPTP (30 µM, 5 days) in the presence or absence of ascorbic acid (Vit C; 200 µM), rasagiline (Rasa; 10 µM), CEP1347 (CEP; 250 nM), zVAD-OMe-fmk (zVAD; 50 µM), oxidized glutathione (GSSG; 100 µM), Y-27632 (10 µM), HA-1077 (10 µM). Cells were fixed, permeabilized and immunostained for β-III-tubulin. Representative images were displayed in b/w and without nuclear stain for optimal contrast.
No protection was offered by the clinically-used MAO inhibitor rasagiline. These data essentially corroborated our earlier observations (Poltl et al., 2012). The situation was exactly reversed in the MPTP model: CEP1347, zVAD and Vit C failed to protect from neurodegeneration. They also failed to rescue the neurons when added several times during the culture period (not shown) or when used in various combinations (Figure 8).

The same differential protection (complete rescue of neurons in monocultures, but not at all in co-cultures) was also observed for the antioxidant iron chelator deferoxamine (Figure 8). Instead, rasagiline was fully protective in co-cultures (and not at all in monocultures), as
expected from its effect on MPTP metabolism. These data showed that the MPTP co-culture model showed pharmacological properties that were strikingly different from the simplified model of direct addition of MPP⁺ to neuron monocultures. The data obtained by quantification of the neuronal area were essentially confirmed for 5 drugs by counting of the cell somata (Figure S5).

### Table: Protection in Mono- and Co-cultures

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Protection in monoculture</th>
<th>Protection in co-culture</th>
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<tbody>
<tr>
<td>L-NMMA (NOS inhibitor)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1400W (NOS inhibitor)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-NNA (nNOS, iNOS inhibitor)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Minocycline</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7-Nitroindazole (nNOS inhibitor)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GSSG (GSH precursor)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Deprenyl (MAC-B inhibitor)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rasagiline (MAC-B inhibitor)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CEP 1347 (MLK inhibitor)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid (antioxidant)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>zVAD-OMe-fmk (caspase inhibitor)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>zVAD-OMe-fmk + Ascorbic acid</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CEP 1347 + Ascorbic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CEP 1347 + zVAD-OMe-fmk</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Deferoxamine (iron chelator)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GBR12909 (DAT inhibitor)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DHQ (PARP inhibitor)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ABT-888 (PARP inhibitor)</td>
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<td>+</td>
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<tr>
<td>Y-27632 (Rock inhibitor)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HA-1077 (Rock inhibitor)</td>
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**Figure 8.** Comparison of potential neuroprotective compounds in LUHMES mono- and co-cultures.

Monocultures (A) were exposed to MPP⁺ (2 µM, 4 days); co-cultures (B) were exposed with MPTP (30 µM, 5 days) in the presence or absence of varying concentrations of N⁶-Methyl-L-arginine acetate salt (L-NMMA; 1-1000 µM), 1400W (0-100 µM), N₂-Nitro-L-arginine (L-NNA, 1-500µM), Minocycline (0-50 µM), 7-Nitroindazole (0-100 µM), oxidized glutathione (GSSG; 100 µM), deprenyl (10 µM), rasagiline (10 µM), CEP1347 (250 nM), ascorbic acid (200 µM), zVAD-OMe-fmk (50 µM), deferoxamine (100 µM), GBR12909 (1 µM), DHQ (50 µM in mono-culture, 200µM in co-culture), ABT-888 (0-200 µM), Y-27632 (10 µM), HA-1077 (10 µM); combinations of ascorbic acid (200 µM) with zVAD-OMe-fmk (50 µM), zVAD-OMe-fmk (50 µM) with CEP1347 (250 nM) and CEP1347 (250 nM) with ascorbic acid (200 µM). Then, cells were fixed, permeabilized and immunostained for β-III-tubulin. The total neurite area was quantified by an automated imaging procedure. (+) = protection; (-) = no protection observed at any concentration.
In order to explore whether preferential protection of neurons in co-cultures can be achieved by compounds that do not block MPTP metabolism, we tested oxidized glutathione (GSSG) (100 µM) based on the idea that this experimental treatment would affect neurons indirectly via neuron-glia metabolic interaction. Indeed, we observed complete protection in co-cultures vs no effect at all in monocultures (Figure 6 and 7). The effect was so far specific for GSSG, as reduced glutathione or cysteine showed no effect in either culture system. These findings provide additional evidence that the MPTP co-culture system developed here has properties different from pure neurons, and that it allows the characterization of drug candidates on human neurons under more complex conditions than offered by pure neuronal cultures.

We also evaluated the behavior of compounds assumed to specifically protect neurites. For this purpose, the two Rho kinase (ROCK) inhibitors Y-27632 and HA-1077 were tested. We found that ROCK inhibitors protected neurons from degeneration in a concentration-dependent manner in co-culture as well as in monocultures (Figure 6, 7 and S9). ROCK inhibitors and PARP inhibitors were the only agents we have identified so far that protected neurons when cultured alone, or together with glial cells.

As PARP activation in dopaminergic cell death has often been associated with prior damage by peroxinitrite or other reactive nitrogen species, we explored whether this may also play a role in human LUHMES cells. Several approaches to block NO generation or to scavenge peroxinitrite failed to show any protective effect (Figure 8). Thus, PARP activation must be able to occur in human neurons independent of NO-triggered DNA damage. This may represent species differences in the regulation of nitric oxide synthases, and is consistent with recent discoveries on PARP activation independent of DNA damage (Lee et al., 2013).

In summary, the pharmacological characterization with more than 15 compounds (Figure 8) showed that (i) the MPTP co-culture system represents a new model of human dopaminergic neuron degeneration that recapitulates key features of MPTP toxicity in vivo, and (ii) that the pharmacological features of the more complex co-culture model are clearly distinct from those of the simplified monoculture model.
5. Discussion and conclusions

To our knowledge, we have presented here the first model that allows pharmacological studies of human dopaminergic degeneration triggered by MPTP. The toxicant has raised considerable scientific attention, since it had been shown to be responsible for triggering Parkinsonian-like symptoms in young drug addicts (Langston et al., 1984b). The discovery that MPP\(^+\) is the metabolite ultimately responsible for neurotoxicity has lead to the widespread use of this agent in cellular models. Such monoculture models neither account for the various modulatory effects of astrocytes (McNaught et al., 1999; Smeyne et al., 2001), nor for the considerably higher complexity of continuous toxicant production within a network of interacting cell populations. Our data provide clear evidence for major functional differences between an MPTP co-culture model, and the acute addition of MPP\(^+\) in monocultures. In parallel, we show that the MPTP model reproduces essential features (drug metabolism, cellular distribution, involvement of PARP and Rho kinase) known from in vivo mouse models. The possibility to study MPTP toxicity in a co-culture model opens new ways of pharmacological tests on human neurons, and to explore many of the more recent mechanistic pathways from animal model (Figure S10).

There is a strong interest in neuroprotective compounds that prevent dopaminergic degeneration in diseases such as PD. As all candidate drugs have failed so far in clinical trials (Kincses et al., 2011; Meissner et al., 2011; Olanow et al., 2009), better, more predictive drug discovery models are under high demand. One newer strategy is the preferential use of human target cells. The availability of DA neurons generated from induced pluripotent stem cells may provide new disease models for drug testing (Sanchez-Danes et al., 2012). However, such cells often show relatively immature phenotypes and are yet difficult to control for biochemical studies (Miller et al., 2013). The use of LUHMES neurons that are highly committed to the dopaminergic phenotype, and that allow generation of homogeneous cultures (Scholz et al., 2011) remedies some of these issues. However, pure neuronal cultures represent a rather artificial situation, since neurons in the brain are in close contact and interaction with their surrounding glial cells. Co-cultures of neurons and astrocytes may better reflect this situation (Allen et al., 2009; Sofroniew et al., 2010). A high content imaging approach addressed the additional analytical complexity of co-cultures and allowed the quantification of specific neuronal damage. The use of murine astrocytes together with the human neurons will allow for the analysis of mRNA levels by PCR in future studies, and also Western blots may be performed in a cell type-specific way if species specific-antibodies for
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the proteins of interest are available. Our examination of PARP inhibitors showed that mechanistic studies are possible in such co-cultures, if changes (such as PAR-formation) can be imaged on the single cell level.

It would be helpful to understand, why several drugs working in the MPTP mouse model were not successful in clinical trials. A new hypothesis suggested by our findings is that the glia-neuron ratio may play a role, as this much smaller in rodent brains than in humans (Tower et al., 1973). Several compounds that protected neurons in mice or in monocultures were not effective in co-cultures (Figure S11). Thus, the most striking finding of our study was the different efficacy of experimental neuroprotectants in monocultures vs. co-cultures (Figure 8). The lack of protection in co-cultures may have several reasons: (i) Some compounds might be metabolized or modified by astrocytes; (ii) the metabolic situation of neurons may be affected by astrocytes; (iii) the relative contribution of cell death mechanisms may change in the presence of astrocytes; (iv) the continuous production of MPP+ may lead to different early stress responses and adaptations. A further explanation may apply to CEP1347. At the high concentrations used here, this drug reduced intracellular GSH level in IMA monocultures (Figure S12). This may account for the loss of neuronal protection in co-cultures, as it has been shown that astrocytes depleted of GSH are not able to protect neurons from neurotoxicants (Pizzurro et al., 2014). This would be in line with a neuroprotective counter-regulatory program triggered in LUHMES by MPP+ (Krug et al., 2014).

Three compounds that were protective in the co-culture model appear of particularly high interest to us for future more detailed characterizations.

First, we provided evidence for the lack of NO / peroxinitrite contribution in the activation of PARP. This shows on the one hand, that PARP can be involved in human neuronal cell death, also when NO synthases are not activated. On the other hand, these findings suggest caution concerning the human transferability of mechanisms discovered in rodents. Previous studies had suggested that generation of NO is a necessary condition for PARP over-activation relevant to neurodegeneration (Grunewald et al., 1999; Mandir et al., 1999). In our co-culture system it became clear that PARP can be activated by other mechanisms, as treatment with inhibitors that completely shut down NO synthase activity did not show any effect.

Second, we found clear protection by two different Rho kinase inhibitors in monocultures and co-cultures. This is consistent with concepts that neuronal degeneration in PD represents
a “dying back” pathology (Burke et al., 2013), and protection of axons may prove to be beneficial (Chong et al., 2014; Tonges et al., 2012). The extent of protection in co-culture was particularly robust.

Third, we identified an experimental treatment that only works in co-cultures, but not in monocultures: GSSG addition. This underlines the importance of astrocyte-neuron interactions beyond simple xenobiotic metabolism, even though we cannot offer a consistent mechanistic explanation. GSSG did not increase the intracellular levels of glutathione in IMA or LUHMES monocultures, but we suppose that it was used by astrocytes to provide glutathione precursors to neurons. This could however not be confirmed by direct measurements, as the content of glutathione in co-cultures was not affected by GSSG addition. The fall of glutathione as a consequence of MPTP addition was prevented by GSSG (not shown), but only complex studies of metabolite fluxes between the cell populations will show whether this effect is a direct consequence of improved GSH re-synthesis in neurons or rather secondary to other mechanisms of neuroprotection.

In summary, our study demonstrated, that glial cells not only play a major role for the biology and metabolism of neurons, but that they can drastically affect the pharmacology of neuroprotection. Moreover, our study paves the road to the construction of complex neurodegeneration models that consist of more than one cell type, but are still amenable to high throughput endpoints and single cell analysis of human neurons. Related systems have been used in the past mainly for the study of neuron-microglia interactions, while considerably less information is available on astrocytes (Bal-Price et al., 2001; Cacci et al., 2008; Frakes et al., 2014; Hirt et al., 2000; Kingham et al., 2001; Lee et al., 2014a; Malchiodi-Albedi et al., 2001; Piani et al., 1992). An important future step will be the combination of neurons, astrocytes and microglia at defined proportions, possibly also considering oligodendrocytes as fourth population.

6. Acknowledgements

This work was supported by the Doerenkamp-Zbinden Foundation, the Land BW, the DFG (RTG1331; KoRS-CB), the BMBF and University of Konstanz funds.
7. Supplementary figures.

Supplemental Material

Chemicals used in this study:

GSSG (L-glutathione oxidized), DHQ (1,5-isoquinolinediol), rasagiline, GBR12909, deprenyl, ascorbic acid, deferoxamine, HA-1077, L-NMMA, 1400W, L-NNA, minocycline, 7-nitroindazole, MPTP and MPP+ were obtained from Sigma (St. Louis, MO); caspase inhibitor I (zVADfmk) from Calbiochem (San Diego, CA, USA); Y-27632 was from Tocris Bioscience (Bristol, UK); CEP1347 (3,9-bis(ethylthio)methyl)-K-252a) was obtained by chemical modification of K252a (Calbiochem, San Diego, CA) according to standard methods (Kaneko et al., 1997).

Antibodies used in this study:

**Primary antibodies:** monoclonal mouse anti-S-100 (β-subunit) antibody, clone SH-B1, isotype IgG1, was used for immunocytochemistry at a dilution of 1:500 (catalog number S2532, Sigma), monoclonal mouse anti-neuronal class III β-tubulin antibody, clone TUJ1, isotype IgG2a, was used for immunocytochemistry at a dilution of 1:500 and for western blot analysis at a dilution of 1:1000 (catalog number MMS435P, Covance); monoclonal mouse anti-GAPDH antibody, clone GAPDH-71.1, isotype IgM, was used for western blot analysis at a dilution of 1:10 000 (catalog number G8795, Sigma); monoclonal rat anti-DAT antibody, clone DAT-Nt, isotype IgG2ak was used for immunocytochemistry at a dilution of 1:500 (catalog number MAB369, Millipore); monoclonal mouse anti-TH antibody, clone LNC1, isotype IgG1k was used for immunocytochemistry at a dilution of 1:500 and for western blot analysis at a dilution of 1:1000 (catalog number MAB318, Millipore); rabbit anti-human VMAT2 antibody (catalog number AB1767, Chemicon) was used for immunocytochemistry at a dilution of 1:500; monoclonal mouse anti-NeuN antibody, clone A60, isotype IgG1 was used for immunocytochemistry at a dilution of 1:200 (catalog number MAB377, Millipore); monoclonal mouse anti-PAR antibody (clone 10H) and monoclonal mouse anti-PARP1 antibody (clone FI-2-3) were obtained as previously described (Fischer et al., 2014; Kawamitsu et al., 1984). They were used for both immunocytochemistry and western blot analysis at a dilution of 1:300.

**Secondary antibodies:** Alexa Fluor 555 goat anti-mouse IgG2a antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21137, Life technologies); Alexa Fluor 488 goat anti-rat IgG antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-11006, Life technologies); Alexa Fluor 488 chicken anti-rabbit IgG antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21441, Life technologies); Alexa Fluor 488 goat anti-mouse IgG2a antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21131, Life technologies) Alexa Fluor 488 goat anti-mouse IgG1 antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21121, Life Technologies), Alexa Fluor 555 goat anti-mouse IgG antibody was used for immunostaining at a dilution of 1:500 (catalog number A-21422, Life Technologies), peroxidase-conjugated goat anti-mouse IgG antibody was used for western blot analysis at a dilution of 1:2000 (catalog number 115-035-174, Jackson Immuno Research.)
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Figure S1. Dopaminergic characteristics of LUHMES differentiated in monoculture and in co-culture with astrocytes.

(A) Predifferentiated LUHMES (day 2) were seeded (12 × 10^6 cells per data point) alone or on top of astrocytes. On day 6, cells were lysed in 0.2% Triton X100, 0.01M HCl, 1 mM EDTA, 4 mM sodium metabsulfite, 1mM ascorbate. The amount of dopamine in supernatants was analysed by using a Dopamine Elisa Kit (Abnova). For comparison, IMA monocultures were analysed alone. Dopamine was not detectable (n.d.). (Here, the same number of astrocytes was analyzed as in co-cultures). Data are means ± SD of triplicates. 100% Dopamine was 30ng/ 12 × 10^6 cells. (B) LUHMES cells were differentiated in monocultures and co-cultures, then stained on days 6 (d6) for the dopaminergic markers DAT and VMAT2 (green). Nuclei were stained with H-33342 (red). Scale bar = 20 µm.
Figure S2. MPTP metabolism by LUHMES, IMA 2.1 and primary glial cells.

IMA 2.1, primary rat glial cells, and differentiated LUHMES (d6) were treated with 30 µM MPTP for the time periods as indicated. MPTP, MPP⁺ and MPDP⁺ were detected by HPLC in the respective supernatants. Data are expressed as means ± SD of triplicate samples.
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Figure S3. Quantification of neuronal area in co-culture.

(A) For illustration of the imaging procedure, a defined set of samples was generated by exposure of co-cultures to solvent control or 30 µM MPTP for 5 days. Then, cells were immunostained for the specific neuronal marker βIIItubulin (using Alexa Fluor 488 as detection dye of the secondary antibody), and an automatic imaging acquisition, segmentation, recognition and quantification process was started. The images in the second row show in blue the pixels recognized by the software as neuronal area (= 'tracing'). In detail, the procedure was as follows: Images were acquired on an automated microscope (Array-ScanII HCS Reader, Cellomics, PA) equipped with an x-y stage (programmed for sequential imaging of all relevant wells of a micro-well plate). Thirty fields per well (330 µm × 330 µm size each) were imaged using a 20x lens according to pre-set co-ordinates, and digital images were recorded with a Hamamatsu ORCA ER camera (resolution 1024 × 1024; run at 2 × 2 binning). A filter set for fluorescence excitation/emission wavelengths of 474 ± 40/535 ± 45 was used, and exposure time was held constant across one experiment. In the image processing phase, an intensity histogram-derived threshold were used for object identification. All βIIItubulin-positive pixels of the field (beyond a given intensity threshold) were recognized and counted by the software (Thermo Scientific Cellomics Spot Detector Bioapplication V4). The threshold was dynamically determined for each field after flat field and background correction and intensity normalization to 512 gray values and was set to 12% of the maximal brightness (channel 63 of 512). In our laboratory, this procedure was performed using Cellomics Bioapplication SpotDetector.V2 on the Array-ScanII HCS Reader. This software automatically performs a segmentation of the image field into areas belonging to one cell (by virtually inflating each cell outline until it meets with the inflated neighboring cell outline). This process is not necessary for the actual calculation of the neuronal area, but we used it to correct for edge effects. Cells that were only partially in the image field were excluded, together with a corresponding part
of neurites lying in this cells’ segmentation area. Corrections for cell numbers per field were not performed, as our cell counting data (based on simultaneous recording of H-33342-positive nuclei in the same imaging fields) showed that cell numbers/field were highly reproducible. All cellular components (somata and neurites) with βIII-tubulin-positive pixels were defined as “neuronal area”. Three wells were analysed for each treatment. Then, the total pixel number was normalised to the total pixel number in untreated controls and presented as percentage. (B) Quantitative representation of total neuronal area. Co-cultures were treated with varying concentrations of MPTP for 5 days. Data are expressed as means ± SEM (n = 2). (C) Representative images of co-cultures treated with various MPTP concentrations for 5 days, and stained for β-III-tubulin.
Figure S4. MPTP toxicity in co-cultures of LUHMES neurons and primary rat glial cells.

Primary rat glial cells were seeded at a density of 15,000/cm². After 24 h, medium was changed to LUHMES differentiation medium. After additional 48 h, pre-differentiated LUHMES cells (d2) were seeded on top of astrocytes at a density of 15,000/cm². On day 6 of LUHMES differentiation, MPTP (30 µM) was added for 5 days. Cells were fixed and immunostained for β-III-tubulin (green), nuclei were stained with H-33342 (red). The neuronal area was quantified as in Figure S3. Data are expressed as means ± SD of quadruplicates.
Figure S5. Comparison of drug effects on neuronal area and on the number of cell bodies.

Co-cultures were exposed to MPTP (30 µM) in the presence or absence of zVAD-OMe-fmk (zVAD; 50 µM), CEP1347 (CEP; 250 nM), HA-1077 (10 µM), deprenyl (10 µM), GBR12909 (1 µM). Cultures were analysed after 5 days for neuronal area and after 6 days for counting of somata. For assessment of neuronal numbers (somata), cells were immunostained for NeuN, which stains exclusively neuronal nuclei. The number of cells per field was counted manually. For neuronal area assessment, cells were fixed, permeabilized and immunostained for β-III-tubulin and the neuronal area was assessed by an automated imaging procedure. These data demonstrate that the number of neuronal somata was decreased when treated with MPTP. However, the pronounced degeneration of neuronal cell bodies took one day more than the neurite degeneration. We found that when neurites were protected, then cell bodies were also protected. When neurites degenerated, this was always followed by death of the somata at one day later.
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Figure S6. Toxicity of MPTP or MPP⁺ in IMA 2.1 monocultures.

IMA 2.1 were exposed to various concentrations of MPTP or MPP⁺ for 5 days. Cell viability was assessed by the (A) resazurin reduction assay and levels of intracellular GSH and ATP were measured in parallel (B) as well as immunostaining (S100β) to visualize general cell morphology. Data are expressed as means ± SD. (C) LUHMES neurons were exposed to 5 µM MPP⁺ for 3 days. Then, conditioned medium from neurons (> 80% cell death) was collected and transferred to astrocytes for 24 h. (D) Astrocytes were exposed to 5 µM MPP⁺ for 24 h. Astrocyte mRNA was isolated and expression levels of 10 selected inflammatory genes were measured via RT-qPCR. Data were normalized to GAPDH and displayed as ∆∆ ct values relative to the respective mRNA expression in untreated cells. IFNβ = interferon-β; IL6 = interleukin 6; TLR2 = toll-like receptor 2; NOS2 = nitric oxide synthase-2; TDK1 = thymidylate kinase family LPS-inducible member; IFIT3 = interferon-induced protein with tetratricopeptide repeats 3; NOD2 = nucleotide binding oligomerization domain 2; COX2 = cyclooxygenase-2; TLR3 = toll-like receptor-3; TNFα = tumor necrosis factor α. *, p < 0.05.
Figure S7. Uptake of MPP⁺ by LUHMES mono- and co-cultures.

(A) Mono- and co-cultures were exposed to 5 μM MPP⁺ for the time intervals as indicated, intracellular levels of MPP⁺ were assessed by HPLC. Intracellular levels of MPP⁺ are expressed as the amount per cell number. Intracellular MPP⁺ levels in IMA 2.1 were under the detection limit. (B) To ensure equal cell numbers under both mono- and co-culture conditions, samples from both conditions were collected after varying differentiation periods as indicated. Cell homogenates were subjected to Western analysis and stained for β-III-tubulin. Data are expressed as means ± SD of triplicates.
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Figure S8. Involvement of PARP in MPP⁺-mediate LUHMES toxicity.

LUHMES mono-cultures (d6) were exposed for 48 h with 5 µM MPP⁺ in the presence or absence of DHQ (50 µM). Then, ATP and NAD⁺ levels were measured. Data were normalized to cells treated with solvent (control) only. They are means of triplicates ± SD.
C. Prevention of human dopaminergic neurodegeneration in an astrocytes co-culture system allowing endogenous drug metabolism

Figure S9. ROCK inhibition protects LUHMES mono- and co-cultures from MPP⁺/MPTP toxicity.

Co-cultures (A) were exposed to MPTP (30 µM, 5 days), monocultures (B) were exposed to MPP⁺ (2 µM, 4 days) in presence or absence of various concentrations of the ROCK inhibitors Y-27632 and HA-1077. After the time intervals as indicated, cells were fixed and immunostained for β-III-tubulin. Neuronal area was quantified by an automated imaging procedure. Data are expressed as means ± SD of triplicates.
Prevention of human dopaminergic neurodegeneration in an astrocytes co-culture system allowing endogenous drug metabolism

<table>
<thead>
<tr>
<th>Ref</th>
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<th>Results/Relation to co-culture</th>
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<tr>
<td>5</td>
<td>HDAC</td>
<td>Valproic acid, the most investigated HDAC inhibitor in PD, did not show a protection.</td>
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<td>16</td>
<td>Glutathione</td>
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<td>2</td>
<td>ROCK pathway</td>
<td>ROCK inhibitors were protective in co-cultures</td>
<td>ROCK inhibitors also protected monocultures</td>
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<td>11</td>
<td>Wnt/b-catenin signaling cascade.</td>
<td>This aspect was not explored</td>
<td>As microglia were absent; astrocytes did not reach the proper reactive state observed in vivo</td>
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<tr>
<td>3</td>
<td>Autophagy mitophagy</td>
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<tr>
<td>23</td>
<td>PARP</td>
<td>Protective effect of PARP inhibitor</td>
<td>Protection also in monoculture (independent of NO)</td>
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<td>14</td>
<td>Mitochondrial fission and fusion</td>
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<td>22</td>
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<td>Treatment with NO inhibitors did not protect</td>
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<td>VMAT overexpression is protective, but this cannot be obtained pharmacologically</td>
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Figure S10. Recent findings on MPTP animal models in relation to our co-culture model.

The literature has been scanned for recent findings concerning the MPTP model. Altogether, more than 5000 references were retrieved, and key findings concerning the animal model have been compiled here. The MPTP model most commonly uses mice as experimental species, but also primates, including humans, are susceptible (but rats are not). MPTP produces a final pathological state, a functional impairment, and a pharmacological responsiveness that closely resembles human Parkinsonism. Some features of the pathogenesis may also be overlapping between the model and the human disease. These include oxidative stress, early mitochondrial impairment, a failure of proteostasis and an involvement/modulation by levels of alpha-synuclein. Dissimilarities of the MPTP animal model to human disease are that the damage occurs rapidly, is hardly age-dependent, and usually does not involve formation of Lewy bodies. The MPTP co-culture model developed here has the advantage that the metabolism and distribution of the metabolite MPP⁺ can be better controlled and followed, and the same applies to cellular processes, that are accessible for microscopic and biochemical endpoints. This advantage is balanced by the shortcomings that only one type of neurons, and no microglia are present in the model. The kinetics of MPTP conversion is slower than in vivo, and this is possibly the reason for the relatively slow cell death process we observe. The latter may also be considered a positive feature, if human disease pathogenesis is to be modelled. Some findings from the MPTP model have been selected for tabular presentation, as they show the extremely broad range of biological pathways involved in the complex pathogenesis. Comparison of the literature findings with information available from the co-culture model indicates that there are still many processes to be explored in more detail, and that our co-culture (possibly supplemented with microglia) could contribute to this. For references, see suppl. Fig. S11.
C. Prevention of human dopaminergic neurodegeneration in an astrocytes co-culture system allowing endogenous drug metabolism

**Figure S12.** Toxicity of candidate compounds used in IMA monocultures.

IMA were exposed to CEP1347 (CEP; 250 nM), ascorbic acid (Vit C; 200 µM), zVAD-OMe-fmk (zVAD; 50 µM), GBR12909 (1 µM); deprenyl (10 µM); rasagiline (Rasa; 10 µM) for 5 days. Cell viability was assessed by the LDH release assay. Intracellular GSH levels were measured in parallel. Data are expressed as means ± SD.
C. Prevention of human dopaminergic neurodegeneration in an astrocytes co-culture system
allowing endogenous drug metabolism

Supplemental methods:

1. Cell culture
LUHMES were grown in flasks pre-coated with poly-L-ornithine (PLO) (50 μg/ml) and fibronectin (1 μg/ml) (Sigma, St. Louis, MO) in proliferation medium, which consisted of Advanced DMEM/F12 (Gibco Invitrogen, Karlsruhe, Germany) supplemented with 2 mM L-glutamine (Gibco), 1 x N2 supplement (PAA) and 40 ng/ml recombinant bFGF (R&D Systems). At 80 % of confluency cells were detached with 2 x ATAMP/Trpentin (136 mM NaCl, 5.4 mM KCl, 6.9 mM NaHCO₃, 5.6 mM D-glucose, 0.54 mM EDTA, 0.5 g/l trypsin from bovine pancreas type-II-S; Sigma–Aldrich), incubated for 3 min and passaged 1:10. For differentiation, 8 x 10⁵ LUHMES were seeded into a T75 flask in proliferation medium and differentiation was started after 24 h by changing medium to differentiation medium consisting of Advanced DMEM/F12 supplemented with 2 mM L-glutamine, 1 x N2 supplement, 1 mM dibutyryl-cAMP (Sigma), 1 mg/ml tetracycline (Sigma) and 2 ng/ml recombinant human glial derived factor (GDNF) (R&D Systems). After 48 h (on day 2 of differentiation) cells were trypsinized as described, counted and seeded in multi-well plates at a density of 1.5 x 10⁵ cells/cm². The differentiation process was continued for additional 4 days to achieve a total differentiation period of 6 days before the cells were used for experiments.

IMA were grown at 37°C (5% CO₂) in medium consisting of DMEM (Biochrom, Berlin) with 10% FCS (fetal calf serum, PAA) and 1% of penicillin/streptomycin. They were trypsinized for 2 min with 0.25% trypsin/DMEM and reseeded at a ratio of 1:10 when 80% of confluence was reached. Astrocytes were seeded (for co-cultures) at a density of 15,000/cm² (that means 30,000 per well of a 24 well plate), and after 3 days we plate neurons on top of them at a density of 150,000/cm² (that means 300,000 per well). Since LUHMES neurons exit cell cycle after addition of tetracycline, their number does not change. IMA still undergo cell division. In order to estimate the neuron-astrocyte ratio at the time point when experimental treatment starts, we counted the amount of astrocytes (nuclei per cm²) on the day of seeding (d0) and on the day when treatment starts (d7). Neuronal and astrocyte nuclei localize in different focal planes and have a different morphology (shape, size). Therefore it is easy to distinguish and count them. The amount of astrocyte nuclei after 7 days was around 195,000 cells/cm². That means there is a astrocytes/neuron ratio of 1.3 in our co-culture system. According to the literature, astrocytes outnumber neurons in the brain up to ten-fold (Magistretti et al., 1999). A ratio of 2 has been found in the cortex (Pakkenberg et al., 2003) and this agrees well with classical older studies (Tower and Young, 1973). The proportion of glial cells surrounding DA neurons in the substantia nigra, the target nucleus for neurodegeneration in Parkinson’s disease (PD), is the lowest compared to any brain area (Damier et al., 1996; Mena et al., 2002). We suppose therefore that the ratio we are using is close to the in vivo situation.

2. HPLC based detection of MPTP and MPP⁺
Following treatment, cell pellets or supernatants (0.5 ml) were acidified with 4.5 μl of perchloric acid (70%) and centrifuged at 10,000g for 15 min. Samples were then filtered through a Chromafil PET-20/15 MS-filter with 0.2 μm pore size from Macherey Nagel (Düren, Germany). MPTP, MPDP⁺ and MPP⁺ concentrations were detected using a Kontron system (Goebel Analytik, Au/Hallertau, Germany) composed of a model 520 pump, model 560 autosampler, models 535 and 430 diode array detectors, set at 245 nm for MPTP, 295 nm for MPP⁺, and 345 nm for MPDP⁺. Separation was carried out on a C8 nucleosil column (250 x 4.6 mm; 5 μm particle size) from Macherey Nagel (Düren, Germany) at RT. The mobile phase consisted of acetoniitrile : distilled water : triethylamine : sulphuric acid (12.50 : 86.18 : 1.04 : 0.28, v/v, pH 2.3). The mobile phase was degassed with an online vacuum degasser and delivered isocratically at a flow rate of 1 ml/min at an average pressure of 145 bar. Data analysis was performed with Geminxy II software (Goebel Analytik, Au/Hallertau, Germany).
5. Dopamine determination by ELISA

Predifferentiated LUHMES cells (day 2) were seeded (12 x 10^6 cells per data point) alone or on top of astrocytes. On day 6, cells were lysed in 0.2% Triton X100, 0.01 M HCl, 1 mM EDTA, 4 mM sodium metabisulfite, 1 mM Ascorbate. Then cells were scraped with cell scraper, sonicated, centrifuged at 3000 g for 5 min, and supernatants were frozen at -20°C. The dopamine content in supernatants was analyzed by using Dopamine Elisa Kit (Abnova) according to the manufacturer’s protocol.

6. RNA extraction, RT-PCR and quantitative RT-PCR was performed as described in Kuegler et al., 2012.

Primers used (see table below)

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Supplemental References


Tower DB, Young OM (1973). The activities of butyrylcholinesterase and carbonic anhydrase, the rate of anaerobic glycolysis, and the question of a constant density of glial cells in cerebral cortices of various mammalian species from mouse to whale. J Neurochem 20: 269-278.
Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation

Liudmila Efremova\textsuperscript{1,2}, Stefan Schildknecht\textsuperscript{1}, Martina Adam\textsuperscript{1}, Petra Chovancova\textsuperscript{1,3}, Simon Gutbier\textsuperscript{1,2}, and Marcel Leist\textsuperscript{1}

Affiliations:
\textsuperscript{1} Doerenkamp-Zbinden Chair for In vitro Toxicology and Biomedicine, University of Konstanz, PO Box M657; D-78457 Konstanz, Germany;
\textsuperscript{2} Research Training Group 1331 (RTG1331), University of Konstanz;
\textsuperscript{3} Konstanz Research School Chemical Biology, University of Konstanz

Manuscript in preparation
1. Abstract

BACKGROUND: Although astrocytes are the largest cell population in the human brain, relatively little is known about their neuropharmacological relevance. Only few experimental studies have examined the interaction of activated astrocytes with human neurons.

EXPERIMENTAL APPROACH: Immortalized murine astrocytes (IMA) were combined with human LUHMES neurons, and stimulated with an inflammatory (TNF, IL-1) cytokine mix (CM). Neuronal survival was studied both in co-cultures and in monocultures after transfer of conditioned medium from activated IMA. Interventions with >20 pharmaceutical compounds were used to profile the model system.

KEY RESULTS: Control IMA supported neurons, and protected them from neurotoxicants. Inflammatory activation reduced this protection, and prolonged exposure of co-cultures to CM triggered neurotoxicity. This neither involved direct effects of cytokines on neurons, nor secretion of NO from astrocytes, but it was prevented by the corticosteroid dexamethasone. The neurotoxicity-mediating effect of IMA was faithfully reproduced by human astrocytes. Moreover, glia-dependent toxicity was also observed, when IMA cultures were stimulated with CM, and the culture medium was transferred to neurons. Such neurotoxicity was prevented when astrocytes were treated by p38 kinase inhibitors or dexamethasone, whereas such compounds had no effect, when added to neurons. Conversely, treatment of neurons with seven mechanistically different drugs, including the ROCK inhibitor Y-27632 prevented toxicity of astrocyte supernatants.

CONCLUSIONS: The sequential IMA-LUHMES neuroinflammation model is suitable for separate profiling of both glial-directed and directly neuroprotective strategies. Moreover direct evaluation in co-cultures of the same cells allows for testing of therapeutic effectiveness in more complex settings, in which astrocytes affect pharmacological properties of neurons.

ABBREVIATIONS: ARE, the antioxidant responsive element; cAMP, cyclic adenosine monophosphate; CEP1347, (3,9-bis[(ethylthio)methyl]-K-252a); CCM, complete cytokine mix; CDNF, cerebral dopamine neurotrophic factors; CM, cytokine mix; CNS, central nerve system; COX2, cyclooxygenase-2; DA, dopaminergic; DAT, dopamine transporter; DHQ, 1,5-isoquinolinediol; DM, differentiation medium; DTNB, 5,5´-dithiobis(2-nitrobenzoic acid); GDNF, glial derived neurotrophic factor; GSH, glutathione; GSSG, L-glutathione oxidized; IFN-γ – interferon-gamma; IkB, NF-kappa-B inhibitor beta; Nrf2, NF-E2-related factor 2; IL-1β, Interleukin-1 beta; IMA, immortalised mouse astrocytes; LDH, lactate dehydrogenase; L-NNA, Nω-Nitro-L-arginine; LPS, lipopolysaccharides; LUHMES, Lund
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human mesencephalic cells; mAGES, mouse astrocytes generated from embryonic stem cells, MPP⁺, 1-methyl-4-phenyl-pyridinium; MS, multiple sclerosis; NEP, neuroepithelial cells; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NHA, normal human astrocytes, NOS, nitric oxide synthase; NSE, neuron-specific enolase; PARP, poly-(ADP-ribose)-polymerase; PD, Parkinson’s disease; PLO, poly-L-ornithine; ROCK, Rho kinase; SNpc; substantia nigra pars compacta; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-alpha; VMAT2, vesicular monoamine transporter.
2. Introduction

Inflammatory situations and several inflammation-related mediators can trigger or aggravate neuronal damage in experimental models (Hunter et al., 2009; Oleszak et al., 1998). They may also be involved in several neurodegenerative diseases, and modulation of inflammation may contribute to current and future treatments (Gao et al., 2011; in’t Veld et al., 2001; Rees et al., 2011; Ruitenbergen et al., 2001). At present, only few drugs are available that reach therapeutically effective levels within the brain and can be tolerated well for prolonged treatment periods. Moreover, most drugs have been developed for other indications, and the inflammatory key players and processes in the brain differ strongly from better-examined situations in the body periphery. Specific target cells in the central nervous system include microglia, a cell type related to peripheral macrophages but of distinctly different developmental origin (Gomez Perdiguero et al., 2015), and astrocytes, an often neglected cell population that has a moderate abundance in rodent brains, but exceeds the number of neurons in human brain by 3-10-fold (Magistretti et al., 1999). Astrocytes have a peculiar double function, as major neuro-supportive cell population in the healthy brain, and as powerful inflammatory cells that may exert detrimental effects in disease situations (Allen et al., 2009; Maragakis et al., 2006; Sofroniew et al., 2010; Volterra et al., 2005).

Besides a general maintenance of brain homeostasis, astrocytes can protect neurons in multiple ways. For instance, they secrete neurotrophins such as GDNF or CDNF (Lindholm et al., 2007), supply antioxidants and other beneficial factors (Cipriani et al., 2012; Pizzurro et al., 2014; Shih et al., 2003), promote synaptogenesis (Allen et al., 2012; Dodla et al., 2010), foster neuritogenesis (Guizzetti et al., 2008), and allow growth under unfavorable conditions (Kuegler et al., 2012).

There is genetic evidence that astrocytes can contribute to neuropathology, e.g. in the RETT syndrome (Lioy et al., 2011; Williams et al., 2014) or in Huntington’s disease (Valenza et al., 2015). However, for most other situations, the role of these cells, as bystander or aggressor, is less understood. It is often assumed that astrocyte functions may get out of the balance in pathological situations, and then compromise neuronal survival (Buffo et al., 2010). In support of this, altered (i.e. activated) astrocytes are found in many neurodegenerative diseases (Forno et al., 1992) and in AD (Medeiros et al., 2013), in intoxications (Breckenridge et al., 2013; Streifel et al., 2012) and after traumatic injury (Hatten et al., 1991).
D. Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation

The different functions of astrocytes may be due to largely different activation states (Falsig et al., 2004a; Henn et al., 2011) triggered by damage, disease or infection. All these conditions are related to neuroinflammation and the majority of research in this field has focussed on microglia. These latter cells are equipped with a full set of toll-like receptors and other damage/pattern recognition receptors, so that they can react to a multitude of stimuli within minutes to few hours. During this first wave of innate immune reaction in the brain, cytokines like IL-1ß and TNF-α are secreted, which are very potent activators of astrocytes. This large pool of cells then reacts with a second wave of mediators (Falsig et al., 2008) and can remain activated and altered in their function for many days after stimulation (Henn et al., 2011), even this consists only of a short peripheral response (Biesmans et al., 2015; Zhang et al., 2009a).

The causal involvement of activated glia is a matter of current discussion. Genetic tools have been developed to deplete microglia (CD11b-positive cells in the brain), but the use of such approaches in mouse models of disease has not supported a role of microglia (Gowing et al., 2008). In the case of astrocytes, the data base is even less clear, as much less experimental tools are available to manipulate and detect them, and their reaction is slow and protracted (Breckenridge et al., 2013; Dusart et al., 1994; Frank et al., 1996). The pathological and toxicological role of astrocytes is further complicated by the facts that they may themselves be targets of toxicity (Yin et al., 2011), they may have a role in xenobiotic metabolism (Schildknecht et al., 2012) and they show a large plasticity, e.g. by taking the role of stem cells (Robel et al., 2011). Moreover, astrocytes and microglia produce a large variety of different mediators that may affect neurons, e.g. lipid mediators/small molecules (Giulian et al., 1993) reactive oxygen species (Ma et al., 2013), proteases (cathepsin B), cytokines (Mattson et al., 1997; Oleszak et al., 1998), complement factors (Pekny et al., 2007) and chromogranins (Kingham et al., 1999), but their respective contribution to human pathology is unclear or not identified at all (Lioy et al., 2011; Williams et al., 2014).

Lack of knowledge of the relevant damage mediators has prevented the development of targeted therapies. Nevertheless, (Carbone et al., 2012) showed that the Riluzole reduced the neurodegeneration via suppression of suppression of reactive astrocytosis, and (Zhang et al., 2009b) showed that administration of SKF83959 resulted in marked protection of dopaminergic neurons from MPTP-induced neurotoxicity via FGF-2 expression in astrocytes via IP3-dependent Ca2+ signalling. However, in both cases the exact mechanisms of astrocyte targeted neuroprotection remains elusive. Moreover, use of CEP1347, a compound that
Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation decreases glial activation (Falsig et al., 2004b; Lund et al., 2005), did not show clinical efficacy for Parkinson’s disease.

Many biological processes and metabolic functions of individual cell types are particularly difficult to study in vivo because of several cell types communicating in space and time. An alternative is provided by newer cell culture models, some of which also allowing the use of human neurons. These could provide an important insight into mechanism and potential pharmacological targets, since they provide a level of control not available in vivo (More et al., 2013; Schlachetzki et al., 2013). One approach uses for instance re-aggregating brain cultures (Sandstrom von Tobel et al., 2014; von Tobel et al., 2014) to study delayed astrocyte responses over very long times, but also classical co-cultures have been used (Zhang et al., 2010a). The traditional in vitro culture systems used primary rodent cultures suffering from a number of limitations: undesired baseline activation, microglia contamination (Saura, 2007), loss of physiological features of astrocytes (Cahoy et al., 2008; Pekny et al., 2014). Some of these problems have been overcome by the development of new methods for astrocytes preparation (Foo et al., 2011) and 3D-dimentional culture systems for astrocytes and neurons (Monnet-Tschudi et al., 2011; Puschmann et al., 2014; Puschmann et al., 2013).

Another approach focuses more on the neurons as target of toxicity, and relies on a simple, but reproducible source of astrocytic factors. For instance, post mitotic human neurons can be differentiated from the LUHMES cell line (Scholz et al., 2011). These cells are conditionally immortalized and assume neuronal morphological, electrophysiological and neurochemical properties upon the switching off of the v-myc transgene by tetracycline. The cells are compatible with cultures on a layer of astrocytes and can therefore be used for layered co-cultures (Efremova et al., 2015). This system has worked particularly robust for a combination of LUHMES neurons with the immortalized murine astrocytic cell line IMA (Schildknecht et al., 2012). The latter cells support drug metabolism, e.g. of MPTP, and are well-characterized in their inflammatory response. The co-culture allowed multiple pharmacological interventions and the level of neurons or astrocytes (Efremova et al., 2015).

In the present study this experimental system was used to study neuron-glia interaction under inflammatory conditions, and to provide a pharmacological characterization of relevant cell responses. In particular, we asked, whether neuronal support by healthy non-activated glia can be identified and quantified. We then proceeded to examine, how this situation changes under inflammatory conditions (cytokine exposure). The prolonged activation of astrocytes (6 days) resulted in neuronal death, mediated by a soluble factor. This system of human neuronal
death due to glial activation allowed for the first time evaluation of several experimental pharmacological approaches targeted either to neurons or glia. The model allows separate testing on the individual cell types (for target and pathway definition), or direct evaluation in co-cultures (for testing of therapeutic effectiveness) in complex settings that allow multiple reciprocal cell interactions, that may lead to responses hard to predict from individual cells (Gantner et al., 1996).
3. Materials and Methods

Chemicals used in this study:

- GSSG, 1400W, KW6002, Brefeldin A, glucose, sodium pyruvate, Indomethacin, Ibuprofen, Mifepristone, Resveratrol, SB202190, SB203580, L-NNA, Dexamethasone, DHQ, HA-1077, and MPP⁺ were obtained from Sigma (Steinheim, Germany); caspase inhibitor I (zVADfmk) from Calbiochem (San Diego, CA, USA); CAY10512 from Santa Cruz Biotechnology (Heidelberg, Germany); ATB-337 from Cayman Chemical (Michigan, USA); Y-27632, Arctigenin, IKK16 were from Tocris Bioscience (Bristol, UK). SIN-1 and SpermineNONOate were from Cayman Chemicals (Ann Arbor, Michigan, USA); CEP1347 (3,9-bis[ethylthio)methyl]-K-252a) was obtained by chemical modification of K252a (Calbiochem, San Diego, CA) according to standard methods (Kaneko et al., 1997). The recombinant mouse and human IL-1β, TNF-α, IFN-γ were from R and D Systems (Minneapolis, USA).

Antibodies used in this study:

**Primary antibodies:** monoclonal mouse anti-S-100(β-subunit) antibody, clone SH-B1, isotype IgG1, was used for immunocytochemistry at a dilution of 1:500 (catalog number S2532, Sigma); monoclonal mouse anti-neuronal class III β-tubulin antibody, clone Tuj1, isotype IgG2a, was used for immunocytochemistry at a dilution of 1:500 (catalog number MMS435P, Covance); monoclonal mouse anti-TH antibody, clone LNC1, isotype IgG1k was used for immunocytochemistry at a dilution of 1:500 (catalog number MAB318, Millipore); rabbit anti-human VMAT2 antibody was used for immunocytochemistry at a dilution of 1:500 (catalog number AB1767, Chemicon); purified anti-NF-kB antibody, clone 20/NF-kB/p65, isotype IgG1, was used for immunocytochemistry at a dilution 1:200 (catalog number 610869, BD Biosciences).

**Secondary antibodies:** Alexa Fluor 555 goat anti-mouse IgG2a antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21137, Life technologies); Alexa Fluor 488 chicken anti-rabbit IgG antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21441, Life technologies); Alexa Fluor 488 goat anti-mouse IgG2a antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21131, Life technologies); Alexa Fluor 488 goat anti-mouse IgG1 antibody was used for immunostaining at a dilution of 1:1000 (catalog number A-21121, Life Technologies); Alexa Fluor 555 goat
anti-mouse IgG antibody was used for immunostaining at a dilution of 1:500 (catalog number A-21422, Life Technologies).

**Cell culture**

All cell cultures used were grown at 37°C (5% CO₂). **Human neuronal cell line (LUHMES):** LUHMES cells were maintained in proliferation medium (Advanced Dulbecco’s modified Eagle’s medium/F12, 1x N2-supplement (Invitrogen, Germany), 2 mM GlutaMax (Gibco, Rockville, MD, USA) and 40 ng/ml recombinant basic fibroblast growth factor (R&D Systems) on poly-L-ornithine (50 μg/ml) and fibronectin (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) coated cell culture plastic (Nunclon). For differentiation, the cells were maintained in differentiation medium (Advanced Dulbecco’s modified Eagle’s medium/F12, 1x N2-supplement, 2 mM GlutaMax, 1 mM dibutyryl cAMP (Sigma Aldrich), 10 μg/ml tetracycline (Sigma Aldrich) and 2 ng/ml recombinant recombinant human GDNF (R&D Systems) for 2 days. LUHMES were then trypsinised and seeded onto the desired format at a density of 1.5*10^5 cells per cm². Unless stated otherwise, co-culture medium (Advanced Dulbecco’s modified Eagle’s medium/F12, 1x N2-supplement, 2 mM GlutaMax, 10 μg/ml tetracycline) was used for further differentiation and in co-cultures. **Immortalized mouse astrocytes (IMA2.1) cells:** IMA were grown in medium consisting of DMEM (Biochrom, Berlin) supplemented with 10% FCS (fetal calf serum, PAA) and 1% of penicillin/streptomycin. They were trypsinized for 2 min with 0.25% trypsin/DMEM and reseeded at a ratio of 1:10 when 80% of confluency was reached. **Normal human astrocytes (NHA) cells:** NHA cells (purchased from LONZA, catalog number CC-2565, Cologne, Germany) were grown in medium consisting of astrocyte basal medium (ABM™, catalog number CC-3187, LONZA) supplemented with AGM SingleQuot Kit Supplement & Growth Factors (catalog number CC-4123, LONZA). Cell culture maintenance procedures were performed according to the manufacturer’s protocol. **Caco-2, human epithelial colorectal adenocarcinoma cells,** (ATCC, HTB-37) cells were kindly provided by Dr. Regina Pape (University of Konstanz, Germany), and were cultured in EMEM (Sigma) supplemented with 20% FBS (PAA), 1 × MEM amino-acid solution (Sigma), 4 mM L-glutamine and 50 μg/ml gentamycin (Sigma). **A-375, human malignant melanoma,** (ATCC, CRL-1619) cells were kindly provided by Dr. Francesco Baschieri (Biotechnology Institute Thurgau, Kreuzlingen, Switzerland), and were cultured in DMEM (Biochrom, Hamburg) supplemented with 10% FBS (PAA). **Primary mixed rat glia:** Primary cortical astrocytes were generated from Wistar rats bred at the animal facility of the University of Konstanz, Germany. All rats were housed
at 22°C and 55% relative humidity in a 12h day/night rhythm with free access to food and water according to international regulations. Primary glia cells were prepared from rat pups at 24h post partum. In brief, brains were removed, cleared of the meninges, the cortices were mechanically homogenized by pipeting the tissue up and down and finally filtered through a nylon sieve (70 µm; BD Falcon, Bedford, MA). Cells were maintained in DMEM with 10% FCS and 1% of penicillin/streptomycin until they reached confluency. After one week experiments were performed. **Mouse astrocytes generated from embryonic stem cells (mAGES)** were kindly provided by Simon Gutbier (University of Konstanz). **Coating:** Neural precursor cells were grown on Nunclon™ (Nunc, Roskilde, Denmark) plastic cell culture flasks. These flasks were pretreated with 0.1 % gelatin in H₂O overnight. For differentiation cells were cultured in Falcon multiwell dishes. These dishes were pretreated with poly-L-ornithine (PLO) 1:100 in PBS overnight washed with PBS and H₂O and incubated with Laminin (1:1000 in PBS) overnight. **AGES differentiation to NPC’s:** 70000 cells/cm² in N2/B27 medium containing BMP4 (1:1000) were seeded into PLO/Laminin coated plates. After 72 hours cells were washed with PBS to remove remaining BMP4 and LUHMES cells were seeded on top for co-culture. **Human astroglioma CCF-STTG1** (ATCC, CRL-1718), cells were kindly provided by Dr. Regina Pape (University of Konstanz), and were grown in medium consisting of RPMI1640 supplemented with 10% FCS (fetal calf serum, PAA), 2% of penicillin/streptomycin, 2 mM Glutamine. NEP, neuroepithelial precursor cells were kindly provided by Nadine Dreser (University of Konstanz). **NEP, neuroepithelial cells.** Human embryonic stem cells (hESC) (H9 from WiCells, Madison, Wi, USA) were differentiated according to the protocol published by Chambers and colleagues (Chambers et al., 2009) with the following modifications. Instead of using 500 µM noggin we used the combination of 35 µM noggin and 600 nM dorsomorphine together with 10 µM SB-431642 for dual SMAD inhibition as described earlier (Balmer et al., 2012; Chambers et al., 2009). This was used to prevent BMP and TGF signaling, and thus to achieve a highly selective neuroectodermal lineage commitment. All differentiations were performed in 6 well plates containing 2 ml of medium each.

**Co-cultures**

For co-cultures, CACO-2 cells (at a density of 15.000/cm²), IMA cells (at a density of 15.000/cm²), NHA cells (at a density of 5.000/cm²), primary mixed rat glia (at a density of 15.000/cm²), human astroglioma (at a density of 15.000/cm²), A-375 cells (at a density of 15.000/cm²). After 24 h, medium was changed to LUHMES differentiation medium. After
additional 48 h, pre-differentiated LUHMES cells (d2) were seeded on top at a density of 150,000/cm². On day 6 of LUHMES differentiation, experimental treatment was performed.

**Cell viability assays**

The resazurin assay was performed as described previously (Falsig et al., 2004a; Schildknecht et al., 2009; Zimmer et al., 2011a). Lactate dehydrogenase (LDH) activity was detected in cell culture medium (supernatant) and cell lysates separately. LDH release was expressed as 100 x LDH\(_{\text{supernatant}}\)/LDH\(_{\text{supernatant}}\) + LDH\(_{\text{lysate}}\).

**Immunocytochemistry**

*Immunostaining for Tuj1, S100β, TH, VMAT2, NFκB:* Cells were grown on precoated plastic plates or glass cover slips (Menzel, Braunschweig, Germany) in 4-well plates. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in 2% sucrose for 15 min at RT. After washing twice with 0.05% PBS-Tween 20, cells were permeabilized with PBS/0.2% Triton X-100 for 10 min at RT, washed with 0.05% PBS-Tween 20 and incubated with PBS/1% BSA (Calbiochem, San Diego, CA, USA) for 1 h at room temperature for blocking. Then, primary antibodies diluted in 1% BSA/PBS were added overnight at 4°C. After 3 washing steps with 0.05% PBS-Tween 20, secondary antibodies diluted in PBS were applied for 1 h at room temperature in the dark. Hoechst-33342 (1 µg/ml, Molecular Probes) was added for 15 min prior to the final washing steps. Cover slips were then mounted on glass slides with FluorSave™ Reagent (Calbiochem). For visualisation, an Olympus IX81 inverted epifluorescence microscope equipped with a F-view CCD camera (Hamburg, Germany) and 10x air, 20x air and 100x oil objectives was used. For image processing, Cell^P software (Olympus) was applied. For confocal microscopy, images were taken with a Zeiss LSM 510Meta confocal microscope equipped with a Plan Apochromat 63x, NA 1.4 oil DIC lens.

**Quantitative image analysis**

Following treatment, cells were stained for the neuronal marker Tuj1. An Array-Scan VTI HCS Reader (Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera was used for image acquisition. Thirty fields per well (330 µm x 330 µm size each) were imaged in one channel using a 20 x objective. Image sets were analysed using the Thermo Scientific Cellomics Spot Detector Bioapplication V4 as described earlier (Krug et al., 2013). The software recognise the Tuj-positive pixels and calculates the total pixel number in 30 fields of one well. Three wells were analysed for each treatment. Then the total pixel number was normalised to the total pixel number in untreated control and presented as percentage.
**Astrocytes nuclei number count:** cells were stained with H-33342. The pictures in ten fields per well were taken and analysed by the software as mentioned above. Cells positive for H-33342 were recognised by the software and calculated in each field. Three wells were analysed for each treatment.

**Protein amount determination**

Cells were lysed in buffer containing 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P 40 substitute, 1% phenylmethylsulfonyl fluoride, 1 mM Na$_3$VO$_4$, pH 7.4; (all from Sigma-Aldrich) for 15 min on ice and scraped from the plates. After removal of cell debris via centrifugation, protein concentrations in supernatants were determined by using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s protocol.

**Release of human specific neuronal enolase**

The human neuronal enolase release in supernatants was analyzed using Human Enolase 2/Neuron-specific Enolase Quantikine ELISA Kit (R and D Systems) according to the manufacturer’s protocol.

**Amino acids content analysis**

Cell culture supernatants were collected, and SSA (sulfosalicylic acid) was added at a final concentration of 2%. Samples were incubated at 4°C for 30 min and then centrifuged at 10,000g for 10 min. Samples were diluted 1:1 with lithium citrate buffer (Sykam Chromatography, Fuerstenfeldbruck, Germany), and amino acid analysis was conducted in a Li-HPLC amino acid analyzer with ninhydrin post-column derivatization (S433, Sykam Chromatography, Fuerstenfeldbruck, Germany) for amino acid detection. The chromatograms were calibrated to an amino acid standard (Sykam Chromatography, Fuerstenfeldbruck, Germany) using the software ChromStar 7 (SCPA, Weyhe-Leeste, Germany).

**Detection of NO**

Nitrite (NO$_2^-$), the stable autooxidation product of •NO, was measured by the Griess assay. Briefly, 30 µl of 12.5 sulfanilamide (Sigma) and 30 µl 6 M HCl were mixed with 200 µl cell culture supernatant at a room temperature and incubated for 5 min. Absorbance was measured before and after the addition of 25 µl N-(1-naphthyl)ethylenediamine (12.5 µM) (Sigma) at 560 nm using a microtiter plate reader. Nitrite concentrations were calculated from NaNO$_2$ standard curve in the range of 0.5-10 µM.
Statistics

All data were confirmed in at least two (usually three) different experiments. Values are expressed as the mean ± SD (n≥3) (unless otherwise stated). Data were analysed by one-way ANOVA, two-way ANOVA or Student’s t-test as appropriate. Differences between treatment groups in multiple comparisons were determined by Bonferroni’s or Dunnet’s post hoc test (Graph Pad software, San Diego, CA, USA).
4. Results

4.1. Astrocytic support of neurons and attenuation of neurotoxicity

We used here a recently-established co-culture model (Efremova et al., 2015) of LUHMES neurons (Scholz et al., 2011) and IMA astrocytes (Schildknecht et al., 2012), that allows growth of the human neurons on top of murine astrocytes. Three-dimensional imaging by confocal microscopy confirmed that neurons are mostly positioned in a layer above the astrocytes, and that both neurites and somata establish large contact interfaces with astrocytes (B). Immunostaining showed that co-cultured LUHMES extended long neurites and expressed tyrosine hydroxylase (TH) as well as the vesicular monoamine transporter (VMAT) like LUHMES monocultures (Figure S1). We also found that neuronal cultures were more easily maintained in the presence of IMA astrocytes (viability close to 100% on d12 of neuronal differentiation) than as mono-cultures (variable viability on d12 with a range of 50-100%).

Therefore, we investigated whether other cell types would generally support LUHMES neurons. Amongst the panel of eight cell lines/populations investigated, we found two major types of behavior. IMA, primary rat glia, stem cell-derived murine astrocytes, and commercially-obtained normal human astrocytes (NHA) allowed LUHMES neuronal precursor attachment, growth of neurites and establishment of intricate networks, while human non-brain tumor cells or human stem-cell derived neuroepithelial precursors did either not allow neuronal attachment or otherwise generated a hostile environment so that plated neuronal precursors clustered together, failed to extend neurites, and died. Human astroglioma (CCF-STTG1) took a somewhat intermediate position, in that attachment of LUHMES was poor (clustered together), but then thick cables of neurite assemblies were extended between the somata clusters (Figure S2). Thus, IMA behaved similar to non-transformed astrocytes, in that they passively supported neuronal growth and viability.

To examine whether IMA provided also a more active form of protection we challenged LUHMES neurons by the peroxynitrite donor SIN-1. A significant decrease of neuronal viability was observed in LUHMES monocultures, whereas neurite integrity and viability of co-cultured neurons was not affected (Figure 1C). Astrocytes are generally much more robust towards peroxynitrite (Leist et al., 1997), and accordingly they were not affected by the SIN-1 concentrations used here. Encouraged by these findings, we tested three different neurotoxicants and quantified neuronal viability/network complexity by βIIIItubulin staining. At a low concentration of 1 µM, the specific dopaminergic neurotoxicant MPP⁺ led to a 50% decrease of neuronal area, while viability was fully maintained in the presence of IMA. At
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higher concentrations (5 µM), IMA provided partial protection, and at even higher concentrations (≥ 10 µM; never reached in vivo) the protective effect was eventually lost. For the peroxynitrite donor SIN-1 and the NO-releasing agent spermineNONOate, full protection by IMA was observed even at concentrations that killed > 90% of monocularly cultured neurons (Figure 1C). These data are in good agreement with many literature findings of astrocytic support of neurons, e.g. by provision of glutathione precursors (Pizzurro et al., 2014) or other mechanisms.

4.2. Switching from neuroprotective to neurotoxic properties of astrocytes exposed to pro-inflammatory cytokines.

Astrocytes and other glia have not only been associated with neuroprotection, but have also been implicated in mediating neurotoxicity. These discrepant properties might be due to different activation states. For instance, astrocyte functions may be altered during brain damage and neuroinflammatory disease, and this may affect neuronal viability (Colangelo et al., 2014a; Hamby et al., 2010) by reduced astrocytic support or by active contribution of inflammatory mediators to damage. As little is known about how human neurons are affected in such situations, we used the IMA-LUHMES co-culture model to address this question.

To model an inflammatory situation, we used a cytokine mix (TNF-α and IL-1β) known to be produced in neuroinflammatory situations mainly by microglia, and established to activate astrocytic cells, including IMA (Falsig et al., 2006; Henn et al., 2011; Kuegler et al., 2012; Schildknecht et al., 2012). This CM (exposure period of up to 6 days) had no effect on the viability and neurite structure of LUHMES monocultures (Figure S3A). The absence of an effect was confirmed, when individual cytokines were tested or even when a third cytokine, IFN-γ, was added to CM (Figure. S3B). Moreover, CM did not affect the toxicity of SIN-1 or NO in LUHMES monocultures (Figure S3C).

Having established, that CM treatment does not affect neurons directly, we tested whether it would alter the protective effect of glia in co-cultures. To this end, we exposed cells to CM at 24 h before the treatment with toxicants (1 mM SIN-1, 100 µM spermineNONOate). The CM reduced the neuroprotective effect of IMA significantly (Figure 2A).
D. Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation

Figure 1. Alteration of neurotoxicity by co-cultivation with glial cells.

(A) Human neurons (derived from LUHMES cells) were cultivated on top of a layer of immortalized murine astrocytes (IMA) as indicated in the scheme. Cultures of LUHMES and IMA were stained on day 4 of co-cultivation i.e. d6 of LUHMES differentiation for the neuronal-specific marker Tuj1 (βIII tubulin, red), the astrocyte-specific marker S100β (green), and with the DNA stain H-33342 (blue). A z-stack of images was recorded on a confocal microscope (scale bar = 20 µm). The x-z and y-z sections show the localization of neurons and astrocytes in different focal planes (as schematically shown). (B) LUHMES monocultures (d6, ‘-IMA’) or IMA-LUHMES co-cultures (d6 of LUHMES, ‘+ IMA’) were exposed to SIN-1 (1000 µM) for 24 h. Then, cells were fixed and stained to visualize βIII tubulin and nuclei (H-33342); scale bar = 100 µm. (C) LUHMES monocultures and co-cultures (d6) were exposed to various concentration of MPP⁺ (0 - 100 µM), SIN-1 (0 - 1000 µM) and spermineNONOate (0 - 100 µM) for 24 h. Then, cells were fixed, stained for βIII tubulin, and the βIII tubulin-positive neuronal area was quantified by an automated imaging procedure. Data are expressed as means ± SD of quadruplicates.* p < 0.05 (co-culture vs. monoculture).

While short exposure (< 3 days) to CM did not reduce neuronal viability in co-cultures (but reduced glial protection from NO), exposure to CM for six days led to pronounced neurodegeneration without any other toxicant added (Figure 2B). As standard endpoint to quantify neurodegeneration in co-cultures, we used specific staining of betaIII tubulin and an
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automated algorithm to quantify the overall neuronal area (Efremova et al., 2015). This end-point correlated with the measurement of the release of neuron-specific enolase (Figure 2C).

Figure 2. Switching from neuroprotective to neurotoxic properties of glia by exposure to inflammatory cytokines.

(A) Co-cultures (d6 of LUHMES, + IMA) and LUHMES monocultures (d6) were exposed for 24h to a cytokine mix (CM) consisting of murine IL1ß (10 ng/ml) + murine TNFα (10 ng/ml) before they were treated with SIN-1 (1000 µM) or spermine NONOate (100 µM) for additional 24 h. Then, cells were fixed, stained for βIIItubulin, and the βIIItubulin-positive neuronal area was quantified by an automated imaging procedure, * p < 0.05 (exposed to CM vs. unexposed). (B) Co-cultures (d6) were exposed to CM for 3 or 6 days. Then, neurons were stained for βIIItubulin, and images were recorded on a fluorescent microscope. (C) IMA-LUHMES co-cultures (d6) in 24 well plate were exposed to CM or to 10 µM MPP+. After 6 days, cultures were stained for βIIItubulin, and the βIIItubulin-positive neuronal area was quantified by an automated imaging procedure. The release of human neuron specific enolase (NSE) was measured in culture supernatants. (D) IMA-LUHMES co-cultures (d6) were exposed to murine IL-1ß (10 ng/ml), murine TNF-α (10 ng/ml), murine IFN-γ (20 ng/ml), to CM, or to a complete cytokine mix (CCM: IL-1ß+TNF-α+IFN-γ) for 6 days. Then, cultures were stained for βIIItubulin, and the neuronal area was measured by an automated imaging procedure. Data are expressed as means ± SD of quadruplicates.

This glycolytic isoenzyme, which is widely used as a specific read out for the assessment of neuronal damage level (Hans et al., 1993; Morishita et al., 1997), is only expressed in
neurons, but not glial cells. In order to find out which cytokine was involved in glial cell-mediated neurodegeneration, we exposed LUHMES-IMA co-cultures to IL-1β, TNF-α and IFN-γ individually, and in combinations. Both, IL-1β and TNF-α were able to trigger the degenerative response, while IFN-γ was without effect (Figure 2D). To ensure a robust response, we selected the combination of TNF-α and IL-1β (= CM) for our further experiments.

4.3. Signalling events occurring in reactive astrocytes exposed to cytokines

Since CM did not affect neuronal viability in monocultures, but led to neurodegeneration in LUHMES-IMA co-cultures, we assumed that the primary target were the astrocytes. To obtain direct evidence, IMA monocultures were exposed to CM. After 2 days, the cultures started to change morphology and first elongated cells were observed. On day four more spindle-shaped cells appeared, and on day 6 all cells were elongated and orientated along parallel lines (Figure 3A). This change was accompanied by other common features of reactive astrocytes, such as increase of protein content and proliferation (Figure 3B,C). The immediate cytokine response (within 30 min) was reflected by NF-kB translocation (Figure 3B-D), similar to the one observed in primary astrocytes (Henn et al., 2011).

As NO is a candidate for glia-dependent neurotoxicity (Brown et al., 2010), we examined the production of this neuromediator, but no such activity was detected by an established analysis method (nitrite detection in cell culture supernatants). For control purposes, IFN-γ was added to the CM, and under these altered conditions, pronounced and continuous (over 5 days) NO production was measured (Figure 3E). We conclude from this, from the data in Figure 2D, and from the absence of any protective effect of very effective (Falsig et al., 2004a; Schildknecht et al., 2012) nitric oxide synthase inhibitors (Figure S4) that NO is unlikely to be involved in the neurodegeneration observed in our model.

4.4. Pharmacological protection of neurons from cytokine toxicity in co-cultures

To obtain additional evidence on the inflammatory nature of neurodegeneration in the LUHMES-IMA-CM model, we tested the effect of the anti-inflammatory corticosteroid dexamethasone. This drug had been shown to protect DA neurons in the in vivo MPTP model via a decrease of the inflammatory reaction of glial cells (Kurkowska-Jastrzebska et al., 2004).
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Figure 3. Astrocyte activation and proliferation by exposure to cytokine mix (CM).

(A) IMA monocultures were exposed to murine CM (IL1ß+TNF-α) for 2, 4 and 6 days. Then, cells were fixed, permeabilized and immunostained for the astrocyte-specific marker S100β (green); nuclei were stained with H-33342 (red), scale bar = 100 µm. (B) IMA cells were exposed to CM for 6 days. Then cells were lysed, and the protein amount in lysates was assessed. (C) For assessment the number of nuclei, IMA monocultures were fixed and stained with H-33342 after 6 days of CM exposure. Three wells of 24 well plates were analysed for each treatment conditions. (D) IMA monocultures were stimulated with CM or solvent for 30 min. Then cells were stained with an anti-NF-κB p65 antibody (green), nuclei were stained with H-33342 (red). Images were taken by using a fluorescent microscope, scale bar = 100 µm. (E) IMA cells were exposed to CM (TNF-α+IL-1β) with or without IFN-γ. The nitrite concentrations in the cell culture supernatant were measured by using the Griess method. Nitrite served as an indicator for NOS activity and subsequent NO release. Data are expressed as means ± SD of quadruplicate samples.

Neurons in co-cultures treated with dexamethasone could be completely protected from CM toxicity (Figure 4A). As the effects were observed only at high micromolar concentrations, we followed up on these findings by testing whether dexamethasone effects were mediated by its nuclear receptor. To this end, we compared the potential neuroprotection
by 100 µM of the synthetic corticosteroid in the presence and absence of the glucocorticoid receptor antagonist mifepristone (0 – 10 µM). Mifepristone alone had no effect on neuronal viability (neither in normal co-cultures nor in degenerating cultures exposed to CM). However, it abolished the protective effect of dexamethasone in a concentration-dependent manner, with maximal effects reached between one and three µM (Figure 4B). In contrast to the broadly-acting corticosteroid, several other treatments that may affect inflammatory responses did not alter neuronal survival. Test compounds included various inhibitors of NF-kB, of cyclooxygenases (indomethacin, ibuprofen), of the adenosine A2a receptor (KW6002), and of protein secretion (brefeldin A). Thus, our model allows exploration of gliomodulatory drug candidates, with dexamethasone as positive control, but our initial screening experiments did not give further clues on the glial signaling pathway involved in the neurodegeneration.

**Figure 4. Protection from cytokine toxicity in co-cultures by dexamethasone.**

(A) Co-cultures of IMA and LUHMES (d6) were pre-treated with various dexamethasone concentrations (0 - 200 µM) for 30 min, followed by the addition of CM (=TNF-α+IL-1β) for 6 days. Then, the cultures were stained for βIII tubulin, and the βIII tubulin-positive neuronal area was quantified by an automated imaging procedure. (B) Co-cultures were pretreated for 30 min with different concentrations of mifepristone (0 - 10 µM) before dexamethasone (0 or 100 µM) and CM was added for 6 days. Then, the neurite integrity was evaluated by staining for βIII tubulin, and quantification of the βIII tubulin-positive neuronal area. Data are expressed as means ± SD of triplicate samples.

4.5. Essential reproduction of model features after substitution of IMA by human astrocytes

Although the use of murine IMA cells for the co-culture model has many advantages (robustness, ease of preparation, easy analysis of specific cell responses on mRNA and protein level (Efremova et al., 2015)), there may be situations that would require human
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Moreover, we wanted to be sure that the effect observed was not an interspecies artifact, but reproducible with human astrocytes. Therefore, we substituted IMA for human astrocytes (NHA) and tested their response to the human version of CM. Upon stimulation, they underwent a morphological change similar to IMA (spindle-shaped, parallel-oriented cells), and triggered degeneration of LUHMES cells (Figure 5A). The response was faster than with IMA, as already after four days, >50% of the βIII tubulin staining was lost (Figure 5B). Concerning the pharmacological properties, also the full protection by dexamethasone was faithfully reproduced (Figure 5C), although at somewhat lower concentrations (low µM range) than in the IMA-LUHMES model. In summary, human astrocytes behaved similar to IMA concerning major features of the co-culture cytokine mediated neurodegeneration.

4.6. Neurotoxicity triggered by activated glia through the activity of soluble factors

In order to assess whether soluble transferrable factors play a role in neurotoxicity mediated by activated glia, IMA monocultures were exposed to CM and conditioned medium was collected after 6 days. It was transferred to d6 differentiated LUHMES monocultures, and neuronal viability was monitored. Immunostaining showed that after 3 days, about 50% of the neurons had died (Figure 6A). Measurements of the viability by resazurin (not shown) or quantification of the neuronal area confirmed neuronal death triggered by CM supernatants, but not control supernatants from IMA (Figure 6B). Time course experiments of supernatant sampling indicated that maximal neurotoxic activity of the IMA medium was reached after about 5 days (Figure 6C). Freezing, and storage for up to 2 weeks had no effect on this activity, but dilution with fresh culture medium led to a reduction of neurotoxicity (Figure 6D). For practical purposes, 3 parts of IMA supernatants were diluted with 1 part of fresh medium for all following experiments. The astrocyte density used for production of conditioned medium did not play a major role in the range of 7.5-30.000 cells/cm². At higher cell densities, also control supernatants became toxic to neurons (Figure S5). At the production conditions used here (15.000 IMA/cm²), we examined whether the composition of conditioned medium changed drastically. Amino acid analysis showed that cytokine-conditioned medium differed for few amino acids from control supernatant, but many constituents remained largely unchanged (Figure S6A). As further control, we checked whether re-addition of factors important for neuronal viability and possibly depleted by IMA would alter the toxicity of the conditioned supernatant. However, no effect was observed for GDNF (2 ng/ml), N2 supplement, pyruvate or glucose (Figure S6B). Thus, presence of a
factor produced by IMA upon cytokine stimulation is the most straightforward explanation for neurotoxicity of the conditioned medium.

Figure 5. Cytokine toxicity in human neuron-astrocyte co-cultures.

(A) Human neurons (LUHMES) were cultivated on top of a confluent layer of normal human astrocytes (NHA). Co-cultures of normal human astrocytes (NHA) and LUHMES cells (d6) were exposed to CM (human 10 ng/ml IL-1ß + human 10 ng/ml TNF-α) for 4 days. Then, co-cultures were stained for the neuronal-specific marker Tuj1 (ßIIItubulin, b/w), the astrocyte-specific marker S100ß (green), and the DNA stain H-33342 (red). Images recorded on a fluorescent microscope. (B) NHA-LUHMES co-cultures were exposed to human CM for 2 or 4 days. Cultures were stained for ßIIItubulin, and the ßIIItubulin-positive neuronal area was quantified by an automated imaging procedure. ** P < 0.01 control vs. human CM exposure for 4 days. (C) Co-cultures (d6 of LUHMES, + NHA) were pre-treated with various dexamethasone concentrations (0 - 100 µM) followed by the addition of human CM for 4 days. Then, cultures were stained for ßIIItubulin, and the ßIIItubulin-positive neuronal area was quantified by an automated imaging procedure. Data are expressed as means ± SD of triplicate samples. * P < 0.05 treated with dexamethasone vs. untreated.

4.7. Pharmacological protection from glia-conditioned neurotoxic medium

The possibility to separate IMA stimulation and neuronal exposure physically allowed now a pharmacological characterization of the neuro-damaging effects of astrocytes on the level of the target cell. We used this big advantage of the new model for some targeted interventions (Figure 7). Boiling of conditioned medium did not decrease its toxic properties. This suggests that factors different from proteins are responsible for the neurotoxic effect. Dexamethasone did not protect LUHMES. Thus, its effect in the co-cultures was most likely due to immunomodulation of the glia. Neither the antioxidant tripeptide GSH nor its oxidized variant (GSSG) showed a protective effect. In this respect, the toxicity of conditioned supernatant differed from MPTP toxicity in LUHMES-IMA co-cultures where GSSG showed a powerful neuroprotection (Efremova et al., 2015). Two p38 inhibitors did not show protection, and
neither did other anti-inflammatory compounds such as NF-kB inhibitors and cyclooxygenase inhibitors (Figure S4). The ROCK inhibitor Y-27632 showed weak protection, also in contrast to the MPTP model, where it was very efficacious. However, several other compounds protected very efficiently from the toxicity of IMA/cytokine-conditioned supernatant: the H₂S donor (ATB-337), high concentrations of GDNF (100 ng/ml), the PARP-inhibitor DHQ, the phenolic plant ingredient and highly bioactive response modifier resveratrol, the caspase inhibitor zVAD, and the mixed-lineage kinase inhibitor CEP1347. More detailed concentration-response studies confirmed all initial findings (Figure 7B). These data suggest the activation of several pathways known to be associated with neurotoxicity. Targeting of such pathways may result in new approached to modify detrimental effects of neuroinflammation.

An important control of the sequential model of IMA activation and supernatant transfer is the effect of dexamethasone. This drug acted in co-cultures, but not on LUHMES cells. It would therefore be expected that the drug would act during the phase of IMA stimulation, and this was tested in a new pharmacological setup. The drug (100 µM) was co-incubated with CM during the phase of IMA stimulation. This conditioned supernatant was not toxic to neurons. A transfer of the drug from IMA onto neurons cannot be avoided in this setup, but as dexamethasone alone had no protective effect, when applied directly on LUHMES, we conclude that it must have acted on IMA. These studies were repeated at multiple dexamethasone concentrations, and half-maximal effects were observed at 1 µM, while partial protection was already observed at 10-100-fold lower concentrations (Figure 8A). Thus, dexamethasone showed it expected pharmacological potency in this sequential model, while the co-culture had right-shifted the concentration-response considerably. Encouraged by these findings, we tested the effect of a new class of anti-inflammatory agents, i.e. p38 MAP kinase inhibitors (Schindler et al., 2007). They were selected, as they did not affect neurons directly, and as they had proven earlier to modify complex astrocyte responses (Falsig et al., 2004b). Both drugs reduced neurotoxicity of glia-conditioned medium in the sub-micromolar range (Figure 8B,C). These data show that drugs targeting specific regulation of inflammatory responses in astrocytes may have neuroprotective potential in neuroinflammatory conditions.
D. Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation

Figure 6. Mediation of neurotoxicity of inflammatory astrocytes by soluble, transferrable factors.

(A) IMA monocultures were exposed to CM for 6 days. Then, conditioned medium was collected, diluted (3+1) with fresh LUHMES differentiation medium and transferred to d6 LUHMES for 3 additional days. Afterwards, cells were fixed and stained for βIIItubulin (green), the DNA stain H-33342 (blue), and images were recorded on a fluorescent microscope. The βIIItubulin-positive neuronal area was quantified by an automated imaging procedure. Data are expressed as means ± SD of triplicate samples (B). (C) IMA monocultures were incubated in presence or absence of murine CM. Each 24 h (within the time period of 6 days) conditioned medium from untreated (dashed line, control supernatant) and treated (black line, CM) IMA was collected, filtered and frozen at -20°C. After, conditioned medium was thawed, 100% of it was transferred to LUHMES monoculture (d6) for following 3 days. Then, the neuronal viability was measured by the resazurin reduction assay. Data are expressed as means ± SD of quadruplicates. (D) IMA were exposed to CM for 6 days, then different amount of conditioned medium (0-100% of total medium) was transferred to d6 LUHMES cells for following 3 days. The viability was measured by the resazurin reduction assay. Data are means ± SEM of three different experiments.
Figure 7. Pharmacological protection from glia-conditioned neurotoxic medium.

(A) Three parts of conditioned medium from control IMA monocultures (control supernatant) or IMA exposed to CM for 6 days were mixed with one part of fresh LUHMES differentiation medium, and used to culture d6 LUHMES monoculture for 3 days. This medium was supplemented with putative neuroprotective compounds: dexamethasone (100 µM), GSH (100 µM), GSSG (100 µM), Y-27632 (10 µM), H₂S donor – ABT-337 (10 µM), glial derived neurotrophic factor (GDNF, 100 ng/ml), the PARP inhibitor DHQ (50 µM), resveratrol (1 µM), the caspase inhibitor zVAD (100 µM), the mixed lineage kinase inhibitor CEP1347 (300 nM). Alternatively, it was boiled (10 min at 100 °C). The neuronal viability was measured by the resazurin reduction assay. Data are means ± SEM of three different experiments. * P < 0.05, control supernatant vs. CM-supernatant. (B) Conditioned medium as in A from control or CM-exposed IMA was transferred to d6 LUHMES monocultures, and various concentrations of drugs were added: dexamethasone, GSSG, DHQ, resveratrol, zVAD, CEP1347. After 3 days, the neuronal viability was measured by the resazurin reduction assay. Data are expressed as means ± SD of triplicate samples from one experiment.
Figure 8. Protection of neurons by prevention of astrocyte activation.

(A) IMA monocultures were pre-treated with two different p38 inhibitors (SB202190 or SB203580); or with dexamethasone for 30 min before stimulation with CM. After six days, conditioned medium was collected, the diluted (3+1) with fresh medium, and transferred to d6 LUHMES monocultures for 3 additional days. Afterwards, the neuronal viability was measured by the resazurin reduction assay. Data are expressed as means ± SD of three experiments.
5. Discussion

For years studies of pathological processes in the brain have been focused on neuronal abnormalities, but it is becoming obvious that other neural cells may also be important players in neurodegenerative diseases. Indeed, normally neuron-supportive glial cells undergo dramatic alterations in pathology and currently emerge as the additional culprits of CNS disorders. For instance, a depletion of activated astrocytes in mouse model of MS ameliorated the disease in the chronic phase (Mayo et al., 2014), indicating their contribution to the pathological progress. Molecular, temporal and functional interconnections amongst various signaling pathways controlling astrocytes reactivity are extremely complex, and still largely unknown. Moreover, an exact contribution of reactive astrogliosis to neuronal demise is still to be studied in detail. However, the lack of appropriate in vitro models hampers studies of molecular mechanisms involved and a search for potential therapeutical approaches.

Our data provide the clear evidence that astrocytes might trigger neuronal death upon activation. The co-culture model established here allowed us: 1- to assess the classic neuroprotective properties of astrocytes; 2- to trigger the transition from the quiescent to the activated state followed by degeneration of human neurons; 3- to test the pharmacological interventions targeting either astrocytes or neurons.

First, in our model the common protection properties of astrocytes were observed. Astrocytes protected neurons against the neurotoxicants apparently through supply of antioxidant molecules (e.g. GSH), since the protective effect was abolished by buthionine sulfoximine, an inhibitor of GSH synthesis (data not shown).

Second, in several experimental inflammation models it was shown that astrocytes react to proinflammatory cytokines (TNF-α, IL-1β) released from microglia, which represents a first line of innate immune response in the brain, (Kohutnicka et al., 1998; Rohl et al., 2007). Using our model we could recapitulate the steps of astrocytes activation upon stimulation. This process involves following morphological and functional features: total protein levels increase; cell proliferation and hypertrophy. The latter results in appearance of the spindle-shaped cells elongated and orientated in parallel to each other that is reminiscent of migration process which astrocytes undergo during the acquisition of the reactive phenotype (Buffo et al., 2010).

Third, we observed that astrocytes reaction led to a neuronal death. Activated astrocytes may mediate a neuronal death both through direct membrane interactions (disruption of
attachment, changes in integrin profiles) and by the release of factors that bind to neuronal receptors. We exclude the first possibility, since human neurons plated on activated astrocytes were able to attach and build a dense network without any degenerative features (data not shown). We confirmed the second hypothesis using the soluble factors transfer approach. We suggest that this is a consequence of increased secretory activity of astrocytes, and the soluble factors released upon cytokine stimulation confer a toxic effect on neurons.

We showed that the neurotoxic effect of conditioned medium from activated astrocytes is caused not by the depletion of the nutrients in the medium, but rather by the neurotoxic soluble factors released into it. Activated astrocytes secrete a number of factors. Some of them create an autocrine loop of astrocytic activation, e.g. ATP (Coco et al., 2003), enormous array of growth factors (Buffo et al., 2010); lipocalin 2 (Lee et al., 2009), cytokines (Oleszak et al., 1998). However, there are also factors secreted by activated astrocytes, which might be harmful for neurons. Several of in vitro models attempted to identify putative astrocytes-derived neurotoxic factors. (Garwood et al., 2011) showed that astrocytes release soluble inflammatory factors such as neutrophil chemoattractant (CINC) 2αβ, IFN-γ, IL-1β, IL-1ra, IL-6, IL-13, IL-17, IP-10 and monokine induced by IFN-γ (MIG). An inhibition of astrocyte activation by the anti-inflammatory agent, minocycline, reduces astrocytic inflammatory responses and the associated neuronal loss. (Bi et al., 2013) showed that lipocalin 2 (lcn2) is secreted by reactive astrocytes and is selectively toxic to neurons.

Since the neurotoxic effect of conditioned medium is preserved upon its freezing, thawing and boiling, we may exclude proteins and short-lived substances as free radicals, ROS. The putative responsible factors might be lipids, glycan/polysaccharide or glycolipids that might also exert neuro-damaging effects. Indeed, the number of active glia-derived nonproteinaceous factors was found. The stable nonproteinaceous neuron-killing factors secreted by microglia were identified by (Giulian et al., 1993). In the mouse model of MS astrocytes secreted a glycosphingolipid - Lactosylceramide (LacCer), and an inhibition of its synthesis suppressed local CNS immunity and neurodegeneration (Mayo et al., 2014). However, the LacCer-mediated molecular pathways resulting in neuronal death are not revealed yet. In the in vitro model of AD astrocytes stimulated with Aβ peptide secreted exosomes enriched with proapoptotic ceramide affecting neuronal viability (Wang et al., 2012). The neurotoxic potential of this factor is also not studied yet. Overall, it is technically challenging to define the precise astrocytes-released neuron-killing factors.
In our study, the analysis of conditioned medium content from activated and quiescent astrocytes might point the existence of the new key players responsible for neuronal demise. To further identify the class of compounds responsible for neurotoxic effect from astrocyte-derived condition medium, several enzymes might be used such as lipase, protein kinase K/peptidase or amylase. Alternatively, the fractionation of conditioned medium might be performed. However, these approaches might lead to a depletion of the essential components required for the survival of human neurons in control conditions impeding than the revealing of responsible factors. Therefore, high-throughput analytical methods, e.g. LC-MS analysis, must be applied to analyze the precise content of supernatants.

However, the neuronal death observed in co-culture conditions might occur due to a combination of soluble factors released and additional causes. Indeed, astrocytes might not only trigger but also exacerbate neuronal damage by a number of additional mechanisms. Since a long time microglia have been considered to be one of the main components of the complement-phagosome pathway in the brain (Bodea et al., 2014; Depboylu et al., 2011). However, it was shown that astrocytes might also express functional receptors of the complement system (Biber et al., 2002) and therefore be involved in phagocytosis. Additionally, they may secrete other molecules (chondroitin sulphate proteoglycan (CSPG)) halting axonal regeneration (Silver et al., 2004). Finally, a wide variety of important/supportive astrocyte functions become transiently or permanently impaired (reduced trophic support, GSH depletion) during brain damage (including diseases and brain injury) and may contribute to decrease of neuronal viability (Hamby et al., 2010). Moreover, the neuron-glial interaction is a reciprocal process. Indeed, neuronal state also strongly influences the astrocytic properties (Heneka et al., 2015; Lee et al., 2010a; Valenza et al., 2015). Therefore, the application of certain neuron-specific degenerative stimuli with followed supernatant transfer to astrocytes will reveal the additional impact of degenerating neurons on astocytic inflammatory status.

Additionally, the pharmacological interventions used in our model defined two targets of manipulation: neurons and astrocytes. Several compounds were applied to IMA during stimulation process (COX-2 inhibitors, A2A receptor antagonist, iNOS inhibitors, NF-kB and protein secretion inhibitors) allowed us to exclude the involvement of these pathways in astrocytes neurotoxicity, since the subsequent conditioned medium transfer to neurons remains toxic.
Other set of compounds seems to “work” rather on a side of neurons than suppress astrocytes reaction (zVAD, CEP; resveratrol, DHQ, GDNF, Rock inhibitor), since they were potent to increase neuronal viability being applied together with neurotoxic conditioned medium to neurons.

Glucocorticoids are potent anti-inflammatory drugs that have long been used in clinical neurology for the treatment of brain inflammation and brain injury. Dexamethasone protects DA neurons in a mouse model of PD (Kurkowska-Jastrzebska et al., 2004), it has also prevented the degenerative effect of a single intranigral injection of LPS on the DA system (Castano et al., 2002), and was also potent in our co-culture model. The exact mechanism of neuroprotection by dexamethasone in our system is not clear. However, it was shown that dexamethasone decreases astrocytes numbers by reducing glucocorticoid receptor expression in vitro and in vivo (Unemura et al., 2012).

A number of compounds still might be tested in our system. In case of astrocytes several attempts were made to reinforce or restore their protective properties. An activation of Nrf2-ARE pathway in astrocytes resulted in elevated GSH production (Steele et al., 2013). The urate application to astrocytes triggers release of molecule(s) that are protective for neurons (Cipriani et al., 2012). Several substances have been shown to exert anti-inflammatory properties especially in astrocytes at extremely low concentrations: naloxone, ouabain, and bupivacaine (Block et al., 2012; Lundborg et al., 2011).

Obviously, a combination of two drugs (one – neuroprotective, second – reducing the astrocytes reaction) would be efficient to decrease glia-mediated neurodegeneration.
6. Supplementary figures.

![Supplementary figures](image)

**Figure S1. Co-culture characterization.**

(A) Co-cultures of LUHMES and IMA were stained for the neuronal-specific marker TuJ1 (βIII tubulin, red) and for the astrocyte-specific marker S100β (green). Nuclei were stained with H-33342 (blue). Images were recorded on confocal microscope, scale bar = 20 µm. (B) LUHMES differentiated in monocultures and co-cultures were stained on day 6 (d6) for tyrosine hydrolase (TH). Nuclei were stained with H-33342. Images were taken by using a fluorescent microscope scale bar = 100 µm. (C) LUHMES differentiated in monocultures and co-cultures were stained on day 6 (d6) for VMAT-2. Nuclei were stained with H-33342. Images were recorded on confocal microscope, scale bar = 20 µm.
Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation

Figure S2. Compatibility and incompatibility of different cell types with neuronal cultures.

Several different cell lines were grown in LUHMES differentiation medium for 2 days before predifferentiated LUHMES cells (d2) were seeded on top of their confluent monolayer, and differentiated further for 4 days in LUHMES differentiation medium. Then, co-cultures were stained for the astrocytes (S100ß) and neuronal (βIII tubulin) specific markers. Nuclei were stained with H-33342. Images were recorded on fluorescent microscope, scale bar = 100 µm. Abbreviations: CACO-2: human epithelial colorectal adenocarcinoma cells; NEP: neuroepithelial precursor cells; A375: human malignant melanoma. IMA: immortalized mouse astrocytes; mAGES: mouse astrocytes generated from embryonic stem cells, LUHMES, lund human mesencephalic cells.
Figure S3. Resistance of LUHMES monocultures to direct cytokine effects.

(A) LUHMES monocultures (d6) were exposed to CM for 6 days. Then, cells were stained for βIII-tubulin, and neuronal area was measured by automated imaging procedure. Data are expressed as means ± SD of triplicate samples. Representative images of LUHMES monocultures exposed to CM. After treatment, cells were fixed, permeabilized and immunostained for βIII-tubulin (green). Nuclei were stained with H-33342 (red). Images were recorded on fluorescent microscope, scale bar = 100µm. (B) LUHMES monocultures (d6) were exposed to cytokine mix (CM): mouse IL1β (10 ng/ml) + mouse TNF-α (10 ng/ml) for 24h. Then, cells were treated with SIN-1 (1000 µM) and spermineNONOate (100 µM). After 24h, cells were fixed, stained for βIII-tubulin, and the βIII-tubulin-positive neuronal area was quantified by an automated imaging procedure. (C) LUHMES monocultures (d6) was exposed to mouse IL-1β (10 ng/ml) or mouse TNF-α (10 ng/ml) or mouse IFN-γ (20 ng/ml), or to combination of IL-1β+TNF-α (CM=cytokine mix), or to combination of IL-1β+TNF-α+IFN-γ (CCM=complete cytokine mix) for 6 days. Afterwards, viability assays: the resazurin reduction assay and LDH release were performed.
<table>
<thead>
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<th>Compounds</th>
<th>Protection on glia (supernatant transfer)</th>
<th>Protection on neurons (supernatant transfer)</th>
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<tr>
<td>ATB-337</td>
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<tr>
<td>H₂S donor</td>
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<tr>
<td>GDNF</td>
<td>-</td>
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<tr>
<td>Neurotrophic factor</td>
<td></td>
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<tr>
<td>DHQ</td>
<td>-</td>
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<tr>
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<tr>
<td>zVAD-OMe-fmk</td>
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<tr>
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<tr>
<td>Y-27632</td>
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<tr>
<td>Rock inhibitor</td>
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<tr>
<td>Resveratrol</td>
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<tr>
<td>KW6002</td>
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<td>-</td>
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<tr>
<td>A2A receptor antagonist</td>
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<tr>
<td>Indometacin/Ibuprofen</td>
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<td>COX-2 inhibitors</td>
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<td>Brefeldin A</td>
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<td>Cay10512</td>
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<td>SB202190</td>
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<td>p38 inhibitor</td>
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Figure S4. Comparison of potential neuroprotective compounds in two different exposure scenarios.

Protection glia column: IMA monocultures were exposed to various compounds for 30 min followed by the addition of CM. After 6 days, was collected, diluted (3+1) with fresh differentiated LUHMES medium, and transferred to LUHMES neurons for 3 following days. Protection on supernatant transfer column: LUHMES monocultures were exposed (for 3 days) to 75% of supernatant from 6 days CM-stimulated astrocytes supplemented with 25% of fresh LUHMES differentiation medium, in presence of putative neuroprotective compounds. Viability of LUHMES monocultures in both approaches was detected by the resazurin reduction assay. (+) = protection effect with the lowest compound concentration; (-) = no protection observed at any concentration.
Different cell numbers of IMA cells were seeded per well of 24 well plate, after 2 days IMA medium was exchanged to LUHMES differentiation medium, and after following 4 days cells were exposed to CM for 6 days. Then, the conditioned medium was collected, diluted (3+1) with fresh differentiation LUHMES medium, and transferred to d6 LUHMES monoculture for 3 following days. Then, the neuronal viability was measured by the resazurin reduction assay. Usual experimental set up (IMA cell density) for all conditioned medium experiments is underlined as black rectangle. Data are expressed as means ± SD of triplicates.
Figure S6. Characterization and replenishment of IMA supernatants.

(A) IMA monocultures were exposed to CM. After 6 days, conditioned medium from treated (CM) and untreated (supernatant) IMA was collected and analyzed by amino acid analyzer. (B) IMA monocultures were exposed to CM. After 6 days, conditioned medium from treated (CM) and untreated (supernatant) IMA was collected and 75% of it was transferred to d6 LUHMES monoculture for 3 days in presence of N2 supplement, GDNF (2 ng/ml), Glucose (20 mM), pyruvate (100 mM) or combination of them (all). The neuronal viability was measured by resazurin reduction assay. Data are expressed as means ± SD of triplicates.
Chapter E

General discussion

Overall the aim of this thesis was the characterization of the established co-culture in vitro systems based on human neurons and astrocytes for modeling of PD-related events, and its application for mechanistic and pharmacological studies.

The thesis contains two publications and one prepared manuscript that all discuss their individual findings in the chapters B, C, D. The following text is a concluding discussion summarizing the major achievements of this thesis and highlighting general aspects that need to be considered for the generation of appropriate human-based alternative co-culture test systems. Additionally, the putative mechanism triggered in glial cells in pathology affecting neuronal viability will be addressed. Finally, the future perspectives of this project considering the potential of both cell types used in this study will be discussed.

1. Studying PD in culture models: urgent need for in vitro test systems, cell lines (neurons, astrocytes) and co-culture models

Neurodegenerative diseases affect millions of people worldwide. Unfortunately, for most of them, including PD, pathogenesis is not fully understood yet. Moreover, specifically for PD there is no treatment available which can halt or slow down the progressive pathological conditions. Therefore, different in vivo, ex vivo, and in vitro models were generated. In vitro models of PD offer advantages over in vivo models in several aspects. First of all, these models allow studying the role of individual cells of one particular type in model disease environment. Second, one can study mechanisms of putative detrimental or defensive effects of particular molecular players and compounds. Third, cells culture models have an advantage of virtually unlimited availability and quick reproducible results which allow rapid screening for disease pathogenesis and drug candidates. Finally, in vitro studies reduce the necessary number of animal experiments. Thus, application of in vitro models for studying of neurodegenerative diseases provides an important insight into mechanism and potential pharmacological targets. Moreover, they are the only option to have a human genetic background. The main biochemical disease pathways participating in PD indeed have been extensively investigated in vitro. One of the main aims of this thesis was to establish co-culture model of neurons and astrocytes, for this purpose one has to carefully select the best appropriate monoculture candidates for successful modeling of required process. (Schlachetzki et al., 2013)
Development of the relevant models for PD in vitro studies relies on the correct identification of DA neurons. Specific markers such as tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), dopamine transporter (DAT), and vesicular monoamine transporter (VMAT2) must be present. Moreover, transcription factors, morphological features, electrophysiological properties, as well as an ability to release dopamine are major criteria that have been developed to define DA neurons for research purposes (Bunney et al., 1991; Perrier et al., 2003; Scholz et al., 2011). LUHMES cell line used in our studies combines numerous features required by an appropriate in vitro system for PD disease modeling due to a number of key issues: 1-LUHMES is well-characterized cell line with human origin, differentiating to highly homogeneous postmitotic DA neurons (Scholz et al., 2011); 2- besides MPP⁺ toxicity mediated by uptake via DAT, LUHMES were also found to be sensitive towards other classical parkinsonian toxins like Methamphetamine/Fe²⁺ and rotenone (Schildknecht et al., 2009; Zhang et al., 2014); 3- LUHMES cells were successfully cultivated with astrocytes from different origin (Schildknecht et al., 2012).

Despite the fact that neuronal monocultures are extensively used in PD, they rather represent non-natural situation, since in vivo they are surrounded by glial cells. The generation of complex models consisting of several cell types in vitro still represents a challenge.

Recently it has been suggested that astrocytes do not only support and modulate neuronal functions but also might play an important role in disease initiation and progression (Maragakis et al., 2006; Verkhratsky et al., 2014). Since neurons in the brain are embedded in a complex interaction network together with astrocytes, a co-culture model of both cell types is supposed to reflect the situation in a brain better. Moreover, development of such model is not only of high interest for basic research on neurodegenerative events, but also for the testing of pharmacological compounds.

Therefore, the second cell type for the co-culture models - astrocytes - was needed. The prevailing method for obtaining astrocytes is isolation from prenatal rodents. These methods are hampered by the relatively low yield of cells, the high demand for laboratory animals and the relatively high inter-laboratory variability. Moreover, the caution should be taken with astrocytes cultures because these cells may be “contaminated” with a high amount of microglia, oligodendrocytes, neurons, and endothelial cells. Therefore, the newly generated pure astrocytes cell lines received much attention. Astrocyte cell cultures of human origin that are currently available either still lack a comprehensive characterization and were not yet
extensively used. Moreover, human astrocytes possessing sufficient MAO-B activity are hardly available in quantities required for our experiments. Therefore, the new immortalized mouse astrocytes cell line IMA 2.1 was introduced. The application of this cell line for studying of different PD aspects in vitro has a number of advantages. It thus combines unique features, and therefore offers PD researchers a rare opportunity to study a broad range of neuron-astrocyte interactions in vitro. First, IMA cells offer a proper MAO-B activity allowing efficient MPTP conversion to MPP⁺ which triggers neurotoxicity in a co-culture. Second, these astrocytes provide neuronal support against the neurotoxic stimuli in a co-culture. Third, IMA cells show classical features of reactive astrogliosis upon cytokine stimulation: morphological changes, increased proliferation, migration and secretory activity alteration, which lead to neuronal death in a co-culture. Generation and characterization of the IMA 2.1 cell line was documented in Chapter B.

One of the key aims of this thesis was an establishment of an in vitro co-culture system that recapitulate in vivo MPTP model of PD. MPTP is one of the best-studied experimental parkinsonian drug that reproducibly triggers DA neuron degeneration with higher specificity for the human nigrostriatal pathway (Langston 1984). Since MPTP is a precursor of the active neurotoxin MPP⁺, it cannot be used in the neuronal monocultures, but instead requires the activation by the enzyme MAO-B that is expressed in astrocytes within the brain (Schulz et al, 1995). Therefore, we used MPP⁺ in neuronal monocultures and MPTP in co-cultures with neurons to trigger the neuronal death. Figure 1 represents the graphical summary of model established. The panel of neuroprotective candidate compounds was tested in both systems demonstrating different potential in monoculture vs. co-culture. The failure to rescue neurons in co-cultures might be accounted for several aspects: (1) the impact of compounds on astrocytes functions which might be followed by reduced astocytic support; (2) modifications/metabolism of compounds by astrocytes; (3) unknown effects of compounds on astrocytes state/functions; (4) the metabolic situation of neurons may be affected by astrocytes; (5) the relative contribution of cell death mechanisms may change in the presence of astrocytes; (6) the continuous production of MPP⁺ may lead to different early stress responses and adaptations. Evidently, understanding of astrocytic-neuronal interactions in PD is still in its infancy, and much work still has to be done. MPTP in vitro model would provide a more balanced view of this interaction and will help to expand the panel of putative pharmacological targets for therapy.
E. General discussion

Figure 1. Schematic representation of putative targets for neuroprotection from MPP⁺-neurotoxicity.

Following compounds/inhibitors showed protection in LUHMES/MPP⁺ model: Vitamin C, GBR12909, DHQ, CEP1347, ROCK inhibitors, Deferoxamine, zVAD. Neuroprotection in MPTP/co-culture model: GSSG, deprenyl, rasagiline, GBR12909, DHQ, ROCK inhibitors.

In most cases neurons in these co-cultures either do not have dopaminergic phenotype or are not of human origin and moreover, the neurotoxicity is triggered by application of extremely high and thus physiologically irrelevant concentrations of MPP⁺. Our MPTP in vitro system (described in Chapter C) offers a unique opportunity to as metabolism of MPTP in it occurs exclusively in astrocytes thus yielding correct and physiologically relevant concentration-time profile of MPP⁺ exposure (Fuller et al., 1989). In summary, our study demonstrated that astrocytes not only play a major role for the biology and metabolism of neurons, but that they can drastically affect the pharmacology of neuroprotection.

The second aspect which was explored in our studies is an inflammatory activation of astrocytes, and its contribution to neuronal degeneration (Figure 2). We established an in vitro co-culture system based on human neurons and astrocytes, which is able to model the key features of astrogliosis and subsequent neuronal death with the ability to test protective
properties of compounds (described in Chapter D). This system allowed us to model an astrocytes reaction, namely that the neuronal support of healthy astrocytes against neurotoxicants might be weakened or even switched to deleterious effects when pro-inflammatory stimuli are around. The potential therapeutic approaches were tested. The attempts to resque neurons with different inhibitors/substances led us to two sites of intervention: (1) the inhibition of astrocytes activation with subsequent reduction of its harmful effect on neurons (p38 inhibitors, dexamethasone); (2) direct neuroprotection of neurons (CEP1347, DHQ, Resveratrol, zVAD). Thus, in our system we showed that neuroinflammation is indeed a process with many unknown players and a complex cascade of events, which is hard to hamper with a single hit focused only one cell type. We strongly believe that our understanding of mechanisms of astrocytes reaction will reveal contribution of astrocyte-specific pathologies to neurodegeneration.

Figure 2. Schematic representation of inflammation in vitro model (described in Chapter D of this thesis).

Molecules and signaling pathways events triggered in astrocytes, stimulated with inflammatory cytokines, and its influence on neuronal viability with indicated sites of pharmacological inhibition of both processes.
2. Astocytes and Parkinson`s disease

In the Chapter C and D we demonstrated that presence of astocytes may drastically affect the pharmacology of neuroprotection against various neurotoxins. Moreover, astocytes under certain conditions may undergo alterations which not only hamper their neuronal support, but even lead to decrease of neuronal viability. The putative mechanisms and molecular players involved will be discussed in this chapter.

Astrocytes are the most abundant cell population within the entire CNS (Chen et al., 2003). They support neuronal function in a multiple ways: provide structural, trophic and metabolic support of neurons. They achieve this through their involvement in regulation of blood flow, provision of energy metabolites; modulation of synaptic function and plasticity; homeostasis of ions, fluids and transmitters (Allen et al., 2009; Volterra et al., 2005). Apart from differences which astocytes have in white and gray matter, they also show regional and intraregional heterogeneity dependent on brain region, local cellular environment, the activation state and age (Bachoo et al., 2004). Depending on a specific brain region of their origin, astocytes differ in neurotransmitter receptors, immune response, opioid receptor expression or gap junction coupling (Yeh et al., 2009). However, the specific characteristics of different regional astocytes population such as SNpc astocytes are not well studied yet. Indeed, the specific properties of astocytes in SNpc, such as vulnerability to mutations or susceptibility to environmental effects, might contribute to increased vulnerability of neurons in this region. However, most of the studies has been so far focused on a role of microglia in PD progression (McGeer et al., 2008), and the contribution of astocytes was long time neglected. Nonetheless, recent studies indicate that astocytes play pivotal role in many neurodegenerative diseases (Maragakis et al., 2006; Verkhratsky et al., 2014). Therefore, one of the main goals of this thesis was to evaluate astocytic contribution to neuronal cell death under certain conditions related to PD and their influence on drug pharmacology in co-culture conditions.

2.1. Triggers of astocytes reaction and its contribution in PD pathogenesis

Astroglia represents an additional putative culprit in PD, which has to be extensively explored. Astocytes may contribute to neuronal vulnerability and therefore disease progression in several ways. For instance, astocytes outnumber neurons in the brain by the factor of 3-10, however SNpc contains the lowest number of astocytes compared to other brain areas (Mena et al., 2008). We considered this aspect while establishing our MPTP- and inflammation-based co-culture models. Evidently, since neurons rely on an astocytic support
in healthy state and especially in disease lower relative numbers of the astrocytes in SNpc may additionally contribute to their susceptibility. Moreover, DA neurons spontaneously generate free radicals during dopamine metabolism and therefore are less protected if surrounded by the smaller number of astrocytes with their radical-scavenging properties. Additionally, it was also shown that striatal astrocytes express relatively high levels of an inflammatory mediator - intercellular adhesion molecule-1 (ICAM-1), which makes this area susceptible to inflammation (Morga et al., 1998).

In addition to abovementioned aspects of astrocyte in a healthy brain which may influence the neuronal viability, they may change their behaviour and contribute to disease development. In PD, astrocytes may undergo several biochemical and structural alterations, called reactive astrogliosis. This triggers certain changes that contribute to neuronal demise even stronger (Colangelo et al., 2014b). Indeed, reactive astrocytes were observed both in MPTP and in PD patients. However, activated astrocytes were identified in many but not in all cases of PD relying on increasing expression of GFAP protein (Forno et al., 1992; Mirza et al., 2000). Generally, astrogliosis is a complex of molecular, cellular and functional changes which is considered to be physiological response to injury in order to reduce the damage. This process includes several steps: enhanced homeostatic and trophic function, gene expression alterations, increased synthesis of intermediate filaments, cell body hypertrophy and proliferation which is followed by increase secretory activity and migration (Figure 3) (Sofroniew et al., 2010). All these aspects we were able to recapitulate these changes in our inflammatory in vitro co-culture model (Chapter D). However, the exact mechanisms of astrogliosis onset and propagating pathological pathways are not extensively explored yet.

There were extensive studies with attempt to reveal the putative ways of astrocytes activation. One hypothesis is coming from mouse MPTP model of PD, where activated microglia appeared in the SNpc much sooner than reactive astrocytes after MPTP injection (Kohutnicky et al., 1998). This indicates that astrocytes depend on stimuli released from microglia (e.g., IL-1β, TNF-α, IFN-γ) known to trigger astrocytes reaction (Giulian et al., 1985). Furthermore, pronounced features of reactive astrogliosis did not precede but rather occurred at the same time as the active phase of DA neurons degeneration. These data suggest that astrogliosis is a secondary event which occurs after neuronal cell death and contribute to disease propagation but not initiation (Liberatore et al., 1999). In our co-culture model as a first step we brought together monocultures of neurons and astrocytes, effects of microglia
were mimicked by addition of mouse cytokines mix (IL-1β, TNF-α, IFN-γ) which stimulated astrocytic activation demonstrating classic features of astrogliosis.

**Figure 3. Hallmarks of astrocytes reactivity.**

In CNS pathology, activated astrocytes increase their protein synthesis e.g., intermediate filaments, proliferate and undergo cell morphological changes. Astrogliosis includes migration towards the lesion site, the production of growth factors and cytokines, as well as the release of nucleotides and toxic compounds. Their secretion is regulated via complex autocrine and paracrine loop. Finally, they decrease their homeostatic and trophic support of neurons.

Ideally, all three cell types should be present in a co-culture. In this case microglia stimulation triggered by LPS will be followed by astrocytes reaction and neurodegeneration. This will be an important challenging point for further project perspectives.

Secondly, apart from microglia, degenerating neurons might as well promote astrocytes reaction recruiting several signalling pathways. Abnormal protein aggregation of α-synuclein protein is a hallmark of PD. Aggregated α-synuclein is a major component of LBs, which are frequently found in PD in neurons as well as in astrocytes (Gelb et al., 1999). It was shown that neuron-derived α-synuclein can be directly transferred to astrocytes by sequential exocytosis and endocytosis and induce inflammatory responses from astrocytes (Lee et al., 2010b) characterized by secretion of proinflammatory cytokines and chemokines, ROS triggering subsequent activation of microglia and further neuronal demise (Lee et al., 2010a).

In order to address this issue, we exposed astrocytes to conditioned medium from monoculture of dead neurons (exposed to MPP+) and observed a slight inflammatory response in astrocytic monoculture after first 24h. In our system LUHMES cells indeed express α-
synuclein that is not mutated or aggregated. However, non-aggregated α-synuclein as well is an effective stimulator of astrocytes and inflammatory processes.

Degenerating neurons might release other damage-associated ligands (e.g. phosphatidylserine, PS), and therefore induce an inflammatory response and recruit glia to promote clearance of damaged neuronal cells. Moreover, it has become evident that viable cells/neurons might also reversibly expose “eat-me” signal PS and undergo unwanted phagocytosis, contributing even stronger to disease progression (Fricker et al., 2012). The PS is recognized by a transient up-regulation of MerTK (Mer receptor tyrosine kinase) and MFG-E8 (Milk fat globule EGF-like factor 8) on glial cells (Brown et al., 2012). Therefore, a deficiency in these receptors, by inhibition, still leaves the chance to prevent neuronal loss and death (Neher et al., 2013). Additionally, several glia-secreted molecules might bind to their target and flag them for destruction either by the complement system or by phagocytosis, e.g. complement receptor 3 (CR3) and 4 (CR4) that upon activation on a cell surface becomes covalently bound (opsonised) on the cell surface as C3b, which is subsequently cleaved to yield a very stable fragment, iC3b. Another soluble factor, C1q produced by glial cells can bind to damaged neurons and is involved in clearance of cell debris in CNS (Elward et al., 2003).

Additionally, it was shown that several chemokine ligands are induced in damaged neurons such as the lymphoid-tissue chemokine (SLC) or the interferon-inducible protein-10 (IP-10). Neuron-derived chemokines binds to chemokine receptor CXCR3 functionally expressed on microglia and astrocytes, and may therefore attract glial cells to the site of damage (Biber et al., 2002; Biber et al., 2001; Wang et al., 1998).

Finally, a wide variety of important astrocyte functions become transiently or permanently impaired during brain damage (including diseases and brain injury) and may simply impact neuronal viability (Hamby et al., 2010). This issue was addressed in our neuroinflammation in vitro model. Neuronal monocultures exposed to high concentrations of reactive nitrogen species (NO) quickly underwent neuronal death, whereas neurons in co-culture were not affected. Most probably this effect is caused by astrocytic supplementation of neurons with classic antioxidant - glutathione. Neurons are not able to synthesize glutathione in a great amount therefore they rely on astrocytes (Smeyne et al., 2013). Indeed, in our system the astrocytes possess at least ten times more concentration of intracellular glutathione as neurons. However, under stimulation with pro-inflammatory cytokines, abovementioned
astrocytes neuroprotective effect was attenuated apparently due to the decrease of GSH and ATP intracellular pools.

Thus, the extensive studies increased our understanding of astrocyte role as well as importance of neuron-glia interaction in disease process. It has become clear that development of target therapies must take into consideration these issues. In this regard, our co-culture models provide an excellent opportunity to test putative therapeutic interventions, since it reflects the key cellular and molecular events related to PD.

2.2. Intracellular transduction pathways of astrogliosis

Although the reactive astrogliosis is a common event in many neurodegenerative diseases, the molecular mechanisms translating injury-derived signals into astroglia reactivity are numerous and their intracellular interactions are in large unclear. Some of morphological and functional alterations in activated astrocytes appear regulated at the cytoplasmic level and others involve an activation of intracellular cascades involving dramatic gene expression changes. Growth factors are able to activate the mammalian target of rapamycin kinase (mTOR) in astrocytes. Treatment with mTOR-selective antagonist rapamycin prevents proliferation, migration and intermediate filament upregulation in activated astrocytes (Codeluppi et al., 2009).

Several lines of evidence suggest that inflammatory mediators such as TNF-α, NO and interleukin-1, derived from microglia, as well as purines/pyrimidines are considered to evoke an initial early astrocytes activation (Abbracchio et al., 2006; Liberto et al., 2004). Indeed, massive amounts of ATP, other purine (i.e., ADP) and pyrimidine (UTP, UDP, and UDP-sugars) nucleotides can be released from dying cells. Astrocytes express the whole panels of purinergic G protein-coupled receptors (P2Y), and their activation was shown to lead to alterations of astrocytes cytoskeleton dynamics, COX-2 up-regulation, modulation of cytokines and inflammatory mediators synthesis (Brambilla et al., 1999; Verkhratsky et al., 2009).

The major source of pro-inflammatory cytokines in injured brain is microglia. Once released, they are able to stimulate astrocytes reaction, resulting in NF-κB nuclear translocation and transcriptional functions by activating complex kinase cascades (Brambilla et al., 2005; Khoroooshi et al., 2008). In turn, NF-κB promotes the production of chemokines and inflammatory molecules including NO, COX-2, IFN- , TNF- , IL-1 & IL-6, chemokines such as MCP-1 , MIP- and CXCL-8, and MAC which have deleterious effect
on neuronal survival. Moreover, they contribute to development of an extensive second wave of inflammation and limit the repair of tissue damage (Buffo et al., 2010; More et al., 2013).

It was demonstrated that \( \alpha \)-synuclein released from neuronal cells can also be transferred to and accumulate in astrocytes and induce expression of genes associated with immune functions. Proinflammatory cytokines that are differentially expressed in astrocytes in response to extracellular \( \alpha \)-synuclein include IL-1, IL-1, IL-6, IL-18, and colony-stimulating factors-1, 2, and 3, suggesting a strong inflammatory response from astrocytes upon exposure to neuron-derived \( \alpha \)-synuclein. Additionally, \( \alpha \)-synuclein may influence a transition from quiescent to reactive state by main MAPK pathway: ERK1/2, JNK and p38 with followed upregulation of proinflammatory ICAM-1 expression and IL-6 secretion (Klegeris et al., 2006).

Protein phosphorylation and gene expression analysis showed an involvement of JAK-STAT pathway to be responsible for astrocyte reaction in MPTP model of PD. Phosphorylation of JAK2 and nuclear translocation of STAT3 was observed after MPTP administration. The gp130-related cytokines (IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM)), induced by MPTP-mediated neuronal damage, might be responsible for this pathway (Sriram et al., 2004).

Damage-related factors induce the activation of several intracellular pathways that control reactive astrocytic features. One of the most important issues is the impact of astrocytes activation on neuronal viability.

2.3. Soluble factors secreted from astrocytes and their effect of neuronal viability

IMA astrocytes presented in this thesis recapitulate many of abovementioned cellular and molecular events occurring during reactive astrogliosis upon cytokine stimulation, such as Nf-kB translocation, iNOS and COX2 activation, upregulation of various pro-inflammatory genes. These events led to impairment of protective properties of astrocytes in co-culture model, and eventually resulted in neuronal death via the soluble factors released. We were not able to identify the exact soluble factors released in our inflammation model. However, we may exclude proteins and short-lived substances such as free radicals, ROS, since we could freeze, thaw, and boil the neurotoxic conditioned medium, and its neurotoxic effect still remained. The putative neurotoxic factors secreted by astrocytes upon inflammatory stimulation are mentioned in Table 1.
A number of *in vitro* models attempted to identify putative astrocytes-derived neurotoxic factors. (Garwood *et al.*, 2011) showed that astrocytes release soluble inflammatory factors such as neutrophil chemoattractant (CINC) 2α/β, IFN-γ, IL-1β, IL-1ra, IL-6, IL-13, IL-17, IP-10 and monokine induced by IFN-γ (MIG), and inhibition of astrocyte activation with the anti-inflammatory agent, minocycline, reduces astrocytic inflammatory responses and the associated neuronal loss. (Bi *et al.*, 2013) showed that lipocalin 2 (lcn2) is secreted by reactive astrocytes and is selectively toxic to neurons.

The putative responsible factors might be lipids, glycan/polysaccharide or glycolipids that might also exert neuro-damaging effects. Indeed, the number of active glia-derived nonproteinaceous factors was found. It was shown that microgila secretes nonproteinaceous (also not short-living molecules) neuron-killing factors (Giulian *et al.*, 1993). In mouse model of MS astrocytes secrete a glycosphingolipid - Lactosylceramide (LacCer), and an inhibition of its synthesis suppress local CNS immunity and neurodegeneration. However, a direct effect of LacCer on neurons is not revealed yet (Mayo *et al.*, 2014). In AD *in vitro* model (Wang *et al.*, 2012) was discovered that astrocytes stimulated with Aß secrete exosomes enriched with proapoptotic ceramide. The neurotoxic potential of this factor is also not studied yet.

Several of these astrocytes-derived factors might trigger the apoptotic pathways inside neurons. It was demonstrated that fibrillar amyloid-beta-activated human astroglia kill primary human neurons by soluble factors released (e.g. NO) via inducing neuronal apoptosis by N-SMase – ceramide pathway (Jana *et al.*, 2010). In the model of amyotrophic lateral sclerosis it was discovered discovered that familial and sporadic ALS-derived human adult astrocytes secrete neurotoxic factors that selectively kill motor neurons through necroptosis (Pirooznia *et al.*, 2014).

Here we demonstrated that astrocytes play an important role in PD progression and therefore represent a very promising target for the disease treatment. There are many factors involved in astrogliosis that could be manipulated to control the astroglial reaction by reducing their detrimental effects and stimulation an increase of their protective properties. Moreover, astrocytes along with neurons should be included in PD *in vitro* models for screening of potential therapeutic drugs.
<table>
<thead>
<tr>
<th>Categories</th>
<th>Astrocyte molecule</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-living molecules</td>
<td>Free-radicals, NO, ROS, RNS</td>
<td>3, 10</td>
</tr>
<tr>
<td>Chemokines</td>
<td>CC-type (CCL-3, 4, 5, 12, 20), CXC-type (CXCL-1, 2, 5, 7, 10, 11, 12, 16)</td>
<td>5, 15</td>
</tr>
<tr>
<td>Cytokines</td>
<td>TNF, IL-1, IL-6, IL-18</td>
<td>7, 8</td>
</tr>
<tr>
<td>Small effector molecules</td>
<td>ATP, PGE2</td>
<td>6, 11</td>
</tr>
<tr>
<td>Growth factors</td>
<td>VEGF, TGFβ, GDNF</td>
<td>1, 14</td>
</tr>
<tr>
<td>Proteoglycans, extracellular matrix molecules</td>
<td>CSPGs (chondroitin sulfate proteoglycans)</td>
<td>9, 12</td>
</tr>
<tr>
<td>Vesicles</td>
<td>Exosomes enriched with proapoptotic ceramide containing mitochondria and lipid droplets together with ATP; membrane vesicles that contain mitochondria, lipid droplets and ATP.</td>
<td>4, 13</td>
</tr>
<tr>
<td>Other proteins</td>
<td>Lipocalin 2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. Factors secreted by activated astrocytes.


3. Benefits of MPTP modeling in vivo and in vitro

For decades scientists were focused on developing neuroprotective drugs capable to halt neurodegeneration in PD, however none of the pharmacological agents has yet been shown convincingly to slow the progression (Meissner et al., 2011). One major hindrance for the development of novel neuroprotective strategies is the limited availability of human cell-
based in vitro neuronal models. Moreover, most of the available ones are lacking active neuron-glial interactions (Schule et al., 2009). Recently, the neurocentric view of brain function and disease has been challenged by the emerging evidence of the physiopathological potential of neuroglia (Glass et al., 2010).

MPTP model is widely used one for clinical trials as well as in fundamental studies. MPTP model most commonly uses mice and primates where MPTP produces a final pathological state, a functional impairment, and a pharmacological responsiveness that closely resembles human Parkinsonism. Some characteristics of the pathogenesis may also be overlapping between the model and the human disease. These include oxidative stress, early mitochondrial impairment, a failure of proteostasis and an involvement/modulation by levels of alpha-synuclein. Dissimilarities of the MPTP animal model to human disease are that the damage occurs rapidly, is hardly progressive, is little age-dependent, and usually does not involve formation of Lewy bodies.

MPTP model is considered to be the best approach in terms of inducing the toxicity of human nigrostriatal pathway (Langston et al., 1984b), is often reproduced by direct application of its toxic metabolite MPP⁺ to neuronal monocultures, whereas the metabolism, distribution and neurotoxicity of MPTP require complex neuron-glial interactions (Dauer et al., 2003). Therefore, we took an advantage of two cell lines - LUHMES (human DA neurons) and IMA (astrocytes with sufficient MAO-B activity for MPTP conversion), in order to build a unique in vitro system allowing endogenous metabolism of MPTP with subsequent neurodegeneration. The kinetics of MPTP conversion is slower than in vivo, and this is possibly the reason for the relatively slow cell death process we observe. The latter may also be considered a positive feature, if human disease pathogenesis is to be modelled. The MPTP co-culture model developed here has the advantage that the metabolism and distribution of the metabolite MPP⁺ can be better controlled and followed, and the same applies to cellular processes, that are accessible for microscopic and biochemical endpoints.

Some findings from the MPTP model (Chapter C) have been selected for tabular presentation, as they show the extremely broad range of biological pathways involved in the complex pathogenesis (Table 2). Comparison of the literature findings with information available from the co-culture model indicates that there are still many processes to be explored in more detail, and that our co-culture (possibly supplemented with microglia) could contribute to this. The possibility to study MPTP toxicity in a co-culture model opens new ways to test pharmacological agents on human neurons and to explore many of the more
recent mechanistic pathways involved in PD suggested by animal models. Several compounds that protected neurons in mice or in monocultures were not effective in co-cultures. Thus, the most striking finding of our study was the different efficacy of experimental neuroprotectants in monocultures vs. co-cultures (Table 3). The lack of protection in co-cultures may have several reasons: (i) Some compounds might be metabolized or modified by astrocytes; (ii) the metabolic situation of neurons may be affected by astrocytes; (iii) the relative contribution of cell death mechanisms may change in the presence of astrocytes; (iv) the continuous production of MPP\(^+\) may lead to different early stress responses and adaptations. In summary, our study demonstrated, that glial cells not only play a major role for the biology and metabolism of neurons, but that they can drastically affect the pharmacology of neuroprotection. Thus, astrocytes along with neurons should be included in establishment of PD in vitro models for screening potential therapeutic drugs.
General discussion

The literature has been scanned for recent findings concerning the MPTP model. Altogether, more than 5000 references were retrieved, and key findings concerning the animal model have been compiled here. The MPTP model most commonly uses mice as experimental species, but also primates, including humans, are susceptible (but rats are not). MPTP produces a final pathological state, a functional impairment, and a pharmacological responsiveness that closely resembles human Parkinsonism. Some features of the pathogenesis may also be overlapping between the model and the human disease. These include oxidative stress, early mitochondrial impairment, a failure of proteostasis and an involvement/modulation by levels of alpha-synuclein. Dissimilarities of the MPTP animal model to human disease are that the damage occurs rapidly, is hardly progressive, is little age-dependent, and usually does not involve formation of Lewy bodies. The MPTP co-culture model developed here has the advantage that the metabolism and distribution of the metabolite MPP+ can be better controlled and followed, and the same applies to cellular processes, that are accessible for microscopic and biochemical endpoints. This advantage is balanced by the shortcoming that only one type of neurons, and no microglia are present in the model. The kinetics of MPTP conversion is slower than in vivo, and this is possibly the reason for the relatively slow cell death process we observe. The latter may also be considered a positive feature, if human disease pathogenesis is to be modelled. Some findings from the MPTP model have been selected for tabular presentation, as they show the extremely broad range of biological pathways involved in the complex pathogenesis. Comparison of the literature findings with information available from the co-culture model indicates that there are still many processes to be explored in more detail, and that our co-culture (possibly supplemented with microglia) could contribute to this. For references, see Table 3.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Modifying factor</th>
<th>Results/Relation to co-culture</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>HDAC</td>
<td>Valproic acid, the most investigated HDAC inhibitor in PD, did not show a protection.</td>
<td>Compound has numerous targets, which might not be present in co-culture.</td>
</tr>
<tr>
<td>16</td>
<td>Glutathione</td>
<td>GSSG, a GSH precursor, was protective in co-culture.</td>
<td>GSSG was not protective in monocultures.</td>
</tr>
<tr>
<td>20</td>
<td>COX-2</td>
<td>Inhibition of COX-2 was not studied.</td>
<td>Microglia are not present in our system.</td>
</tr>
<tr>
<td>2</td>
<td>ROCK pathway</td>
<td>ROCK inhibitors were protective in co-cultures.</td>
<td>ROCK inhibitors also protected monocultures.</td>
</tr>
<tr>
<td>11</td>
<td>Wnt/b-catenin signaling cascade.</td>
<td>This aspect was not explored.</td>
<td>As microglia were absent; astrocytes did not reach the proper reactive state observed in vivo.</td>
</tr>
<tr>
<td>3</td>
<td>Autophagy</td>
<td>This aspect was not explored.</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>PARP</td>
<td>Protective effect of PARP inhibitor.</td>
<td>Protection also in monoculture (independent of NO).</td>
</tr>
<tr>
<td>14</td>
<td>Mitochondrial fission and fusion</td>
<td>This aspect was not explored.</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>NADPH oxidase</td>
<td>This aspect was not explored.</td>
<td>Microglia are not present in our system.</td>
</tr>
<tr>
<td>22</td>
<td>NO and ROS</td>
<td>Treatment with NO inhibitors did not protect.</td>
<td>Rodent mechanism may not apply to humans.</td>
</tr>
<tr>
<td>15</td>
<td>D2/D3 agonist</td>
<td>This aspect was not explored.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Adenosine A2A receptor antagonists</td>
<td>This aspect was not explored.</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Protein kinase C delta</td>
<td>PKC delta inhibitor (Rottlerin) did not show a protective effect in our model.</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>JNK Pathway</td>
<td>This aspect was not explored in detail.</td>
<td>MLK inhibitor CEP1347 (upstream inhibitor of JNK) did not protect.</td>
</tr>
<tr>
<td>9</td>
<td>VMAT-2 level</td>
<td>This aspect was not explored.</td>
<td>VMAT overexpression is protective, but this cannot be obtained pharmacologically.</td>
</tr>
</tbody>
</table>

Table 2. Recent findings on MPTP animal models in relation to our co-culture model.

The literature has been scanned for recent findings concerning the MPTP model. Altogether, more than 5000 references were retrieved, and key findings concerning the animal model have been compiled here. The MPTP model most commonly uses mice as experimental species, but also primates, including humans, are susceptible (but rats are not). MPTP produces a final pathological state, a functional impairment, and a pharmacological responsiveness that closely resembles human Parkinsonism. Some features of the pathogenesis may also be overlapping between the model and the human disease. These include oxidative stress, early mitochondrial impairment, a failure of proteostasis and an involvement/modulation by levels of alpha-synuclein. Dissimilarities of the MPTP animal model to human disease are that the damage occurs rapidly, is hardly progressive, is little age-dependent, and usually does not involve formation of Lewy bodies. The MPTP co-culture model developed here has the advantage that the metabolism and distribution of the metabolite MPP+ can be better controlled and followed, and the same applies to cellular processes, that are accessible for microscopic and biochemical endpoints. This advantage is balanced by the shortcoming that only one type of neurons, and no microglia are present in the model. The kinetics of MPTP conversion is slower than in vivo, and this is possibly the reason for the relatively slow cell death process we observe. The latter may also be considered a positive feature, if human disease pathogenesis is to be modelled. Some findings from the MPTP model have been selected for tabular presentation, as they show the extremely broad range of biological pathways involved in the complex pathogenesis. Comparison of the literature findings with information available from the co-culture model indicates that there are still many processes to be explored in more detail, and that our co-culture (possibly supplemented with microglia) could contribute to this. For references, see Table 3.
Table 3. Synopsis of drug effects in vivo (animals), in humans, and in co-cultures.

4. Outlook and project prospective

An establishment of in vitro co-culture system still represent a challenge for researchers, since a vast number of characteristics must be taken into account in order to more accurately recapitulate in vivo situation. For example, the original neuronal and astrocyte phenotypes must be preserved while they are brought together in the same well. Generation of our in vitro system took us lots of time and effort for optimization which then was rewarded by successful and fruitful application of this model to study PD. One of the next important future steps will be the combination of neurons, astrocytes and microglia at defined proportions, possibly also considering oligodendrocytes as fourth population. It might be useful, with this multicellular system in hand, to uncover new interconnections between cells in different models of disease.

Moreover, PD is characterized by several classical pathological phenomena, such as oxidative stress, protein aggregation and neuroinflammation. We were able to model two aspects mentioned above in separate experimental set up: MPTP model (oxidative stress) and application of pro-inflammatory cytokines (neuroinflammation). Therefore, bringing these models together will definitely allow us to reveal putative differences in pharmacological activities of mechanistically diverse neuroprotective experimental compounds compared to modelling of single events. Additionally, a protein aggregation aspect might be also addressed in our co-culture system via comparison of the pharmacological behaviour of compound in co-culture astrocytes with “wild-type” LUHMES and LUHMES overexpressing α-synuclein, or LUHMES expressing mutated forms of α-synuclein.

Astrocytes with their immense protective potential as well as detrimental effects occurring during astrogliosis constantly receive an interest in PD field, since their precise contribution to neuronal death remains to be explored in detail. One crucial point which further might be revealed with the help of our system is the secretory activity of astrocytes and precise soluble factors released upon cytokine stimulation, as well as particular treatment capable to impede this process. The analysis of conditioned medium content from activated and quiescent astrocytes might reveal the new key players responsible for neuronal demise.
An application of human astrocytes would be of a great advantage for both in vitro co-culture models established in this study. Since human astrocytes expressing MAO-B with sufficient activity level are hardly available, than human astrocytes overexpressing MAO-B might be generated and used.

Moreover, the neuron-glia interaction is bidirectional process. Indeed, neuronal state also strongly influences the astrocyclic properties and functions. Therefore, the application of certain neuron-specific degenerative stimuli with followed supernatant transfer to astrocytes will reveal the impact of neuronal degenerating stage on astocytic inflammatory status.

Thus, our study paves the road to the construction of complex neurodegeneration models that consist of more than one cell type, but are still amenable to high throughput endpoints and single cell analysis of human neurons. The other methods of co-culturing (transwell approach, sandwich cultures, supernatant transfer) will extensively expand the potential of this model.
## F. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AADC</td>
<td>Aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotropic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nerve system</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyl transferase</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep brain stimulation</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinases</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leucocyte antigen DR</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington disease</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IMA</td>
<td>Immortalised mouse astrocytes</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-inducible protein-10</td>
</tr>
<tr>
<td>LacCer</td>
<td>Lactosylceramide</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy bodies</td>
</tr>
<tr>
<td>lcn2</td>
<td>Lipocalin 2</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>L-dopa</td>
<td>L-3,4-Dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat serine/threonine kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LUHMES</td>
<td>Lund Human Mesencephalic cells</td>
</tr>
<tr>
<td>MAC</td>
<td>Macrophage-1 antigen</td>
</tr>
<tr>
<td>MANF</td>
<td>Mesencephalic astrocytes-derived neurotrophic factor</td>
</tr>
<tr>
<td>MAO-B</td>
<td>Monoamine oxidase B</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
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### F. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MIG</td>
<td>Monokine induced by IFN-γ</td>
</tr>
<tr>
<td>MIPα</td>
<td>Macrophage inflammatory proteins</td>
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<tr>
<td>MPP*</td>
<td>1-Methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin kinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>N-SMase</td>
<td>Neutral sphingomyelinase</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase1</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>P2Y</td>
<td>Receptor purinergic P2Y receptor</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SLC</td>
<td>Secondary lymphoid-tissue chemokine</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia Nigra parca compacta</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydrolase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UCHL1</td>
<td>Ubiquitin carboxy-terminal hydrolase L1)</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinsons’s disease Rating Scale</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
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</tbody>
</table>
G. References


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G. References


G. References


G. References


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G. References


G. References


Tower DB, Young OM (1973). The activities of butyrylcholinesterase and carbonic anhydrase, the rate of anaerobic glycolysis, and the question of a constant density of glial cells in cerebral cortices of various mammalian species from mouse to whale. J Neurochem 20: 269-278.


G. References


H. Record of contribution

Chapter B

The chapter is published in ALTEX.

Experiments were designed and the manuscript was written by Stefan Schildknecht and Marcel Leist. I designed, performed and analyzed experiments leading to figure 8. I was involved in the performance and repetition of experiments, as well as in the finalization and revision process of the manuscript.

Chapter C

The chapter is accepted for publication in British Journal of Pharmacology.

I performed and analyzed experiments leading to figures 1, 3, 4, 5, 6, 7, 8, S1, S3, S4, S5, S6, S9, S10, S11, S12. All other experiments were performed by Martina Adam (cell culture), Regina Pape (HPLC), Simon Gutbier (several monoculture experiments). Experiment describing PARP1 and NAD⁺ involvement are performed in collaboration with Benjamin Hanf and Alexander Bürkle. I wrote the manuscript in collaboration with Stefan Schildknecht and Marcel Leist.

Chapter D

This chapter is manuscript in preparation.

I performed most experiments (leading to figures 1-8, as well as S1, S3, S4, S5) analyzed data and wrote the manuscript. All other experiments were performed by Stefan Schieldknecht, Martine Adam, Petra Chovancova, Simon Gutbier. Marcel Leist designed experiments and wrote the manuscript.
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