Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation

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Abstract Astrocytes, the largest cell population in the human brain, are powerful inflammatory effectors. Several studies have examined the interaction of activated astrocytes with neurons, but little is known yet about human neurotoxicity under such situations and about strategies of neuronal rescue. To address this question, immortalized murine astrocytes (IMA) were combined with human LUHMES neurons and stimulated with an inflammatory (TNF, IL-1) cytokine mix (CM). Neurotoxicity was studied both in co-cultures and in monocultures after transfer of conditioned medium from activated IMA. Interventions with >20 drugs were used to profile the model system. Control IMA supported neurons and protected them from neurotoxicants. Inflammatory activation reduced this protection, and prolonged exposure of co-cultures to CM triggered neurotoxicity. Neither the added cytokines nor the release of NO from astrocytes

were involved in this neurodegeneration. 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 five different drugs, including resveratrol and CEP1347, prevented toxicity of astrocyte supernatants. Thus, 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.

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Keywords LUHMES · Astrocyte · p38 kinase · Neuroinflammation · Neuropharmacology

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Abbreviations

PD	Parkinson's disease
DA	Dopaminergic
MPP ⁺	1-Methyl-4-phenyl-pyridinium
PARP	Poly-(ADP-ribose)-polymerase
PLO	Poly-L-ornithine
GDNF	Glial-derived neurotrophic factor
IMA	Immortalized mouse astrocytes
LDH	Lactate dehydrogenase
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
GSH	Glutathione
GSSG	L-Glutathione oxidized
DHQ	1,5-Isoquinolinediol
CEP1347	(3,9-Bis[(ethylthio)methyl]-K-252a)
ROCK	Rho kinase
DM	Differentiation medium
DAT	Dopamine transporter
LUHMES	Lund human mesencephalic cells
TNF- α	Tumor necrosis factor alpha
IL-1 β	Interleukin-1 beta
IFN- γ	Interferon gamma
CM	Cytokine mix
CCM	Complete cytokine mix
L-NNA	N $^{\omega}$ -nitro-L-arginine
CNS	Central nerve system
VMAT2	Vesicular monoamine transporter
TH	Tyrosine hydroxylase
NHA	Normal human astrocytes
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
I κ B	NF-kappa-B inhibitor beta
COX2	Cyclooxygenase-2
NOS	Nitric oxide synthase
cAMP	Cyclic adenosine monophosphate
NEP	Neuroepithelial cells
mAGES	Mouse astrocytes generated from embryonic stem cells
SNpc	Substantia nigra pars compacta
LPS	Lipopolysaccharides
NSE	Neuron-specific enolase

Introduction

Inflammatory situations and several inflammation-related mediators can trigger or aggravate neuronal damage in experimental models (Hashioka et al. 2015; Hunter et al. 2009). They may also be involved in several neurodegenerative diseases, and modulation of inflammation may contribute to current and future treatments (Gao et al. 2011; Heneka et al. 2014, 2015; In't Veld et al. 2001; Rees et al. 2011; Ruitenberget 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 three-fold to tenfold (Cahoy et al. 2008; Volterra and Meldolesi 2005). Astrocytes have a peculiar double function, as major neurosupportive cell population in the healthy brain, and as powerful inflammatory cells that may exert detrimental effects in disease situations (Allen and Barres 2009; Margaakis and Rothstein 2006; Orre et al. 2014; Sofroniew and Vinters 2010).

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 (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), amyotrophic lateral sclerosis (ALS) (Nagai et al. 2007), or 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; Medeiros and LaFerla 2013; Orre et al. 2014), in intoxications, and after traumatic injury (Silver and Miller 2004).

The different functions of astrocytes may be due to largely different activation states (Avendano et al. 2015; Falsig et al. 2004a; Henn et al. 2011) triggered by neuroinflammatory conditions linked to damage, disease, or infection. The first glial cells to be activated in such conditions are usually microglia. These secrete cytokines like IL-1 β and TNF- α , 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 a long time after stimulation (Biesmans et al. 2015; Henn et al. 2011).

The causal role of astrocytes in disease has been probed by several pharmacological interventions (Kohutnicka

et al. 1998; Kurkowska-Jastrzebska et al. 2004), and also genetic tools exist to deplete such cells from the brain (Mayo et al. 2014), but the interpretation of such studies is difficult. The pathological and toxicological role of astrocytes may be highly model-specific, as they contribute to the metabolism of some toxicants (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., direct excitotoxicants, like glutamate, or indirect excitotoxic mediators, such as NO (Bal-Price and Brown 2001; Bal-Price et al. 2002; Gandelman et al. 2010; Gegg and Clark 1036; Mander et al. 2005), lipid mediators/small molecules (Mayo et al. 2014; Simon et al. 2002; Wang et al. 2012), reactive oxygen species (Ma et al. 2013), proteases (cathepsin B), cytokines (Lee et al. 2013a; Mattson et al. 1997), complement factors (Pekny et al. 2007; Walker et al. 1998), but their respective contribution to human pathology needs further clarification (Lioy et al. 2011; Williams et al. 2014). Lack of knowledge of the relevant damage mediators has prevented the development of targeted therapies for modulation of astrogliosis, but some drugs like riluzole (Carbone et al. 2012) or CEP1347 (Falsig et al. 2004b) have astrocyte-modulating properties besides their main mode of action.

In vitro models allow biochemical studies at cellular-level analysis, often difficult in complex in vivo situations (Fig. S1). Traditional approaches to study astrocyte–neuron interactions include re-aggregating brain cultures, more defined co-cultures in 2D (Garwood et al. 2011; Sandstrom von Tobel et al. 2014), or 3D formats (Puschmann et al. 2013). An added benefit of defined co-cultures is that human neurons can be studied.

For some applications focusing on the neurodegeneration in co-cultures, immortalized astrocyte cell lines provide a simple, but robust source of astrocytic factors or metabolic capacity (Efremova et al. 2015; Lee et al. 2013a, b) to be tested on associated neurons. In cases where the toxic factors are non-proteinaceous, cells from two different species can be used, and they provide some analytical advantages. For such purposes, IMA can be combined with post-mitotic human neurons, differentiated from the LUHMES cell line (Scholz et al. 2011). These cells are conditionally immortalized, and they assume neuronal morphological, electrophysiological, and neurochemical properties upon the switching off of the v-myc transgene by tetracycline. The cells can be cultured on a layer of astrocytes (Efremova et al. 2015; Kuegler et al. 2012), and they form highly reproducible co-cultures when combined with the immortalized murine astrocytic cell line IMA (Schildknecht et al. 2012). The latter cells can metabolize drugs, such as 1-methyl-4-phenyl-tetrahydropyridine (MPTP), and are well characterized in their inflammatory

response. The LUHMES-IMA co-culture allowed multiple pharmacological interventions on the level of neurons and astrocytes (Efremova et al. 2015).

In the present study this experimental system was used to study neuron–glia interactions under inflammatory conditions and to provide a pharmacological characterization of relevant cell responses. In the beginning, we asked whether protection of neurons from toxicants by healthy non-activated glia can be confirmed and quantified. We then proceeded to examine how this situation changes under inflammatory conditions (cytokine exposure). A prolonged activation of astrocytes 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 to human neurons. 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.

Materials and methods

Cell culture

LUHMES cells were used exactly as described earlier (Scholz et al. 2011). They were maintained in poly-L-ornithine (50 µg/ml) and fibronectin (10 µg/ml, Sigma-Aldrich, St. Louis, MO, USA)-coated flasks (Nunc) in proliferation medium containing 40 ng/ml basic fibroblast growth factor (R&D Systems). For differentiation, the cells were seeded at a density of 1.5×10^5 cells per cm² in differentiation medium containing 1 mM dibutyl cAMP (Sigma-Aldrich), 10 µg/ml tetracycline (Sigma-Aldrich), and 2 ng/ml human GDNF (R&D Systems). The immortalized murine astrocytes (IMA2.1) were passaged in DMEM supplemented with 10 % FCS (fetal calf serum, PAA) and 1 % of penicillin/streptomycin (Schildknecht et al. 2012). Normal human astrocytes (NHA) were purchased from LONZA (catalog number CC-2565, Cologne, Germany) and 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 cultures were maintained according to the manufacturer's protocol. For co-cultures, IMA (15.000/cm²) or NHA cells (5.000/cm²) were seeded in their proper medium. After 24 h, medium was changed to LUHMES differentiation medium (no FCS, no antibiotics). After additional 48 h, pre-differentiated LUHMES cells (d2) were seeded on top of the now confluent glia layer (counted cell density: 60.000/cm²)

at a density of 150,000/cm². On day 6 of LUHMES differentiation, experimental treatment was started.

Cell viability assays

Cellular resazurin reduction capacity was determined by addition of 10 µg/ml reagent to the cells, incubation for 30 min, and measurement of fluorescence at $\lambda_{\text{ex}} = 530$ and $\lambda_{\text{em}} > 590$ nm; lactate dehydrogenase (LDH) release was measured enzymatically (NADH consumption): LDH activity was detected in cell culture medium (supernatant) and cell lysates separately. The percent LDH release was expressed as $100 \times \text{LDH}_{(\text{supernatant})} / (\text{LDH}_{(\text{supernatant})} + \text{LDH}_{(\text{lysate})})$ (Krug et al. 2014). Release of human-specific neuronal enolase into co-culture supernatants was analyzed using the 'Neuron-specific Enolase' Quantikine ELISA Kit (R&D, Wiesbaden) according to the manufacturer's protocol. Quantification of neuronal area was based on selective immunostaining (see below) of neuronal β III tubulin (Tuj1 antibody) and high-content imaging: An Array-Scan VTI HCS Reader (Cellomics, PA) was used for image acquisition. Thirty fields per well (330 µm × 330 µm size each) were imaged and analyzed (Efremova et al. 2015). The software recognizes the Tuj-positive pixels and calculates the total pixel number per well. Three wells were analyzed for each treatment, and the total pixel number was normalized to the total pixel number of untreated controls. Astrocyte nuclei were counted in monocultures after staining with H-33342. The pictures in ten fields per well were analyzed after observer blinding, and three wells were analyzed for each treatment. In parallel, cells were lysed for protein determination using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

NO release

Nitrite (NO₂⁻), the stable autooxidation product of NO, was measured by the Griess assay exactly as described earlier (Schildknecht et al. 2012). Nitrite concentrations were calculated from a NaNO₂ standard curve.

Immunocytochemistry

For confocal imaging, cells were grown on poly-ornithine/fibronectin-coated glass cover slips, washed with PBS, and fixed with 4 % paraformaldehyde (PFA) in 2 % sucrose for 15 min. Cells were permeabilized with 0.2 % Triton X-100 in PBS, washed with 0.05 % Tween 20 in PBS, and blocked with PBS/1 % BSA (Calbiochem, San Diego, CA, USA) for 1 h at room temperature (RT). Primary antibodies were incubated overnight at 4 °C. Secondary antibodies were applied for 1 h (RT) in the dark. Hoechst-33342 (1 µg/ml, Molecular Probes) was added for 15 min prior to the final

washing step. Cover slips were mounted on glass slides with FluorSave™ reagent (Calbiochem). The same procedure was also used for high-content imaging of cells grown directly on coated (poly-ornithine/fibronectin) cell culture plastic, using the murine monoclonal Tuj1 (β III tubulin; #MMS435P, Covance; 1:500), and the secondary goat anti-mouse IgG2a antibody (Alexa-Fluor-555-coupled; 1:1000; #A-21137, Life technologies). All other antibodies were used here according to the same protocol. They are standard reagents offered by multiple suppliers in often changing qualities. The details on the particular material used here are given in supplemental methods.

Statistics

Where not indicated otherwise, data are mean ± SEM of triplicates from different cell preparations. Statistics were done by ANOVA followed by an appropriate post hoc test.

Results

Astrocytic support of neurons and attenuation of neurotoxicity

We used here a recently established co-culture model (Efremova et al. 2015) of LUHMES neurons and IMA 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 (Fig. 1a, b). Immunostaining showed that co-cultured LUHMES extended long neurites and expressed tyrosine hydroxylase (TH) as well as the vesicular monoamine transporter like LUHMES monocultures (Fig. S2). 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 monocultures (variable viability on d12 with a range of 50–100 %). A comparison of IMA with seven other cell types showed that only glial cells (murine, rat or human astrocytes) supported neurons, while other cells (e.g., astrocytoma or human neuroepithelial cells) did not (Fig. S3).

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 monocultures, whereas neurite integrity and viability of co-cultured neurons were not affected. Astrocytes were not affected by the SIN-1 concentrations used here. Encouraged by these findings, we tested three different neurotoxins and quantified neuronal viability/network complexity by β III tubulin staining. At a low concentration of 1 µM, the specific dopaminergic

Fig. 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 (β III tubulin, red), the astrocyte-specific marker S100 β (green), and with the DNA stain H-33342 (blue). A Z-stack of images was recorded on a confocal microscope (scale bar = 20 μ m). 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 μ M) for 24 h. Then, cells were fixed and stained to visualize β III tubulin (green) and nuclei (H-33342, blue); scale bar = 100 μ m. **c** LUHMES monocultures and co-cultures (d6) were exposed to various concentrations of MPP+ (0–100 μ M), SIN-1 (0–1000 μ M), and spermineNONOate (0–100 μ M) for 24 h. Then, cells were fixed and stained for β III tubulin, and the β III tubulin-positive neuronal area was quantified by an automated imaging procedure. Data are expressed as mean $\pm$ SD of quadruplicates; * p < 0.05 (co-culture vs. monoculture) (color figure online)

neurotoxicant MPP⁺ led to a 50 % decrease of neuronal area, while viability was fully maintained in the presence of IMA. At higher concentrations (5 μ M), IMA provided partial protection, and at even higher concentrations (≥ 10 μ M; 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 monocultured neurons (Fig. 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.

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

To model an inflammatory situation, as it occurs during brain damage and neurodegenerative disease, we used a cytokine mix (TNF- α and IL-1 β) known to be produced in neuroinflammatory situations (mainly by microglia) and known to activate astrocytic cells, including IMA (Falsig et al. 2006; Henn et al. 2011; Kuegler et al. 2012; Schildknecht et al. 2012). This CM had no effect on the viability and neurite structure of LUHMES monocultures (Fig. S4a). The absence of an effect was confirmed even when a third cytokine, IFN- γ , was added to CM (Fig. S4b). Moreover, CM did not affect the toxicity of SIN-1 or NO in LUHMES monocultures (Fig. S4c).

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 μ M spermineNONOate). The CM reduced the neuroprotective effect of IMA significantly (Fig. 2a).

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 6 days led to pronounced neurodegeneration without any other toxicant added (Fig. 2b). As standard endpoint to quantify neurodegeneration in co-cultures, we used specific staining of β III tubulin and an automated algorithm to quantify the overall neuronal area (Efremova et al. 2015). This endpoint correlated with the measurement of the release of neuron-specific enolase (Fig. 2c). This glycolytic isoenzyme, which is widely used as a specific read out for the assessment of neuronal damage level, is only expressed in neurons, but not in glial cells.

In order to find out which cytokine was involved in glial cell-mediated neurodegeneration, we exposed LUHMES-IMA co-cultures to IL-1 β , TNF- α , and IFN- γ individually, and in combinations. Both IL-1 β and TNF- α were able to trigger the degenerative response, while IFN- γ was without effect (Fig. 2d). To ensure a robust response, we selected the combination of TNF- α and IL-1 β (=CM) for our further experiments.

Signaling events occurring in reactive astrocytes exposed to cytokines

To obtain direct evidence on the stimulation of IMA by CM, they were examined in the absence of neurons. After 2-day exposure, the cultures started to change morphology. On day 6 all cells were elongated and orientated along parallel lines (Fig. 3a). This change was accompanied by other common features of reactive astrocytes, such as increased

protein content and proliferation. The immediate cytokine response (within 30 min) was reflected by NF- κ B translocation (Fig. 3b–d).

As NO is a candidate for glia-dependent neurotoxicity (Brown and Neher 2010), we examined the production of this mediator, but nitric oxide was not produced. 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 (Fig. 3e). We conclude from this, and from the absence of any protective effect of nitric oxide synthase inhibitors (Fig. S5), that NO is unlikely to be involved in the neurodegeneration observed in our model.

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). We also observed complete protection from CM toxicity (Fig. 4a). As this required high micromolar concentrations, we tested whether the protection was glucocorticoid receptor-dependent or may involve, e.g., antioxidant steroid effects (Hansson et al. 2000). To this end, we used the glucocorticoid receptor antagonist mifepristone. Mifepristone alone had no effect at all on neuronal viability, but it fully abolished the protective effect of dexamethasone (Fig. 4b).

In contrast to the broadly acting corticosteroid, several other treatments that specifically affect certain inflammatory pathways did not alter neuronal survival: inhibitors of NF- κ B, of cyclooxygenases, or of protein/cytokine secretion. However, concentration-dependent protection was observed, when the p38 MAP kinase inhibitor SB202190 was used (Fig. 4c). Thus, our model allows exploration of glia-modulatory drug candidates, with dexamethasone and SB202190 as positive control.

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 astrocytes (e.g., characterization of certain biologics drugs). Moreover, we wanted to be sure that the effect observed was not an interspecies artifact, but reproducible with human astrocytes. Therefore, we

Fig. 2 Switching from neuroprotective to neurotoxic properties of glia by exposure to inflammatory cytokines. **a** Co-cultures of d6 LUHMES and IMA (+IMA) and d6 LUHMES monocultures (–IMA) were either left untreated (–CM) or exposed for 24 h to a cytokine mix (+CM) consisting of murine IL-1 β (10 ng/ml) + murine TNF- α (10 ng/ml) before they were treated with solvent (control), SIN-1 (1000 μ M), or spermineNONOate (100 μ M) for additional 24 h. Then, cells were fixed and stained for β III tubulin, and the β III tubulin-positive neuronal area was quantified by an automated imaging procedure; * p < 0.05 (exposed to CM vs. unexposed). **b** IMA-LUHMEs co-cultures (d6) were exposed to CM for 3 or 6 days. Then, neurons were stained for β III tubulin, and images were recorded on a fluorescent microscope; scale bar = 100 μ m. **c** IMA-LUHMEs co-cultures (d6) in 24 well plate were exposed to CM or

to 10 μ M MPP+. After 6 days, cultures were stained for β III tubulin, and the β III tubulin-positive neuronal area was quantified by an automated imaging procedure. The release of human neuron-specific enolase (NSE) was measured in culture supernatants. The total amount of NSE in culture supernatants of control cultures was 8.5 ± 1 ng/ml culture supernatant; t test, * p < 0.05 (CM vs. solvent). **d** IMA-LUHMEs co-cultures (d6) were exposed to murine IL-1 β (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-1 β + TNF- α + IFN- γ) for 6 days. Then, cultures were stained for β III tubulin, and the neuronal area was measured by an automated imaging procedure. Data are expressed as mean $\pm$ SD of quadruplicates; Dunnett's multiple comparison test, * p < 0.05 (all bars vs. solvent)

Fig. 3 Astrocyte activation and proliferation by exposure to cytokine mix (CM). **a** IMA monocultures were exposed to murine CM (IL-1 β + TNF- α) for 2, 4, and 6 days. Then, cells were fixed, permeabilized, and immunostained for the astrocyte-specific marker S100 β (green); nuclei were stained with H-33342 (red), scale bar = 100 μ m. **b** IMA cells were exposed to CM for 6 days. Then cells were lysed, and the protein amount in lysates was assessed; *t* test, $*p < 0.05$ (solvent vs. CM). **c** For assessment of the number of nuclei, IMA monocultures were fixed and stained with H-33342 after 6 days of CM exposure. Ten fields (each 330 μ m $\times$ 330 μ m size each) per well were imaged in one channel using a 20 $\times$ objective. The pictures were then analyzed by software, which recognized the H-33342-positive nuclei

and calculated their number in each field. Three wells of 24 well plates were analyzed for each treatment conditions; *t* test, $*p < 0.05$ (solvent vs. CM). **d** IMA monocultures were stimulated with CM or solvent for 30 min. Then cells were stained with an anti-NF- κ B p65 antibody (green), and nuclei were stained with H-33342 (red). Images were taken by using a fluorescent microscope, scale bar = 100 μ m. **e** IMA cells were exposed to CM (TNF- α + IL-1 β) 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 mean $\pm$ SD of quadruplicate samples; Bonferroni's test, $*p < 0.05$ (solvent vs. CM) (color figure online)

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 the degeneration of LUHMES cells (Fig. 5a). Already after

4 days, >50 % neurodegeneration was observed (Fig. 5b). As in the IMA model, full protection by dexamethasone was observed (Fig. 5c). In summary, human astrocytes behaved similar to IMA concerning major features of the co-culture cytokine-mediated neurodegeneration.

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. It was transferred to LUHMES monocultures, and immunostaining showed that after 3 days, about 50 % of the neurons had died (Fig. 6a). Measurements of the viability by resazurin (not shown) or quantification of the neuronal area confirmed neuronal

Fig. 4 Protection from cytokine toxicity in co-cultures by anti-inflammatory treatment. **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) or left untreated (*control*) 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 pre-treated for 30 min with different concentrations of mifepristone (0–10 μM) and left untreated (*control*) or treated with CM in the absence (CM– dexamethasone) or presence of 100 μM dexamethasone (CM+ dexamethasone) for 6 days. Then, the neurite integrity was evaluated by staining for βIII tubulin and quantification of the βIII tubulin-positive neuronal area. **c** Co-cultures were pre-treated as in **a** with various concentrations of SB202190 for 30 min and exposed to CM for 6 days. Then, viability was measured. Data are expressed as mean ± SD of triplicate samples

death triggered by CM supernatants, but not control supernatants from IMA (Fig. 6b). Time course experiments of supernatant sampling indicated that maximal neurotoxic activity of the IMA medium was reached after about 5 days (Fig. 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 (Fig. 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 (Fig. S6). At the production conditions used here (15.000 IMA/cm²), we examined whether the composition of conditioned medium changed drastically. As elevated extracellular L-glutamate (L-Glu) content has been reported in previous inflammation models (Castillo et al. 2002; Ward et al. 2009), we also investigated whether this could be the cause of neurotoxicity in our model. Amino acid analysis showed that cytokine-conditioned medium differed for few amino acids from control supernatant, but many constituents, including L-Glu, remained largely unchanged (Fig. S7a). Application of exogenous L-Glu to the medium had no effect on LUHMES viability (Fig. S7b), and the absence of any neuroprotection from CM-treated IMA supernatant after treatment with glutamate receptor antagonist MK801 further suggested that L-Glu is not likely to play a role in CM-glia-induced neurotoxicity in our model (Fig. S7c). We further 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, N2 supplement, pyruvate, or glucose (Fig. S7d). Thus, presence of a factor produced by IMA upon cytokine stimulation is the most straightforward explanation for neurotoxicity of the conditioned medium.

Fig. 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 NHA and LUHMES cells (d6) were exposed to CM (human 10 ng/ml IL-1 β + human 10 ng/ml TNF- α) for 4 days. Then, co-cultures were stained for the neuronal-specific marker Tuj1 (β III tubulin, *b/w*), the astrocyte-specific marker S100 β (*green*), and the DNA stain H-33342 (*red*). Images were recorded on a fluorescent microscope. **b** NHA-LUHMES co-cultures were exposed to human CM for 2 or 4 days. Cultures were stained for β III tubulin, and the β III tubulin-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 μ M) followed by the addition of human CM for 4 days. Then, cultures were stained for β III tubulin, and the β III tubulin-positive neuronal area was quantified by an automated imaging procedure. Data are expressed as mean $\pm$ SD of triplicate samples; $^{*}p < 0.05$ (treated with dexamethasone vs. untreated) (color figure online)

Pharmacological protection from glia-conditioned neurotoxic medium

The possibility to separate IMA stimulation and neuronal exposure physically allowed now a pharmacological characterization of the neurodamaging effects of astrocytes on the level of the target cell. We used this big advantage of the new model for some targeted interventions (Fig. 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 from glia-conditioned supernatants. Thus, its effect in the co-cultures was most likely due to immunomodulation of the glia. The same was observed for the p38 kinase inhibitor SB202190.

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). Anti-inflammatory compounds such as NF- κ B inhibitors and cyclooxygenase inhibitors (Fig. S5) did not show protection; neither did the ROCK inhibitors Y-27632 (Fig. 7a) and HA-077 (not shown). The latter finding was in contrast to the MPTP model, where both compounds were very efficacious (Efremova et al. 2015).

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; N.B.: 50 $\times$ higher than the normal medium concentration), the phenolic plant ingredient resveratrol, the caspase inhibitor zVAD, and the mixed-lineage kinase inhibitor CEP1347 (Fig. 7a). More detailed concentration–response studies confirmed all initial findings (Fig. 7b). These data suggest the activation of several pathways known to be associated with neurotoxicity. Targeting of such pathways may result in new approach to modify detrimental effects of neuroinflammation.

Pharmacological prevention of glial neurotoxicant production

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 was co-incubated with CM during the phase of IMA stimulation (Fig. 8a). 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. In these studies, the effects of the steroid were observed already in a submicromolar range (Fig. 8b). Thus, dexamethasone showed its 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 p38 MAP kinase inhibition, which also did not affect neurons alone (medium transfer model), but had protected LUHMES in co-cultures. Two p38 inhibitors reduced neurotoxicity

Fig. 6 Mediation of neurotoxicity of inflammatory astrocytes by soluble, transferable factors. **a** IMA monolayers 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. Afterward, cells were fixed and stained for β III tubulin (green) and the DNA stain H-33342 (blue), and images were recorded on a fluorescent microscope. Scale bar = 100 μ m. **b** The β III tubulin-positive neuronal area from **a** was quantified by an automated imaging procedure. Data are expressed as mean $\pm$ SD of triplicate samples; t test, *** p < 0.001 (-CM vs. +CM). **c** IMA monolayers were incubated in the presence or absence of murine CM. Each 24-h (within the time period of 6 days) conditioned medium from untreated (dashed line, control super-

natant) and treated (black line, CM) IMA was collected, filtered, and frozen at -20°C . After, conditioned medium was thawed, and 100 % of it was transferred to LUHMES monolayer (d6) for following 3 days. Then, the neuronal viability was measured by the resazurin reduction assay. Data are expressed as mean $\pm$ SD of quadruplicates; two-way ANOVA followed by a post hoc Bonferroni's test, * p < 0.001 (control supernatant vs. CM). **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 mean $\pm$ SEM of three different experiments; two-way ANOVA followed by a post hoc Bonferroni's test, * p < 0.05 [control supernatant (d6) vs. CM supernatant (d6)] (color figure online)

Fig. 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 μ M), GSH (100 μ M), GSSG (100 μ M), the p38 kinase inhibitor SB202190, Y-27632 (10 μ M), the H₂S donor ABT-337 (10 μ M), glial-derived neurotrophic factor (GDNF, 100 ng/ml), resveratrol (1 μ M), the caspase inhibitor zVAD (100 μ M), 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 mean $\pm$ 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, resveratrol, zVAD, CEP1347. After 3 days, the neuronal viability was measured by the resazurin reduction assay. Data are expressed as mean $\pm$ SD of triplicate samples from one experiment

of glia-conditioned medium in the submicromolar range (Fig. 8c). These data show that drugs targeting specific regulation of inflammatory responses in astrocytes may have neuroprotective potential in neuroinflammatory conditions.

Discussion

Our study provides clear evidence that activation of astrocytes not only reduces their neuroprotective properties, but

Fig. 8 Protection of neurons by prevention of astrocyte activation. **a** Experimental schematic representation. IMA monocultures were pre-treated with **b** dexamethasone; *t* test, $*p < 0.05$ (dexamethasone untreated vs. treated in the presence of CM) or **c** two different p38 inhibitors, SB202190 or SB203580, $*p < 0.05$ (SB202190 or SB203580 untreated vs. treated in the presence of CM) for 30 min before stimulation with CM. After 6 days, conditioned medium was collected, then 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 mean $\pm$ SD of three experiments. Statistics were performed using one-way ANOVA followed by a Dunnett's multiple comparison post hoc test

also can lead to active killing of human neurons by soluble factors. The effect described here in detail for a murine astrocytic cell line was also reproduced by human primary astrocytes. As neurotoxic factors were transferrable by stored and frozen supernatants, and as the toxicity required considerable time (days) to develop, we conclude that the mediators responsible for the neurodegeneration observed in our model system are different from reactive oxygen/nitrogen species, elevated extracellular glutamate levels, or excitotoxins described in other models (Fig. S1). Therefore, the astroglia-dependent neurotoxicity, occurring in the co-culture model established here, differs also significantly from several forms of microglia-dependent neurotoxicity observed *in vitro* and *in vivo* (Block et al. 2007; Brown and Neher 2010).

When we explored the suitability of our system to investigate potentially neuroprotective agents, we identified seven types of intervention that can be grouped according to the target cell involved. The first set of compounds acted

on astrocytes. For instance, the inhibition of p38 MAP kinases was not beneficial, when restricted to neurons, but it prevented formation of the neurotoxic activity by astrocytes. The same was observed for dexamethasone, a glucocorticoid used in clinical neurology for the treatment of brain inflammation. Five other types of drugs blocked cell death and neurite degeneration directly on the level of neurons. Thus, the 'astrocyte inflammation—LUHMES model' expands the available options (Fig. S1) for efficacy and target finding assays in drug discovery.

Research on neuronal dysfunctions in neurodegenerative diseases has revealed many important cell autonomous mechanisms, such as neuronal programmed cell death and disturbances of proteostasis, often linked to disturbed calcium regulation, oxidative stress, or amyloid formation. More recent research has shown that also non-neuronal cells may play a role in neurodegeneration. For instance, genetic or non-genetic alterations in astroglial cells may adversely affect the survival of neurons in models of human diseases related to the RETT syndrome, Huntington's disease, Parkinson's disease, multiple sclerosis, or amyotrophic lateral sclerosis (Gallardo et al. 2014; Kohutnicka et al. 1998; Lukovic et al. 2015; Mayo et al. 2014; Valenza et al. 2015; Williams et al. 2014).

Studies on the role of microglia toxicity to neurons have shown that *in vitro* models and co-cultures are valuable for identification of potential mechanisms, although the confirmation of their relevance *in vivo* is still under debate, and may be limited to more chronic models (Biber et al. 2014). Studies in this field have shown that the use of co-cultures provides new information due to the sometimes unpredictable reciprocal interactions of the cells. It is, for instance, known from the interactions of immune cells that inflammatory reactions may be dramatically boosted in feed-forward loops, and that they can therefore not be predicted from reactions of individual cells (Gantner et al. 1996). Something similar may apply to the cycle of activated astrocytes affecting neurons and stressed neurons further activating astrocytes (Valenza et al. 2015; Volterra and Meldolesi 2005).

The co-culture system described here allowed the observation of three situations. In the ground state, the astrocytes protected neurons from external toxic stimuli. This is an established feature of astrocyte–neuron interaction (Dodla et al. 2010; Gupta et al. 2012; Lukovic et al. 2015), and it involves, for instance, thiol transfer from astrocytes to neurons. Accordingly, block of GSH synthesis in our model also prevented the protective effect of astrocytes (not shown), as described in similar approaches earlier (Chen et al. 2001; Gupta et al. 2012; Lee et al. 2010).

The second situation involves the inflammatory stimulation. Activation of the astrocytes by pro-inflammatory cytokines (TNF- α , IL-1 β) is well established (Falsig et al.

2004a, b, 2006, 2008; Henn et al. 2011; Kohutnicka et al. 1998), and our model recapitulated many of the well-known features, such as NF- κ B translocation, and morphological alterations reminiscent of the one of the migrating activated astrocytes (Buffo et al. 2010). The activation of astrocytes is not fully understood. Therefore, we do not know which of the many early signals affected by dexamethasone is responsible for the long-term consequences concerning neuronal survival. Only specific inhibitors of p38 kinases, out of several other anti-inflammatory drugs tested, were also protective. This agrees well with an earlier finding that only p38 inhibitors affected the endpoint of activation-induced cell death in primary murine astrocytes (Falsig et al. 2004a, b). The activation process and production of the neurocytotoxic supernatant depend also on the initial state of IMA (proliferation; passage number). This may imply that different astrocyte populations or brain regions in vivo may react differently.

The third situation involved neuronal death triggered by the astrocytes. A major role of soluble factors is suggested by the transferability of the killing activity by supernatant transfer and by defined pharmacological interventions that protected the neurons in this situation.

Concerning the factors released by astrocytes, our preliminary experiments suggest that it is neither a short-lived species (e.g., ROS) nor a type of protein that would lose activity upon boiling. This distinguishes our model from most other model systems based on rodent primary neurons or on neuroblastoma cells (Fig. S1). Further characterization and possibly identification will require extensive experimentation and substantial modifications of the system, so that higher concentrations of the factor are obtained. The latter is a precondition for fractionation experiments. At present, this is not possible, as this would deplete also essential components of the medium required for basic survival of the neurons.

Several candidate factors responsible for astrocyte-mediated toxicity are already known. They comprise cytokines (Buffo et al. 2010; Falsig et al. 2004a; Garwood et al. 2011); lipocalin 2 (Bi et al. 2013), components of the complement system (Bodea et al. 2014; Pekny et al. 2007), or changes due to altered ATPase expression (Gallardo et al. 2014). Besides these mostly proteinaceous molecules, also some small molecules with cell death-inducing properties have been described. They comprise the glycosphingolipid–lactosylceramide (LacCer) (Mayo et al. 2014), the ganglioside GD3 (Simon et al. 2002), or their basic core component, the pro-apoptotic mediator ceramide (Wang et al. 2012). Possibly, also chondroitin sulfate proteoglycan, which is secreted by astrocytes and impedes axon regeneration (Silver and Miller 2004), may take a toxic role for neurites in cell cultures.

Altogether, the astrocyte inflammation model adds a new, yet unexplored option to test neuroprotective compounds. The pharmacological profile observed here differed from the one observed, e.g., in LUHMES neuron monocultures exposed to MPP⁺ or from LUHMES-astrocyte cultures exposed to MPTP (Efremova et al. 2015). In these two toxicant-driven models, ROCK inhibitors showed a potent neuroprotection, not observed here. Vice versa, the robust protection observed here by high-dose GDNF or of ATB-337 was unique, as it was not observed in the MPTP (MPP⁺) model.

In summary, the versatile co-culture model established here will allow future studies (1) on the basic neuroprotective properties of astrocytes; (2) on the transition from the control condition to a slowly developing neurodegenerative state affecting human neurons; (3) pharmacological interventions targeting either astrocytes or neurons; (4) on identification of glia-derived neurodegenerative factors. A better definition of the soluble neurotoxic activity derived from astrocytes, or possibly the identification of a defined factor responsible for it, would greatly help a more direct study of the specific inflammatory signals involved in its production and possibly yield a new biomarker of brain inflammation. Moreover, it will be highly interesting to study mechanisms that might restore, or even boost, the neuroprotective properties of astrocytes, even in a neuro-inflammatory environment. A promising approach may be the triggering of the Nrf-2 pathway (Dodla et al. 2010; Gupta et al. 2012) or utilization of the properties of urate in glia–neuron interactions (Cipriani et al. 2012). Moreover, our model might support the quest for specific anti-inflammatory compounds that dampen astrocyte reactions, but not the protective capacity of the peripheral innate immune response.

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Author's contribution Liudmila Efremova and Petra Chovancova performed most experiments, analyzed data, and wrote the manuscript; Stefan Schildknecht, Martina Adam, and Simon Gutbier performed experiments and proofread the manuscript; Marcel Leist designed experiments and wrote the manuscript.

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