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RESEARCH PAPER

Anti-inflammatory effects of the anticonvulsant drug levetiracetam on electrophysiological properties of astroglia are mediated via TGFβ1 regulation

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BACKGROUND AND PURPOSE

The involvement of astrocytes as immune-competent players in inflammation and the pathogenesis of epilepsy and seizure-induced brain damage has recently been recognized. In clinical trials and practice, levetiracetam (LEV) has proven to be an effective antiepileptic drug (AED) in various forms of epileptic seizures, when applied as mono- or added therapy. Little is known about the mechanism(s) of action of LEV. Evidence so far suggests a mode of action different from that of classical AEDs. We have shown that LEV restored functional gap junction coupling and basic membrane properties in an astrocytic inflammatory model *in vitro*.

EXPERIMENTAL APPROACH

Here, we used neonatal rat astrocytes co-cultured with high proportions (30%) of activated microglia or treated with the pro-inflammatory cytokine interleukin-1β to provoke inflammatory responses. Effects of LEV (50 $\mu g \cdot mL^{-1}$) on electrophysiological properties of astrocytes (by whole cell patch clamp) and on secretion of TGFβ1 (by ELISA) were studied in these co-cultures.

KEY RESULTS

LEV restored impaired astrocyte membrane resting potentials via modification of inward and outward rectifier currents, and promoted TGFβ1 expression in inflammatory and control co-cultures. Furthermore, LEV and TGFβ1 exhibited similar facilitating effects on the generation of astrocyte voltage-gated currents in inflammatory co-cultures and the effects of LEV were prevented by antibody to TGFβ1.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that LEV is likely to reduce the harmful spread of excitation elicited by seizure events within the astro-glial functional syncytium, with stabilizing consequences for neuronal–glial interactions.

Abbreviations

ACSF, artificial cerebrospinal fluid; AED, anti-epileptic drug; CARAT, Copy Number Analysis with Regression and Tree; CSF, cerebrospinal fluid; Cx43, connexin 43; GFAP, glial fibrillary acidic protein; IFN-β, interferon β; IL1-β, interleukin-1β; M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia; MRP, membrane resting potential; PBS, phosphate-buffered saline; pHi, intracellular pH; RRT, resting ramified type; RPT, round phagocytic type; TGFβ1, transforming growth factor β1; TNF α, tumour necrosis factor α

Introduction

Recent studies provide ample evidence of a glial component in the initiation and persistence of inflammatory processes in brain tissues. Astrocytes and microglia, as resident immune effector cells (Nelson *et al.*, 2002; Kim and de Vellis, 2005; Pivneva, 2008), have also frequently been suggested to be involved in the pathogenesis of epilepsy (Tian *et al.*, 2005; Vezzani and Granata, 2005; Seifert *et al.*, 2006; Jabs *et al.*, 2008; Wetherington *et al.*, 2008). Until recently, neurons, as the functional substrate of seizure generation, have received most of the attention in biomedical research. The renaissance of glia, in particular their contribution to neural activity in the form of checkpoints for the regulation of local neurotransmitter-, ion- and immune-homeostasis has fostered the concept of a glial component in epileptogenesis (Tian *et al.*, 2005; Choi and Friedman, 2009; Witcher *et al.*, 2010). This concept has been attributed to the ability of astrocytes to build a functional syncytium mediated by gap junctions (Soroceanu *et al.*, 2001; Meme *et al.*, 2006; Retamal *et al.*, 2007) which in astrocytes is basically constituted by connexins (Cx)43 and 30 (Dermietzel *et al.*, 1991; Giaume *et al.*, 1991; Kunzelmann *et al.*, 1999). Gap junction coupling allows astrocytes to distribute potentially neurotoxic metabolites, mainly extracted from the synaptic clefts within the glial syncytium or to discharge them into the blood vessels (Volterra and Meldolesi, 2005). In order to perform bilateral interactions with local and peripheral immune effector cells, astrocytes employ and react to pro- and anti-inflammatory cytokines as signaling molecules (Turrin and Rivest, 2004; Vezzani *et al.*, 2008).

Earlier, we and others have shown the influence of some cytokines, among them endothelial tumour necrosis factor alpha, Interleukin-1β (IL-1β), interferon β (IFNβ) and transforming growth factor β (TGF-β) on astrocytic gap junctional coupling, suggesting a contribution of glial gap junctions to inflammatory processes (Rouach *et al.*, 2002; Faustmann *et al.*, 2003; Hinkerohe *et al.*, 2005; Haghikia *et al.*, 2008; Hinkerohe *et al.*, 2010). In patients with recent generalized convulsions, acute inflammatory reactions following seizures have been suggested due to clinical observation of aseptic pleocytosis and increase of inflammatory cytokines in the CSF (Peltola *et al.*, 2002; Avignone *et al.*, 2008; Choi and Friedman, 2009). The contribution of the cellular and humoral immune system, including the activation of microglia and astrocytes (Bauer *et al.*, 2007), production of pro-inflammatory cytokines (De Simoni *et al.*, 2000; Vezzani *et al.*, 2000; Jankowsky and Patterson, 2001; Gorter *et al.*, 2006; Rao *et al.*, 2008; Riazi *et al.*, 2008) and related molecules to the pathogenesis of seizures (Peltola *et al.*, 2000) have been described in epilepsy as well as in experimental models of epilepsy. As a major inflammatory mediator, IL-1β has been shown to exert a proconvulsive impact by itself and to alter neuronal network excitability in several rodent forebrain regions, while its endogenous receptor antagonist (IL-1βRa) inhibited seizures (Vezzani *et al.*, 1999; 2000; De Simoni *et al.*, 2000).

The [S]-α ethyl-2-oxo-1-pyrrolidine acetamide [levetiracetam (LEV)] is a clinically established antiepileptic drug (AED) and has proven to be effective in the prevention of various forms of epilepsy. On the neuronal level, there is some evidence of the antiepileptic mechanisms used by LEV, that is, kindling-induced alterations in gene expression in the temporal lobe of rats were modified by LEV (Gu *et al.*, 2004). Furthermore, intracellular pH (pHi) modulation via inhibition of Na^+-dependent Cl^-/HCO_3^- exchange was described in adult hippocampal CA3 neurons of the guinea-pig (Leniger *et al.*, 2004). However, GABAergic currents were not influenced by LEV in a paired-pulse study of field potentials (Margineanu and Klitgaard, 2003) and LEV also failed to block voltage-gated Na^+ and low voltage-activated Ca^{2+} currents as well as NMDA receptors (Zona *et al.*, 2001; Gorji *et al.*, 2002). There was an incomplete inhibition of voltage-operated K^+ currents (Madeja *et al.*, 2003) and N-type Ca^{2+} channels of hippocampal CA1 neurons whose high-voltage-activated calcium currents were reduced by about 20% (Lukyanetz *et al.*, 2002). Thus, although its anti-convulsive properties have been approved for more than 10 years, the exact mechanism of action of LEV in brain tissues remains to be clearly defined.

Using an *in vitro* approach, we have shown that LEV restored the functional astrocytic syncytium, when disrupted by inflammatory conditions

(Haghikia *et al.*, 2008). This effect was comparable with the ability of the anti-inflammatory cytokine TGFβ1 to restore functional properties of astroglia after an induced inflammatory response (Hinkerohe *et al.*, 2005). The aim of our present study was to gain a better mechanistic understanding of the previously observed effects of LEV on the astroglial syncytium under inflammatory conditions.

Methods

Cell Culture

All animal care and experimental procedures were carried out according to the guidelines of the German Animal Protection Law in its present version (1998) and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Efforts were made to reduce the number of animals to a minimum. All experiments were performed on animals bred in the Institute of Neuroanatomy and Molecular Brain Research of the Ruhr-University Bochum (Germany). Primary cell cultures of glial cells were prepared from brain hemispheres of post-natal (P0–P2) Wistar rats as described by Dermietzel *et al.* (1991).

After careful removal of the meninges and the choroid plexuses, the brain tissue was stored in phosphate-buffered saline (PBS) containing 0.1% trypsin (Invitrogen, Karlsruhe, Germany) at 37°C, washed with Dulbecco's minimal essential medium (DMEM) with 1% glucose containing 10% fetal calf serum, followed by a 5 min incubation with 1% DNAse I (bovine pancreas; Serva, Heidelberg, Germany) in PBS at room temperature.

After blocking enzyme activity, whole brain tissues were diluted and dispersed in culture medium [DMEM, 10% fetal calf serum, 1% nonessential amino acids (Invitrogen), penicillin (50 $\mu g \cdot mL^{-1}$), streptomycin (50 $\mu g \cdot mL^{-1}$), and glutamine (2 mM)] by trituration with a pipette and subsequent passage through a 60 µm nylon mesh. Cells were first plated at a density of two brains per culture flask (Becton Dickinson, Heidelberg, Germany). Cultures were maintained in a 5% CO2, 95% air atmosphere at 37°C and nearly 100% relative humidity. After 4–5 days in DMEM culture medium, the astroglial cell layer achieved confluency. Co-cultures were further removed from the culture dishes by trypsin-EDTA (0.1%) treatment and passaged onto poly-L-lysine–coated glass coverslips (12 mm^2), reaching a density of 40.000–50.000 cells per coverslip. A confluent monolayer was again achieved 2–3 days after the passaging of the co-cultures onto glass coverslips. Non-adjacent microglial cells and oligodendroglia, which are conveniently found to grow on top of the astrocytes, were isolated and removed from the culture by shaking the flasks manually. The fraction of microglial cells remaining in the co-cultures can be altered by the extent of shaking. Here, we have used concentrations of microglia of 5% (M5; concentrations equivalent to healthy brains) and of 30% (M30; representing inflammatory conditions).

Before the ELISA and electrophysiology procedures, single co-culture samples were analysed for the fraction of activated and non-activated microglia by double immunocytochemistry using anti-ED1 and anti-glial fibrillary acidic protein (GFAP) antibodies (see next). Cells were used for experiments after a total of 6–8 days after brain preparation.

Immunocytochemistry and quantification of microglia

The proportion of activated microglia was quantified in all experiments using combined immunocytochemistry against ED1 and GFAP according to Faustmann *et al.* (2003). GFAP was detected using a polyclonal antibody (1:100; Sigma G9269, Taufkirchen, Germany). Microglial cells were labelled by applying a monoclonal antibody to the ED1 marker (1:250; Serotec MCA 341R, Eching, Germany) (Dijkstra *et al.*, 1985; Polman *et al.*, 1986). For quantification of the microglial fraction, immunocytochemically labelled cells were counterstained with 4,6-diamidino-2-phenyl-indol (DAPI; 1:2500; Sigma D 9542) to visualize nuclei. After three additional washes with PBS, the coverslips were embedded in ProLong antifade kit (Molecular Probes, Leiden, the Netherlands) and subjected to immunofluorescence microscopy with an Axiovert 35 microscope (Zeiss, Jena, Germany). The ratio of microglia to astrocytes was determined by comparing the number of ED1-stained microglia with the total number of DAPI-labelled cells by two independent examiners. Co-cultures containing a ratio of approximately 5 and 30% of microglia (termed M5 and M30) were determined in five independent experiments. Subpopulations of microglia were differentiated by visually counting 50–100 microglial cells for each experiment at a primary magnification of 40×.

Ratios were expressed as percentage of each subpopulation in the entire microglial cell population. The significance of differences between the median of the three microglial subpopulations (Figure 1) in the M5 and M30 co-cultures was tested using the Mann–Whitney test.

Electrophysiology

Membrane resting potentials (MRP) and astrocytic transmembrane currents were assessed by whole cell patch clamp recordings. Cells on a cover slip were transferred to an open cell chamber (2 mL)

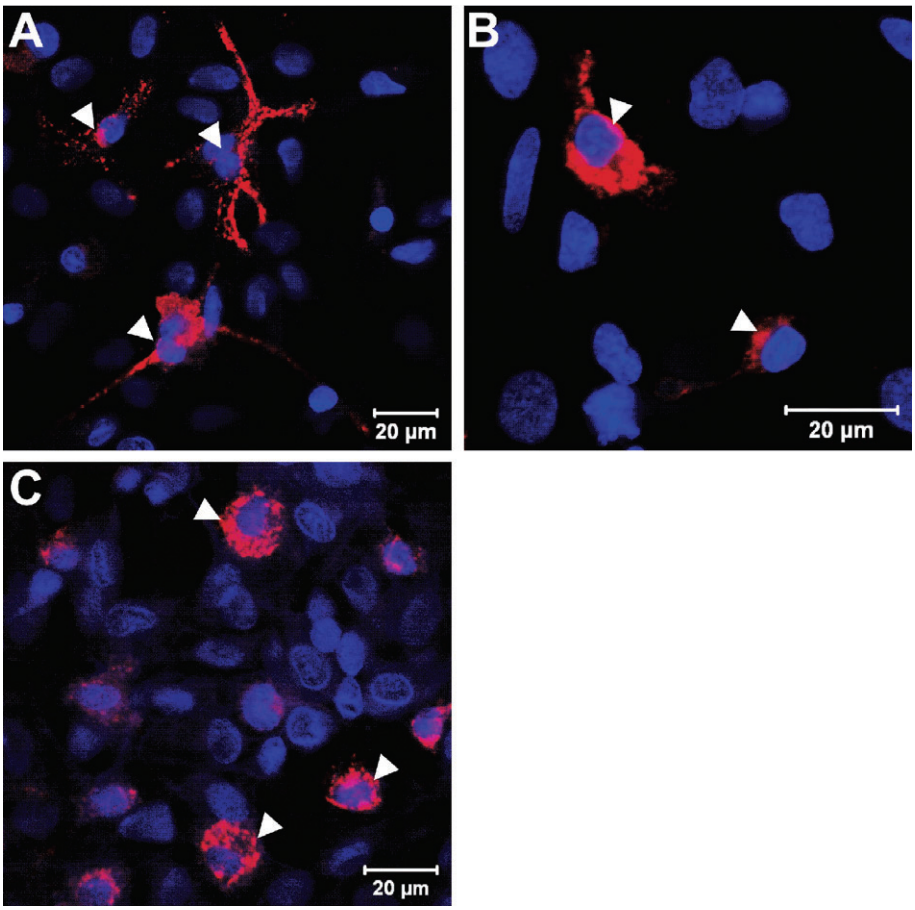

Figure 1

Basic microglial phenotypes of the astroglial co-culture system used in this study. Primary astrocytes were co-cultured with either 5% (M5) or 30% (M30) microglial cells. Figure 1 represent fluorescence immunocytochemistry of the microglia-specific protein ED1 in combination with DAPI counterstaining to visualize astrocytic and microglial nuclei, for quantification and review of microglial activation in accordance to each individual experiment. Immunocytochemistry allows classification of the following different microglial phenotypes: resting ramified type (A), intermediate (B) and activated, round phagocytic type (C). A and B: M5 microglial fraction. C: M30 microglial fraction.

mounted on a fixed-stage microscope and continuously superfused at room temperature with artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM): 127 NaCl, 2 KCl, 0.9 NaH_2PO_4, 11 glucose, 1 MgSo4, 1 $CaCl_2$, 26 $NaHCO_3$. To maintain the pH of 7.4, the external solution was continuously superfused with carbogen gas (5% CO_2/95% O_2). The internal solution of the recording pipette was as follows (mM): 130 K-gluconate, 2 Na-G-gluconate, 20 HEPES, 4 $MgCl_2$, 4 Na2ATP, 0.4 NaGTP and 5 EGTA. pH was adjusted to pH 7.3 and the final solution was filtered through 0.22 µm filters (Millipore Corp., Billerica, MA, USA).

Patch pipettes were pulled from borosilicate glass capillaries (code 7056; Hugo Sachs Elektronik/Harvard Apparatus, March-Hugstetten, Germany) with a vertical puller (PIP5; HEKA, Lambrecht, Germany). Patch resistance was in the range 5–8 MΩ. Currents from astrocytes were recorded in the voltage clamp mode, amplified and filtered at 5 kHz by an Axopatch 200B amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Data were digitized at 10 kHz sampling rate, displayed, stored and analysed using WinWCP software (Strathclyde; Biologic, Knoxville, TN, USA). A 16 bit analogue to digital converter (BNC 2110 connected to Ni-PCi 6229, National Instruments; Munich, Germany) was used to digitize the signals.

All recordings were corrected for junction potentials of 10 mV. Leak currents were not subtracted from the records. Capacitance and series resistance compensation (40–50%) were applied to emend voltage clamp control.

To investigate putative inward and outward rectifier currents in astrocytes, we established the following paradigm for the whole cell voltage clamp/patch clamp technique in the voltage clamp mode: the membrane potential was initially held at

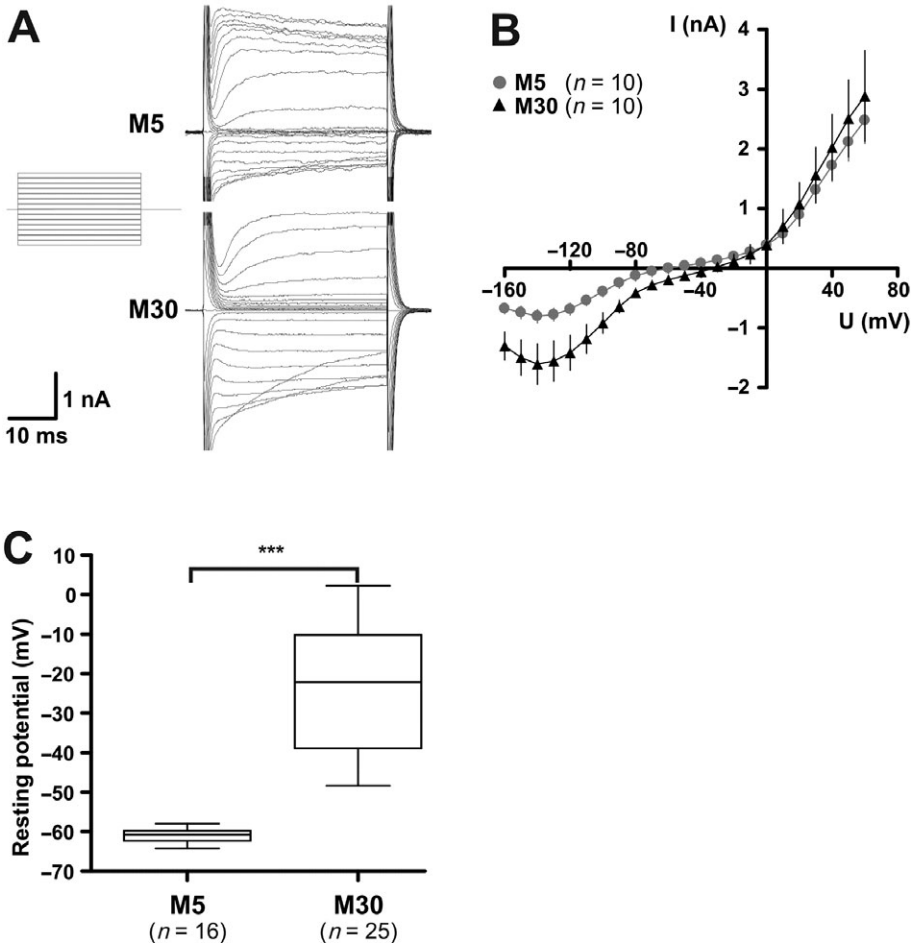

Figure 2

Electrophysiological properties of M5 and M30 co-cultures. (A) Representative examples of holding potential-evoked, voltage-gated membrane currents of M5 and M30 astrocyte co-cultures in whole cell patch clamp recordings. Depolarization steps from –160 to 60 mV (increment = 10 mV) as indicated by the voltage protocol (inset on the left) were used to activate membrane currents. Note that voltage-gated activity was significantly increased in M30 co-cultures. This is also reflected by characteristic current–voltage relations (IV curves) of M5 and M30 cultured astrocytes (B). The main difference between the two conditions is a significant increase of inwardly rectifying currents in the M30-cultured astrocytes at hyperpolarizing holding potentials. (C) Median membrane resting potential (MRP) in M5-cultured astrocytes of –60.77 mV (median; range –57.99 mV to –64.26 mV; n = 16) was in the range of normal astrocytic MRP. In M30 co-cultures, MRP shifted significantly towards depolarization (median –22.40 mV; range +2.04 mV to –48.65 mV; n = 25). ***P < 0.0005. M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia.

–60 mV, then consequently processed stepwise from –160 to +60 mV, with increments of 10 mV and step durations of 50 ms. The interval between sweeps was 1000 ms (Figure 2B). Currents for the establishment of current–voltage relation plots were measured between the 47th and 49th ms of the 50 ms lasting holding potential step evoked by the voltage clamp protocol. Membrane resting potentials were measured in the current clamp mode with the holding current clamped to 0 pA.

Statistical comparisons of the data were performed using a Mann–Whitney *U*-test to evaluate significance between two group means. Analysis of variance was used for multiple groups. The significance of difference between median of the astroglial MRP was tested using the Mann–Whitney test (one-tailed) and displayed in box plots. Input resistances of the different groups are presented in Table 1 as the mean ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

Drug application

To match the serum concentrations found in successfully treated patients after 4 weeks of LEV administration (Grim *et al.*, 2003), cultured astrocytes were incubated with 50 µg LEV for 24 h (5% CO2, 95% air atmosphere at 37°C and nearly 100% relative humidity) prior to electrophysiological recordings. We chose this long incubation period in order to make this *in vitro* approach comparable

Table 1
Input resistance of different groups

Type of culture	Input resistance (MΩ)
M5	41.9 ± 15.4 (n = 16)
M30	23.8 ± 8.6 (n = 25)*
M5 + LEV	39.7 ± 20.0 (n = 16)
M30 + LEV	40.3 ± 8.1 (n = 19)†
M5 + TGFß1	38.5 ± 10.7 (n = 10)
M30 + TGFß1	37.2 ± 15.8 (n = 12)#

*P < 0.005, signficantly different from values in M5 cultures.
†P < 0.005, significant effect of levetiracetam (LEV).
#P < 0.05, significant effect of incubation with TGFβ1.
M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia.

with the prophylactic application of LEV in patients with chronic epilepsy. IL-1β (R&D Systems, Minneapolis, MN, USA) was applied at a concentration of 500 U·mL^{-1} to the primary M5 co-cultures for 2 h. In a second series of experiments, M5 co-cultures following treatment with 50 µg·mL^{-1} LEV for 22 h received an additional incubation with IL-1β (500 U·mL^{-1}) for 2 h. Co-cultures were also incubated for 24 h with 10 ng TGFβ1 mL^{-1} (R&D Sytems) prior to the experiments to mimic a possible anti-inflammatory effect of LEV.

Biological activity of TGFβ1 was blocked by 400 ng·mL^{-1} anti-TGFβ1 antibody (R&D Systems) before the electrophysiological recordings. In case of co-incubations with LEV, the antibody was applied 1 h prior to the LEV treatment of the co-culture.

ELISA

TGFβ1 levels in cell culture supernatants (M5, M30, M5 + LEV, M30 + LEV) were quantified by Sandwich-ELISA (Promega, Madison, WI, USA) according to the instructions of the TGFβ$_1$ E$_{max}$® ImmunoAssay System (Promega, Madison, MI, USA). Optical density of each well was determined by using a microplate reader (Bio-Rad 550, Hercules, CA, USA) set to 450 nm.

TGFβ1 concentration of the supernatants was calculated by normalized standard twice-diluted series. Each individual sample was determined in triplicate. Values are displayed as mean ± SEM. Significance of differences between untreated and with LEV incubated co-cultures were tested using the Mann–Whitney test (GraphPad Software, San Diego, CA, USA).

Measurement of pHi

To analyse changes of pHi, astroglial cell cultures were loaded with the pH sensitive fluorescent indicator 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM, 0.5–1.0 µM; Molecular Probes). Measurements were carried out using a 60× water-immersion objective (Olympus, Hamburg, Germany). Cells were illuminated with alternating light (440 and 490 nm) provided by a halogen lamp and a computer-operated filter wheel (Sutter Instruments, Novato, CA, USA). Light of both wavelengths was dimmed by an appropriate neutral density filter to obtain a BCECF excitation ratio 440/490 of about 1.0 at pH 7.0. Fluorescence images were captured every 20 s by an intensified CCD camera (PTI, Surbiton, Surrey, UK). Background correction and image processing were performed with a Copy Number Analysis with Regression and Tree System (Dr O. Ahrens, Bargteheide, Germany). At the end of each experiment, the ratio 440/490 was calibrated by a standard curve that was obtained by the *in vitro* calibration method (Boyarsky *et al.*, 1996) adapted to water-immersion optics.

After transfer to the optical recording chamber, astroglial cell cultures were superfused with CO_2/HCO_3-buffered solution for at least 20 min to remove free dye. Excitation light was reduced to a minimum to enable optical recordings of up to 3 h. The optical plane was controlled throughout the experiments. Care was taken to apply exactly the same dose of NH_4Cl to make pHi regulation curves comparable. The amplitude of the acidotic peak (ΔpHi) was determined from the difference between baseline pHi (averaged from three values immediately before wash-in of ammonium) and acidotic peak pHi averaged from three consecutive values including the maximum value. The pHi recovery rate (ΔpHi/min) was estimated from the slope of the pHi increase subsequent to intracellular acidification. Data points from a 10 min interval starting at the acid peak were linearly extrapolated on the basis of a least-square regression fit. This slope offers a relative measure for the net acid extrusion (Schwiening and Boron, 1994).

Data analysis of pHi

All data were expressed as mean ± SD. In experiments where ammonium prepulses were used to study pHi regulation, a pHi recovery rate was defined, calculated on the basis of least-squares regression fit to data points after converting ratio recovery 440/490 values to pHi values. The data points for the recovery rate were collected 10 min after the acid peak.

The *t*-test for paired samples was used to compare differences in pHi recovery rates. Differences were considered significant when P < 0.05 (SPSS 11.0, SPSS Inc., Chicago, IL, USA).

Materials

LEV was a kind gift from UCB Pharma.

Results

Morphological characteristics

Fluorescent immunocytochemistry allowed the classification of co-cultured microglia as resting ramified (RRT; Figure 1A), intermediate (INT; Figure 1B) and activated, round phagocytic (RPT; Figure 1C) phenotypes. The microglial phenotypes could easily be detected and characterized with the immunolabelling approach. The RRT microglia possessed relatively small cell bodies (5–10 μm) with only a small perinuclear and submembrane cytoplasmic rim, and thin branching processes, which were longer than the diameter of the cell body (Figure 1A). The activated RPT form was characterized by rare short processes, a large cellular diameter and several cytoplasmic vacuoles (Figure 1C), while the INT microglia possessed only some thick pseudopodia longer than the diameter of the cell body, and a perinuclear cytoplasmic rim containing only a few vesicles and vacuoles (Figure 1B) (Booth and Thomas, 1991; Slepko and Levi, 1996). Specifically, the degree of microglial activation within the M5 co-culture condition in Figure 1A is shifted towards the RRT and INT types. This is in contrast to co-cultures derived from the M30 condition (Figure 1C), dominated by the RPT type (Faustmann *et al.*, 2003).

Voltage-gated membrane current responses of co-cultured astrocytes

To investigate the effect of LEV on astrocytes, we used co-cultures with either 5% or 30% (M5, M30) microglial fractions (according to Faustmann *et al.*, 2003; Hinkerohe *et al.*, 2005; Haghikia *et al.*, 2008). The M5 condition was considered as an inflammation-free environment of the astrocytes and thus served as control. Stepwise depolarization of the holding potential from –160 to 60 mV (increment = 10 mV; duration = 50 ms) evoked characteristic voltage-gated membrane current responses in both M5 and M30 cultured astrocytes (Figure 2A,B). The difference in the evoked membrane current responses of the cultured astrocytes was a major increase in inwardly rectifying currents in M30 cultured astrocytes at hyperpolarized holding potentials between –70 and –160 mV (Figure 2B). This rectification differed significantly from the M5 condition between –120 and –100 mV ($P < 0.05$), and between –100 and –70 mV ($P < 0.005$). At holding potentials positive to –40 mV, a tendency for an increase in voltage-gated inward currents was observed, which did not reach statistical significance. Under the M5 conditions, astrocytes were characterized by a median of MRP of about –60 mV (Figure 2C), corresponding to previous findings (Hinkerohe *et al.*, 2005). A significant depolarization of the MRP was measured after co-culturing astrocytes with 30% microglia (M30; Figure 2C).

LEV treatment exerted a marked effect on M30 astrocytic membrane currents when evoked by stepwise depolarization of the holding potential (Figure 3A). This phenomenon is displayed by a general increase in hyperpolarization-evoked currents (arrows in Figure 3A). This finding was further corroborated by the current-voltage IV-relation plot in Figure 3C. Here, a marked increase in inward current rectification was observed for LEV-treated M30 co-cultured astrocytes due to hyperpolarization between –100 and –160 mV. Following holding potential depolarization positive to –40 mV, the membrane current response of the M30 co-cultured astrocytes was characterized by prominent outward rectification. Significant increases in the membrane current responses of LEV-treated cells under inflammatory conditions could be observed between –20 and 20 mV ($P < 0.005$ for –20 mV to 0 mV; $P < 0.05$ for 10 mV and 20 mV), indicating a probable potentiation of outward currents. In contrast, LEV-pretreated M5 astrocytes presented only slight changes in their voltage–current relations (Figure 3B,D).

As already indicated in the IV relations, LEV treatment of M30 co-cultured astrocytes shifted the astrocytic MRPs towards physiological levels (Figure 3E), when compared with the untreated M30 group. The MRPs of LEV-treated M30 co-cultured astrocytes were comparable with resting potentials in M5 control cultures (Figure 3F). Note that the LEV treatment of M5 co-cultures did not affect their MRP.

LEV restores IL-1β-mediated MRP depolarization to physiological levels

The inflammatory condition provoked by incubating M5 astrocytes with IL-1β (500 $U \cdot mL^{-1}$, for 2 h; Dinarello, 1990; Hinkerohe *et al.*, 2005) revealed significant membrane current alterations compared with the control conditions (M5, Figure 4A) in the range between –110 and –70 mV, and between –50 and –40 mV ($P < 0.05$ for –110, –100 and –40 mV; $P < 0.005$ for –80 mV, –70 and –50 mV; $P < 0.0005$ for –90 mV). In the presence of IL-1β, M5 astrocytes increased their evoked inward rectifying membrane currents at hyperpolarized holding potentials (–80 to –160 mV). The depolarization-driven, inward current responses elicited at holding potentials positive to –10 mV showed a trend towards increase (Figure 4A). At holding potentials of 50 and 60 mV,

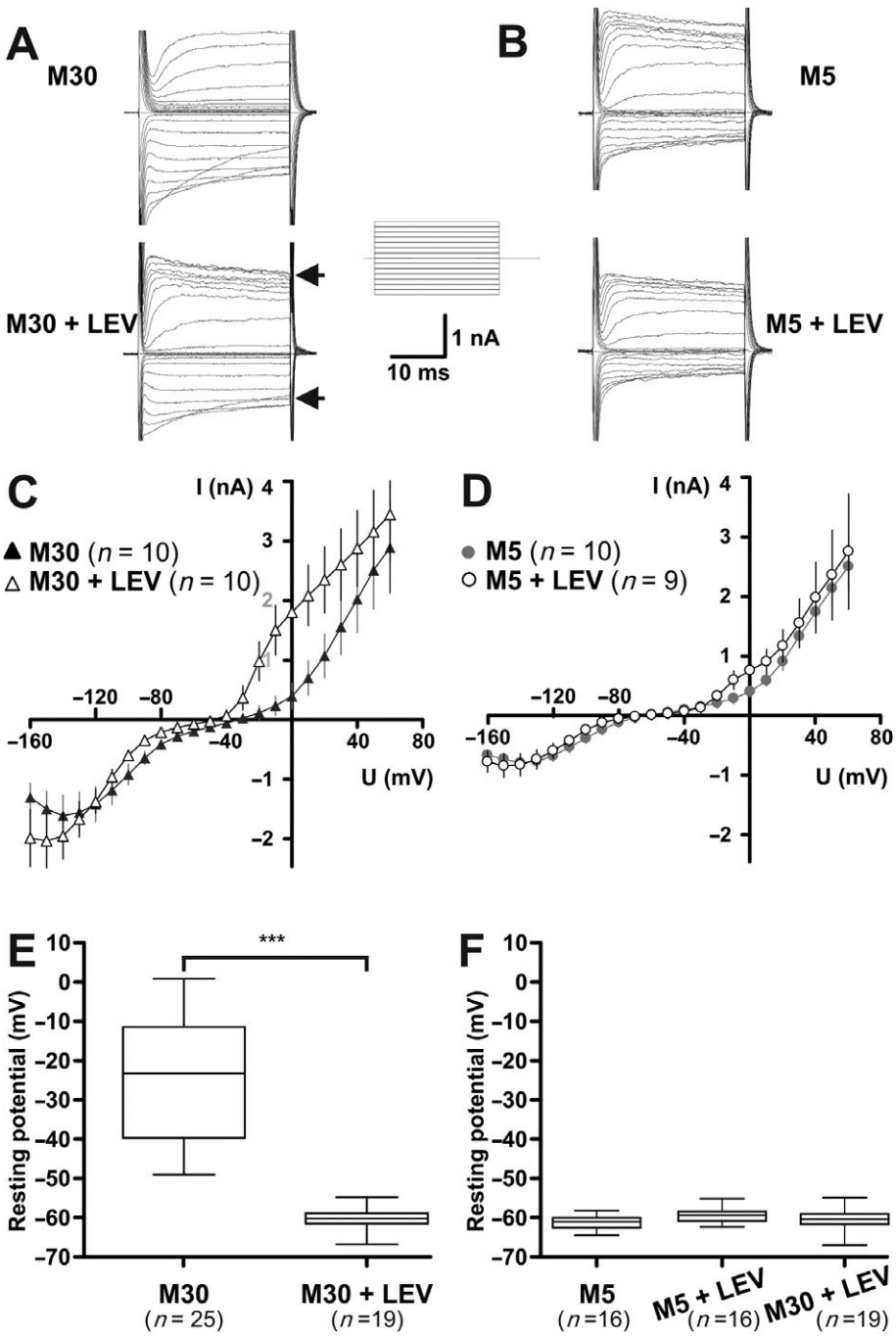

Figure 3

Effects of levetiracetam (LEV) on electrophysiological properties of M5 and M30 co-cultures. Characteristic examples of single cell recordings from M30 and M5 co-cultured astrocytes are shown in A and B respectively. Equivalent recordings from astrocytes following 24 h pre-incubation with 50 $\mu g \cdot mL^{-1}$ LEV (M30 + LEV, M5 + LEV) are displayed in the lower traces. Arrows in (A) indicate the increase in hyperpolarization-evoked membrane currents and a regression of voltage-gated membrane currents at highly depolarizing holding potentials in the M30 + LEV-treated group. This is in contrast to the M5 co-cultured astrocytes in (B) which show only minor changes in evoked membrane currents after incubation with LEV. Comparison of the state specific IV relations in (C) clearly demonstrates that LEV pre-incubation exerts a significant effect on the in- and outwardly rectifying component of the membrane current in M30 co-cultured astrocytes. This is particularly displayed in the significant shift of the cell membrane resting potentials (MRPs) towards hyperpolarization (E). Only minor changes were observed in the IV relations of M5 co-cultured astrocytes as depicted in (D). (F) The MRPs of M5 + LEV ($n = 16$) and M30 + LEV cultures ($n = 19$) did not differ ($P > 0.05$). ***$P < 0.0005$. M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia.

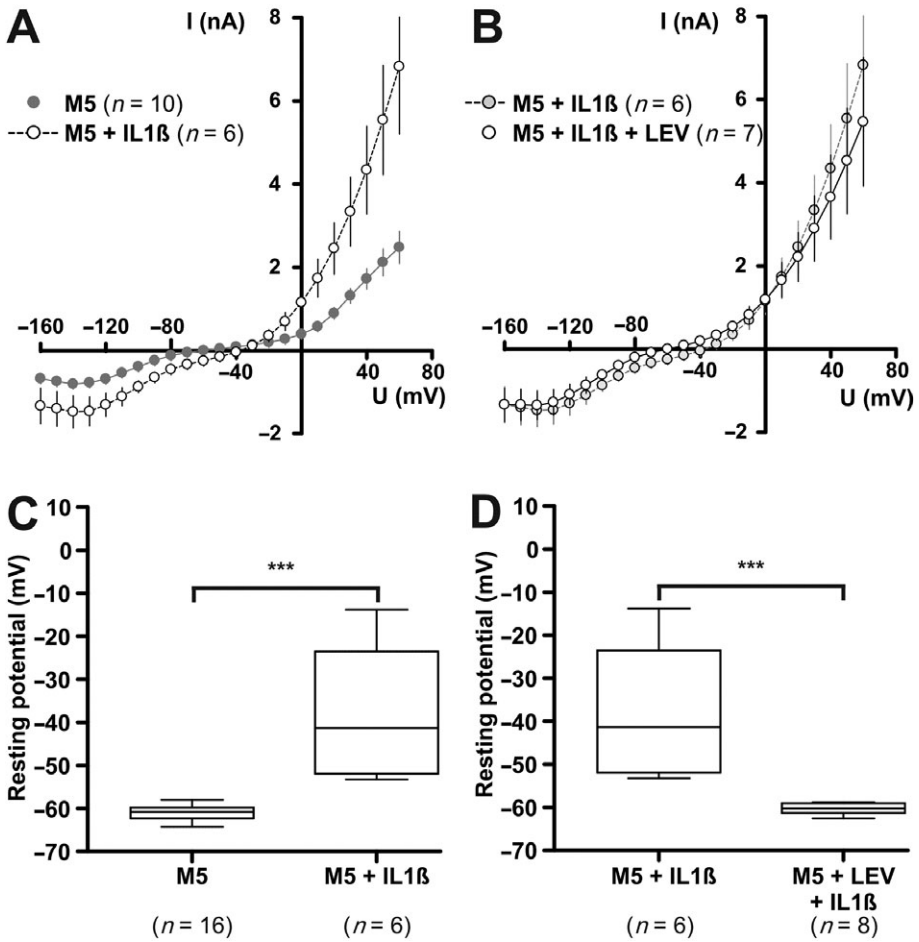

Figure 4

The pro-inflammatory cytokine IL-1β (500 U·mL^{-1} for 2 h) induces an acute inflammatory state by activation of the microglial fraction in the M5 co-cultures. As observed in the M30 co-cultures, a significant increase in membrane currents could be detected in the M5 co-cultures under both hyper- and depolarization of the cells (A). This effect was accompanied by a subsequent significant depolarization of the membrane resting potentials (MRPs) (C). MRPs of levetiracetam (LEV) pretreated, and IL-1β-stimulated M5 astrocytes exhibited a significant shift back towards values of the untreated (physiological) M5 co-cultures (D), which is also reflected by the IV relation in (B). ***$P < 0.0005$. M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia.

membrane currents were significantly increased in the inflammatory condition ($P < 0.05$ for 50 and 60 mV; Figure 4A). In addition, M5 astrocytes stimulated with IL-1β showed a significant shift of their MRPs (Figure 4C) to more depolarized levels. For testing the restoring effect of LEV on these altered currents, we compared the former group (M5 + IL-1β) with a LEV-pretreated group of IL-1β-stimulated M5 astrocytes (Figure 4B). Whereas minor effects were observed with regard to the astrocytic membrane current amplitudes (Figure 4B), the MRPs were markedly shifted towards more negative values after LEV pretreatment (Figure 4D). These data show that one of the major effects of LEV is to prevent the cell from MRP depolarization induced by IL-1β.

LEV and TGFβ1 induce comparable effects in astrocytes

As illustrated in Figure 5A, the courses of the IV relations of TGFβ1 and LEV-treated M30 co-cultured astrocytes are mostly congruent. With respect to the shift of resting potentials towards more negative potentials, LEV and TGFβ1 treatment of M30 co-cultures induced comparable changes (Figure 5C). The IV relations in M5 astrocytes were also unaffected by treatment with LEV or TGFβ1 (Figure 5B). A comparison of LEV or TGFβ1-treated M5 astrocytes with the M5 control group revealed no differences in MRPs. These findings were similar to those of LEV and TGFβ1 on the differently conditioned co-cultures.

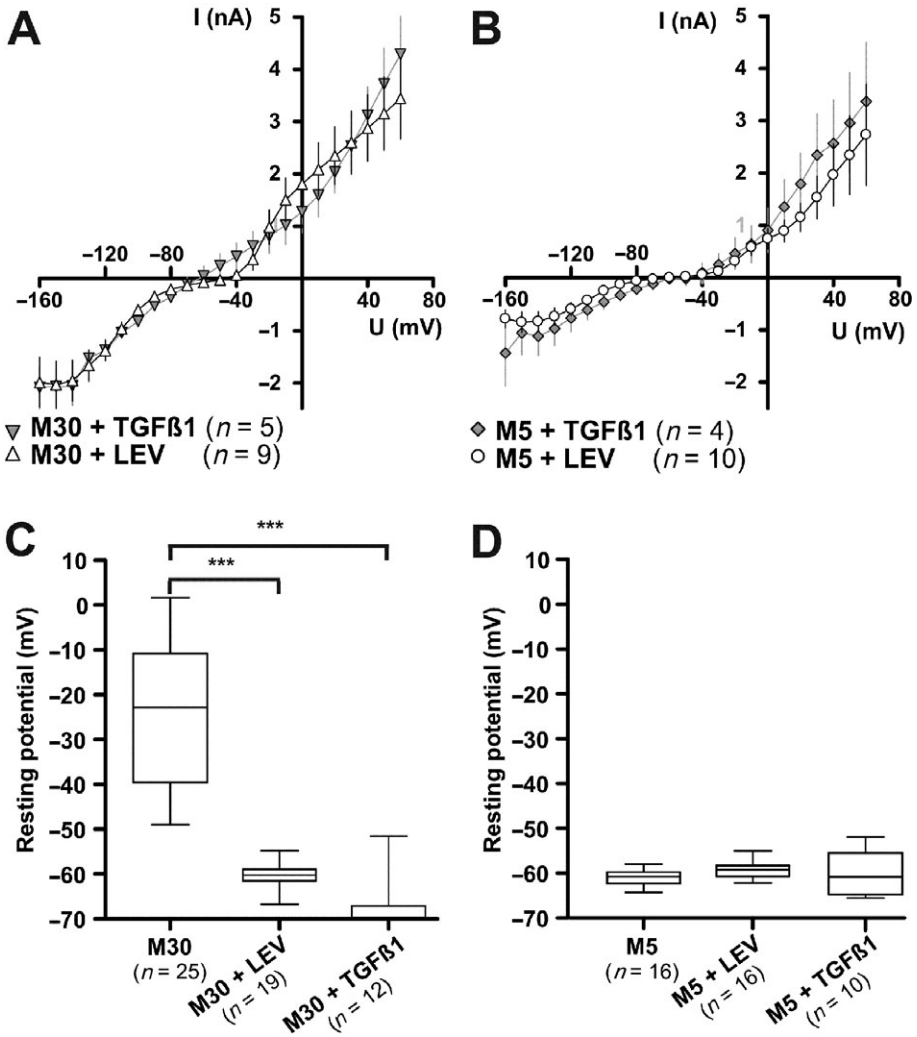

Figure 5

The effects of levetiracetam (LEV) treatment and the anti-inflammatory cytokine TGFβ1 on the IV-relations are similar. Voltage–current-relations of TGFβ1 and LEV treated groups were not affected by the co-culturing conditions: M30 (A) or M5 (B). This is best shown in the restoring shift of the membrane resting potentials (MRPs) in (C), where a comparison of the M30 + LEV and M30 + TGFβ1-treated group with the M30 only co-cultures is shown. Both M30 groups reveal significant shifts towards more negative resting potentials, comparable with those of the M5 control condition (see also D). (D) The M5 samples did not differ in their MRPs, indicating that LEV, as well as TGFβ1, was without effect on astroglia during resting (inflammation-free) conditions. ***$P < 0.0005$. M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia.

LEV incubation alters the TGFβ1 concentration in M30 and M5 astroglial co-cultures

To address the question of whether treatment with LEV affected concentrations of TGFβ1 in the co-cultures, we used two concentrations of LEV (50 or 100 μg·mL^{-1}). As shown in Figure 6, TGFβ1 concentrations were increased concentration-dependently by LEV in M5 (Figure 6A), as well as in M30 (Figure 6B) supernatants. Untreated M5 samples showed similar TGF-β1 concentrations as in M30 co-cultures.

To strengthen the correlation between the effects of LEV on membrane currents and MRPs, and the TGFβ1 concentration in M30 co-cultures, we used an antibody of TGFβ1, which was either applied 1 h in advance of LEV incubation, or in addition to the 24 h lasting LEV incubation. If LEV was effective through increasing TGFβ1 secretion, neutralizing TGFβ1 with the appropriate antibody should prevent the effects of LEV on the IV relations and on the restoration of MRP (Tsang *et al.*, 1995).

Whole-cell patch clamp electrophysiology revealed equivalent effects on the IV-relations of M30 only and LEV-treated M30 co-cultured astrocytes after neutralization of TGFβ1 (Figure 7A). The IV relations of both antibody-treated co-cultures were mostly overlapping. In comparison with

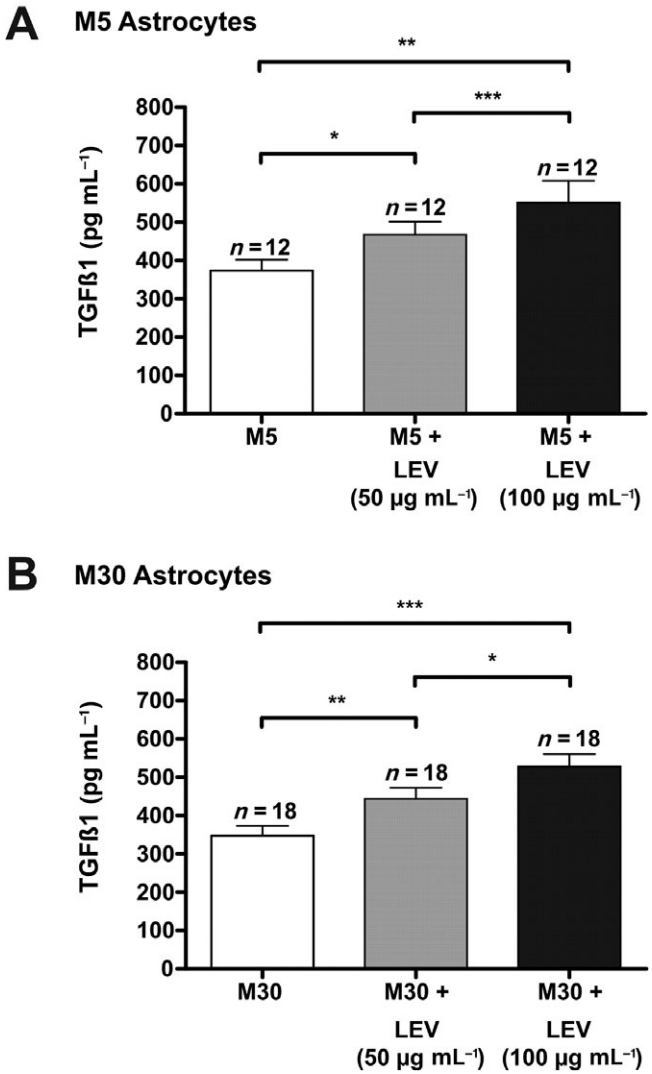

Figure 6

ELISA analyses of TGFβ1 in M5 (A) and in M30 (B) co-cultures following incubation with different concentrations of levetiracetam (LEV). LEV application causes a concentration-dependent increase in TGFβ1 in the co-culture system. Although electrophysiological recordings did not reveal functional changes in LEV or TGFβ1-treated M5 groups (see Figures 3D and 5D), it is evident that the levels of TGFβ1 were increased concentration dependently by LEV (A). A similar dose-dependent increase in TGFβ1 concentrations can be obtained from the M30 co-cultures (B). In contrast to the M5 condition, M30 astrocytes physiologically respond to elevated TGFβ1 levels (see Figure 5C). A: *$P < 0.05$; **$P < 0.005$; ***$P = 0.0969$. B: *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. M5, astrocytes co-cultured with an amount of 5% activated microglia; M30, astrocytes co-cultured with an amount of 30% activated microglia.

untreated cultures, a marked decrease in membrane currents at holding potentials negative to –50 mV occurs. At holding potentials positive to –50 mV, current courses of the antibody treated M30 and M30 + LEV astrocytes were mostly aligned (Figure 7A). After TGFβ1 neutralization, LEV-treated M30 astrocytes and M30 non-treated co-cultured astrocytes revealed similarly depolarized MRPs ($P > 0.05$). In contrast, strongly significant differences were found for all three ($P < 0.0005$), when compared with LEV-pretreated M30 co-cultured astrocytes.

The correlation between LEV and the presence of TGFβ1 was also reflected in the microglial phenotypes found in the co-cultures. Quantification of the fractions of the activated, round shaped, microglial phenotype (RPT; for morphology, see Figure 1C) revealed the highest portion of RPT microglia in M30 co-cultures, followed by the antibody-treated M30 only. Astrocytes from the LEV treated group contained the lowest proportion of RPT microglia. Assay of the RRT (data not shown) in the same LEV group underlined the shift in microglial activation towards this ramified type. The INT microglial fraction remained mostly unchanged under all conditions studied here.

Effects of LEV on pHi in the astroglial co-culture model

LEV (25–50 µM) was unable to influence steady-state pHi of BCECF-AM-loaded astroglial cell cultures in the M5 co-cultures (n = 21, from 11 astroglial cell cultures). The mean steady-state pHi amounted to 7.04 ± 0.16. Acute exposure to LEV (25–50 µM) for 20–30 min had no effect on steady-state pHi (n = 14); also a prolonged wash-in (>50 min) and/or increasing the LEV concentration to 100 µM did not change the pHi (n = 7) (data not shown).

Potential effects of LEV (25–50 µM) on buffer capacity and pHi regulation were also tested by applying ammonium prepulses in the absence and presence of LEV (n = 14). Neither the size of the acid peak [ΔpHi: 0.19 ± 0.04 (control) vs. 0.20 ± 0.03 (LEV)], nor pHi regulation [ΔpHi/min: 0.009 ± 0.002 (control) vs. 0.0086 ± 0.003 (LEV)] was influenced by LEV.

LEV (25–50 µM) also failed to alter the steady-state pHi within 20–30 min of application in IL-1β stimulated astroglial cell cultures (n = 7). Again, a prolonged wash-in (>50 min) and/or raising the concentration to 100 µM did not alter pHi (n = 7), excluding the possibility of a pH-dependence of the observed findings.

Discussion and conclusions

An anti-inflammatory effect of the anti-convulsive drug LEV in non-neuronal tissue was first described by Kim *et al.* (2007) in a macrophage culture model after lipopolysaccharide-induced inflammation. Haghikia *et al.* (2008) showed a LEV-mediated

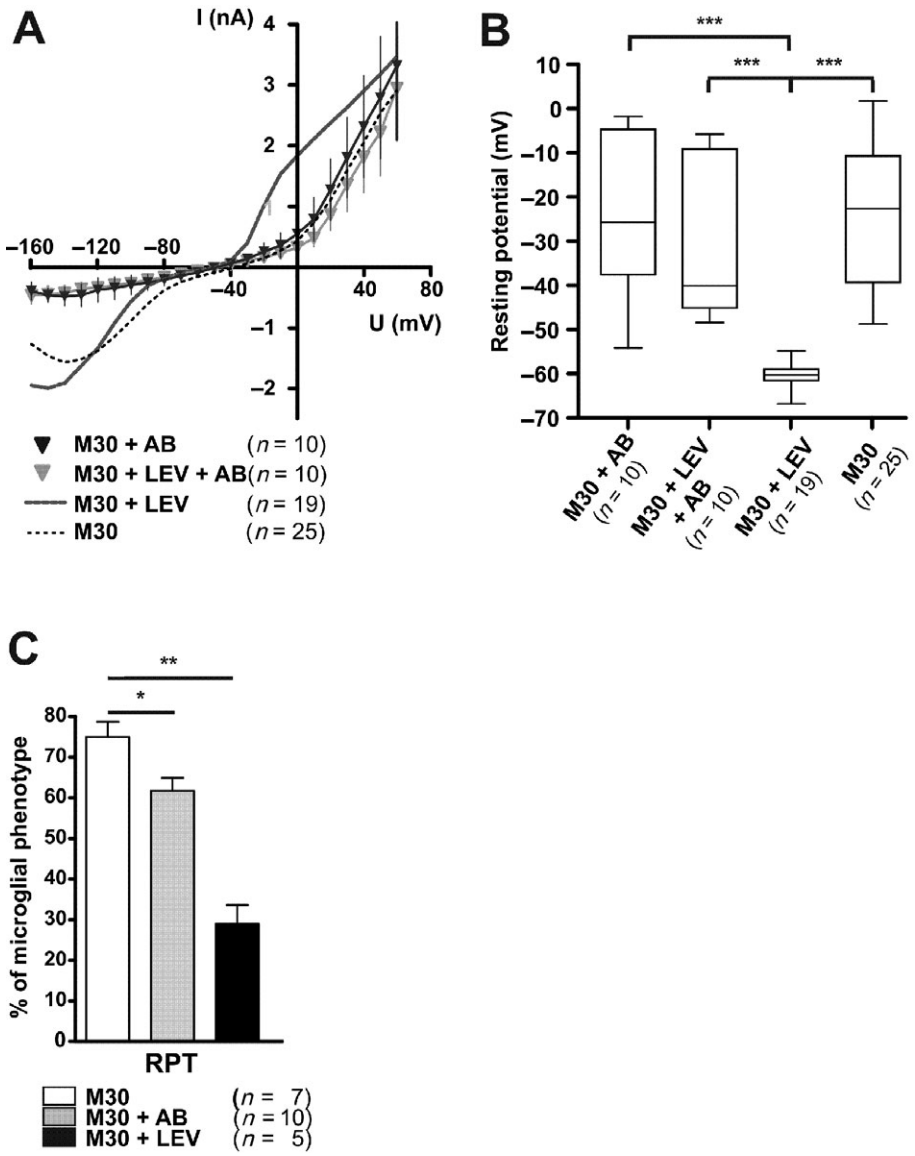

Figure 7

Antibody inhibition of TGFβ1 in M30 astrocytes and levetiracetam (LEV) pretreated M30 co-cultures. (A) Depicts the effect of antibody (AB) treatment on IV relations. Independent of the treatment (AB only; LEV + AB), M30 co-cultures exhibit similar IV correlations characterized by a lack of hyperpolarization-driven membrane current at holding potentials ≤50 mV. The black dashed line indicates the course of currents under LEV-only treatment; the grey dotted line represents the currents of the completely untreated M30 condition for general comparison. (B) Antibody inhibition of TGFβ1 is reflected in the marked shift of resting potential of AB treated groups towards positive values, compared with the LEV-only treated M30 group. (C) Quantification of the fraction of activated round phagocytic type (RPT) revealed the highest amount of RPT microglia in M30 co-cultures, whereas the level of microglial activation within the LEV group is significantly reduced. B: ***$P < 0.0005$; C: **$P < 0.005$; *$P < 0.05$. M30, astrocytes co-cultured with an amount of 30% activated microglia.

reconstitution of impaired astroglial gap junction coupling, along with repolarization of MRP under inflammatory conditions in the microglial/astrocytes co-culture model. In this study, we followed this line of an anti-inflammatory effect of LEV, which can be considered a functional effect of its anti-convulsant actions.

In a basic astroglial/microglial co-culture model, we first provoked a pro-inflammatory status with a fraction of 30% microglia as introduced previously (Hinkerohe *et al.*, 2005; Haghikia *et al.*, 2008), enabling us to demonstrate robust effects of LEV on the evoked membrane current response. At this point it has to be mentioned that currents through gap junctions also contribute to the passive membrane conductance of astrocytes in syncytitial distributions (Blomstrand *et al.*, 2004; Haghikia *et al.*, 2008; Seifert *et al.*, 2009). These currents cannot be

fully excluded from affecting the quality of voltage clamp control. Detailed information about the individual astrocyte's membrane current generation mechanisms can most probably be attained in isolated cell recordings (Steinhauser *et al.*, 1990; 1994; Seifert *et al.*, 2009).

Besides an increase in voltage-gated currents, a significant shift of the depolarized astrocytic MRPs towards values of the M5 control condition could be observed, which confirmed our previous observations (Hinkerohe *et al.*, 2005; Haghikia *et al.*, 2008). The voltage–current relations of LEV-treated M30 astrocytes suggest a significant effect on the outward rectifier activity, voltage-gated inward current generation and subsequent rectification.

Rectifier activity, basically consisting of K^+ current, is necessary to keep the cellular resting potential in the physiological range during strong hyper- and de-polarization (Sontheimer, 1994). As such, this current may compensate strong depolarization, which occurs under inflammatory conditions due to increased Ca^{2+} and via Na^+-influx during cellular activation (Bevan *et al.*, 1987; Steinhauser *et al.*, 1994; Kressin *et al.*, 1995). Under inflammatory conditions, activated microglia are capable of producing pro-inflammatory cytokines (Kloss *et al.*, 1997; Ledeboer *et al.*, 2000), including IL-1β (Pearson *et al.*, 1999), which leads to changes in astrocytic MRP and gap junction coupling (Hinkerohe *et al.*, 2005). In our co-culture model, an increased level of activated microglia in the M30 co-cultures is most likely responsible for the presence of sixfold elevated levels of IL-1β (Haghikia *et al.*, 2008). IL-1β elicits immune responses by activation of microglia and lymphocytes (Dinarello, 1990). In a previous study, we demonstrated that incubation with IL-1β induced an acute inflammatory state in M5 co-cultured astrocytes, which was reversed by an application of known anti-inflammatory mediators such as IFN-β and TGFβ1 (Hinkerohe *et al.*, 2005). Under these conditions, astrocytes change their structural and functional phenotypes (Schilling *et al.*, 2001), which can be regarded as a switch from a resident state towards a reactive and ultimately gliotic state as seen in many primary and secondary inflammatory disorders of the CNS (Kim *et al.*, 2010). Our present findings have provided substantial evidence for an anti-inflammatory effect of LEV on the astrocytic MRPs.

In neurons, LEV modulates the MRP, via the protein kinase A-mediated phosphorylation and activation of ROMK1 potassium channels (K_{IR} 1.1a; channel nomenclature follows Alexander *et al.*, 2009) (Lee *et al.*, 2008). The resting potential, as well as some of the K^+ buffering mechanisms of astrocytes, is established through the activity of inwardly rectifying K^+ channels (K_{IR}), which account for most of the transmembrane current in the hyperpolarizing site of the current–voltage relationship (Sontheimer, 1994). These channels are characterized by a large open probability close to and below the astrocytic resting potential (Kuffler *et al.*, 1966; Dennis and Gerschenfeld, 1969; Ransom and Goldring, 1973), while open probability is near zero at more depolarized potentials. Defects of the glial inward rectifier may be one possible factor in the development of seizure activity (Bordey and Sontheimer 1998). We found that currents elicited in the K_{IR} operation range are increased under inflammatory conditions (M30 and M5; IL1-β by tendency). This observation indicates a mechanism for the cells to restore the membrane resting potential more efficiently. There is evidence that LEV-induced restoration of resting potentials is not only restricted to an increase in membrane currents of the single cell, but also depends on the integrity of the gap junctional coupling properties in the astro-glial syncytium (Haghikia *et al.*, 2008). These, in turn, influence K^+ homeostasis and subsequent resting potential regulation (Volterra and Meldolesi, 2005). The data suggest that the restoration of the MRP by LEV, elicited in M5 astrocytes after IL1-β-only incubation, was dominated by effects of the AED on the gap junctional coupling properties in the syncythium. During epilepsy, impaired astrocytic K^+ buffering might be a possible cause of slower K^+ clearance from the extracellular space, which in turn would lower seizure threshold and thereby contribute to seizure generation in the neuro-glial complex. LEV would appear to enhance uptake and distribution of K^+ within the glial network by improving K^+ channel function and restorating gap junction coupling (Haghikia *et al.*, 2008).

The effects of TGFβ1 on MRP in M5 and M30-treated astrocytes have already been shown by Hinkerohe *et al.*, (2005) and they were comparable with those of LEV. This similarity allowed us to propose a causal link between LEV treatment and subsequently augmented secretion of this anti-inflammatory cytokine. To further explore similarities between LEV and TGFβ1 effects, we studied the direct influence of a 24 h incubation with 10 $ng \cdot mL^{-1}$ TGFβ1 on the IV relations of M30 co-cultured astrocytes and compared these results with those from the LEV-treated M30 group. The same experimental conditions were tested on M5 co-cultured astrocytes. The results of LEV incubations of M30 and M5 astrocytes strongly resemble the outcome of TGFβ1 treatments as both treatments provide comparable effects on astrocytic membrane currents and resting potentials. These data imply a facilitating effect of TGFβ1 on the hyperpolarization-driven current response of M30 astrocytes under pro-inflammatory conditions,

which can be blocked by antibody neutralization. This possibility was substantiated by the ELISA analysis, which indicated a LEV-dependent increase in TGFβ1 concentrations in the co-cultures. The LEV-dependent increase in TGFβ1 secretion in M30 astrocytes clearly underscores the close correlation between LEV treatment and TGFβ1 action. This conclusion is augmented by the MRP distributions of the differently treated groups (Figure 7B). We assume that LEV augments TGFβ1 secretion and that its autocrine/paracrine effect on astrocytic resting potentials is responsible for the shift towards physiological levels, which ultimately may contribute to reduced excitability. A recent work by Cacheaux *et al.*, (2009) showed involvement of TGFβ-associated epileptogenesis *in vitro*, involving the measurement of epileptiform neuronal activity in a brain slice model which mimics trauma-induced acute epilepsy. However, these results would appear to contradict the anti-inflammatory effect observed in our model following LEV-induced TGFβ1 enhancement. The induction of conflicting pathways in response to TGFβ have already been described in the context of autoimmune disorders, in which the combined effect of TGFβ with IL-6 resulted in an inflammatory polarization of T helper cell subsets (TH17), in clear contrast to the anti-inflammatory effect exerted by TGFβ alone (Korn *et al.*, 2007). Furthermore, the TGFβ concentration used by this work is roughly 30-fold higher than the concentrations we measured after LEV treatment.

Our study was performed using co-cultures model to gain information about LEV effects and interactions in the astro-glial syncytium. This artificial neuron-free environment might be one reason for the slightly depolarized MRPs of M5 co-cultured astrocytes, compared with the K^+ equilibrium potential of the solutions or with the MRP of cortical astrocytes in adult rats *in vivo* (Mishima and Hirase, 2010). In addition, it has to be taken into account that cellular electrophysiological properties in this system are strongly influenced by the developmental state of the astrocytes after primary culture preparation, which particularly affects the expression of K^+ channels (Kressin *et al.*, 1995; Kafitz *et al.*, 2008). This indicates that astrocytes used in this system should not be regarded as mature.

A further outcome of this study is the stability of astrocytic intracellular pH which was not found to be modulated under control and inflammatory conditions. The rationale for performing pHi measurements was to exclude pH effects as a possible explanation for the described phenomena, as astrocytes exhibit a number of pH regulatory mechanisms including Na^+ -dependent $Cl^-/HCO3^-$ exchange (Shrode and Putnam, 1994). Likewise, pHi modulation by LEV, through inhibition of the Na^+-dependent $Cl^-/HCO3^-$ exchange, has been described in adult hippocampal CA3 neurons of the guinea pig (Leniger *et al.*, 2004). For nearly all events related to pH changes in nervous tissue, the presence of CO_2 and HCO_3^- is critical (Chesler, 1990). For neural regulation of the pH, HCO_3^- determines the buffering capacity in brain tissues to a large extent. In neurons, HCO_3^- flows through $GABA_A$ receptor channels and thereby modifies the inhibitory synaptic potentials. Furthermore, the presence of HCO_3^- stimulates the powerful glial electrogenic Na^+–HCO_3^- co-transporter and other carriers such as Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchanger (Deitmer and Rose, 1996). A possible explanation for the pH stability we have found might be the steady-state counterbalancing of astrocytes against pH changes via strong, endogenous buffering capacities, as has been described in detail by Boron (2001; 2004).

As a general consequence of our findings, the anti-convulsant drug LEV might be regarded as a prototype for future anti-epileptic therapeutic approaches, where not only modification of neuronal excitability is taken into account, but also aspects of an inflammatory, glia-induced, component of epileptogenesis.

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Conflicts of interest

No conflicts of interest are stated.

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