

## Neuroprotective effects of the anti-inflammatory compound triflusal on ischemia-like neurodegeneration in mouse hippocampal slice cultures occur independent of microglia

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### ABSTRACT

Microglial cells, known to play key roles in neuroinflammation, can be immunotoxically eliminated from hippocampal slice cultures by treatment with saporin coupled to the microglial receptor Mac1. Considering microglial cells as a target for anti-inflammatory treatment we studied the effects of microglial depletion on anti-inflammatory treatment of mouse hippocampal slice cultures subjected to ischemia-like neurodegeneration, induced by oxygen-glucose deprivation (OGD).

Hippocampal slice cultures, derived from 7-day-old mice and grown for 2 weeks, were divided into 8 groups: (1) control cultures; (2) sham-OGD cultures; (3) OGD cultures; (4) OGD cultures treated with triflusal during OGD; (5) microglia-depleted control cultures; (6) microglia-depleted sham-OGD cultures; (7) microglia-depleted OGD cultures; and (8) microglia-depleted OGD cultures treated with triflusal during OGD. The resulting neurodegeneration was quantified by densitometric measurements of cellular uptake of propidium iodide (PI), with focus on the hippocampal CA1 subfield.

Subjection of regular cultures to OGD for 30 min induced a significant increase in PI uptake in the CA1 pyramidal cell layer, compared to control cultures. The presence of 100 μM triflusal during OGD protected against OGD-induced neurodegeneration, and reduced the number of OGD-induced NFκB positive-cells correspondingly. Cultures treated with the Mac1-saporin complex for 7 days displayed an almost total loss of microglial cells. When subjected to OGD after microglial depletion, these cultures displayed a significant increase in OGD-induced PI uptake compared to non-depleted cultures. The presence of triflusal during OGD of these cultures reduced neurodegeneration of the irrespective absence of microglia. In accordance with that, the presence of triflusal during OGD significantly inhibited the increase in the number of reactive microglia and proliferative cells in the CA1 pyramidal and dentate granule cell layers.

We conclude that immunotoxic microglia depletion significantly increases the susceptibility of CA1 pyramidal cells to neurodegeneration and that the anti-inflammatory drug triflusal still can exert its neuroprotective role following depletion of microglia.

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### Introduction

Stroke (cerebral ischemia) is a leading cause of disability and death worldwide. Although several mechanisms are involved in ischemia-induced neurodegeneration, inflammatory reactions play a major role for injury progression and outcome (Barone and Feuerstein, 1999, Samson et al., 2005, Chamorro and Hallenbeck, 2006) as exemplified by reduced damage and improvement of outcome after anti-inflammatory treatment in experimental stroke (Han and Yenari, 2003). Microglial cells are key neuroinflammatory mediators in the central nervous system (CNS). After microglia activation by cerebral ischemia (Streit et al., 1999), they undergo a number of phenotypic changes including—a) structural changes with transformation of their

slender processes into shorter and coarser processes (Jensen et al., 1997, Streit et al., 1999)—b) accumulation at the site of injury, resulting from both migration and local proliferation (Chechneva et al., 2006, Imai et al., 2007), and —c) increased or *de novo* expression (and release) of neurotrophic factors like brain-derived neurotrophic factor (BDNF) (Imai et al., 2007) and transcription factors like nuclear factor-kappa B (NFκB), which regulates inflammatory processes (Baeuerle and Henkel, 1994). Although microglial activation is well described, conditions determining whether microglial activation is protective or damaging for neurons are still unclear (Streit et al., 1999). The role that microglial cells play in the actions of anti-inflammatory compounds, like triflusal (2-acetoxy-4-trifluoromethylbenzoic acid) is therefore of interest. Triflusal is a fluorinated derivative of acetylsalicylic acid and is widely used for the prevention and/or treatment of vascular thromboembolism (McNeely and Goa, 1998). Triflusal has moreover been shown to downregulate the expression of NFκB and inducible

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nitric oxide synthase (iNOS), when applied in relation to excitotoxic lesions of postnatal rat brain (Acarin et al., 2000; 2002).

In this study we examined the potential neuroprotective effect of triflusal on organotypic, mice hippocampal slice cultures subjected to ischemia-like oxygen and glucose deprivation (OGD), and to what extent this effect depended on the presence of microglia and affected the expression of the transcription factor NFκB.

## Materials and methods

### Mouse hippocampal slice cultures

For the preparation of hippocampal slice cultures 7-day-old C57BL/6J mouse pups were used. Following instant cut of the brain stem, the brain was removed and the two hippocampi isolated and cut in transverse coronal sections at 350 μm by a McIlwain tissue chopper. After removal of excess tissue the slices were placed as 6 slices with equal distance on a semiporous (0.4 μm) transparent membrane of inserts (30 mm in diameter) (Millipore Corp., Bedford, MA, USA, Cat. No. PICM 03050), whereafter the inserts were placed in 6 well culture trays (Corning Costar, Corning, NY, USA) with 1 ml of medium in each well. The medium was composed of 50% Opti-MEM (Cat. No. 31985-047), 25% horse serum (Cat. No. 26050-047), 25% Hank's BBS (HBSS; Cat. No. 24020-091) (all from Gibco BRL), supplemented by D-glucose to a final concentration of 25 mM. The 6 well trays were placed in an incubator with 5% CO<sub>2</sub> and 95% atmospheric air at 33 °C. Medium was replaced first time after 3–4 days and then twice a week for the next 2 weeks. The day before the start of experiments the medium was changed to a chemically defined, serum-free Neurobasal medium (Gibco BRL, Cat. No. 21103-049) with 25 mM D-glucose, 1 mM L-glutamine (Sigma, Cat. No. 25030-024), 2% B27 supplement (Gibco BRL, Cat. No. 17504-010) and the temperature increased to 36 °C. No antibiotics or antimetabolic drugs were used at any stage.

### Oxygen-glucose deprivation (OGD)

For oxygen-glucose deprivation (OGD) a modification of the submersion protocol of Frantseva et al. (1999) was used (Bonde et al., 2002a). The medium used for OGD was an artificial cerebrospinal fluid (ACSF) containing the following (in mM): 120 NaCl, 5 KCl, 1.25

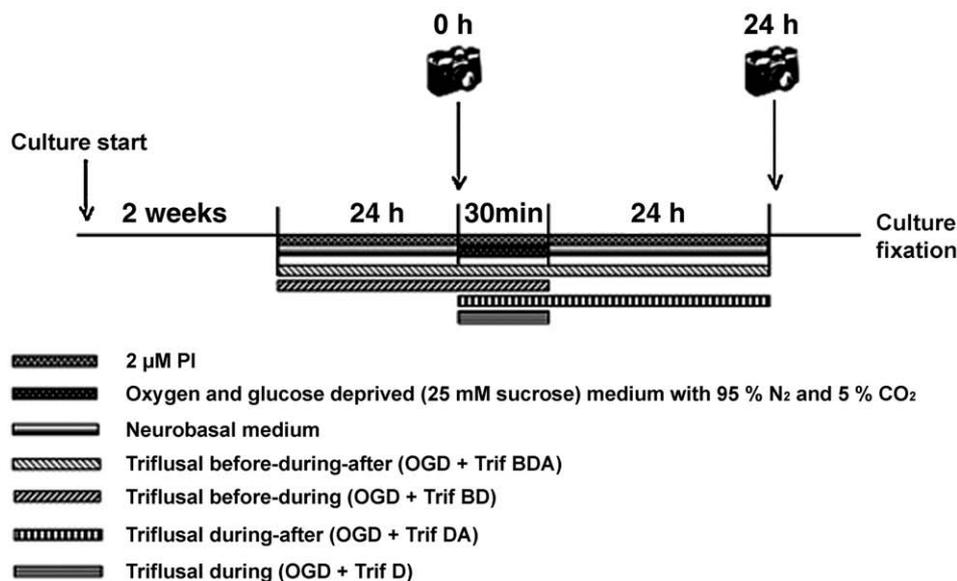
NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 2 MgSO<sub>4</sub> · 7H<sub>2</sub>O, 2 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 20 HEPES and 25 mM sucrose (or glucose in case of sham OGD). To remove oxygen, the medium was bubbled with a gas mixture of 5% CO<sub>2</sub> and 95% N<sub>2</sub>, resulting in an O<sub>2</sub> gas pressure measured to be close to zero. After a single wash of the culture well with the OGD medium, 1 ml of this medium was placed below and 1 ml on top of the semiporous membranes, covering the cultured slices. The culture trays were then immediately wrapped in airtight plastic foil and placed in the incubator at 36 °C for 30 min, which from previous experiments is known to induce cell death in 50% of the CA1 pyramidal cells. Sham-OGD cultures were handled and submersed in the same way, using ACSF with 25 mM glucose instead of sucrose and no removal of oxygen. One series of (control) cultures was not manipulated at any time. After OGD, the OGD experimental and OGD sham control cultures were washed once with regular normal oxygenated Neurobasal medium, and transferred to culture trays confirming regular Neurobasal medium and thereafter returned to the CO<sub>2</sub> incubator at 36 °C for 24 h until PI-uptake measurements (see below) and fixation.

### Quantification of cell death by propidium iodide (PI) uptake

Spontaneous and induced cell death, and accordingly neuronal viability in the slice cultures was determined by cellular uptake of the fluorescent dye PI (Sigma). PI was added to the medium of control and experimental cultures 24 h before OGD to yield a 2 μM concentration. Recordings of PI uptake by fluorescent microphotographs were performed immediately before the implementation of OGD and 24 h after using a tetramethyl rhodamine isothiocyanate filter (510–560/590 nm) and a Sensys KAF 1400 G2 (Photometrics, Tucson, AZ) digital camera (Norberg et al., 1999). Correspondingly recordings of PI uptake were performed on the respective controls. PI uptake in the different hippocampal subfields (CA1, CA3, DG and all the hippocampal slices) was quantified densitometrically, using NIH Image software (version 1.64).

### Oxygen-glucose deprivation (OGD) and triflusal treatment protocol

Hippocampal slices, derived from one-week-old (P7) C57BL/6J mice and cultured for 2 weeks, were treated according to the experimental protocol outlined in Fig. 1. 24 h before exposure to



**Fig. 1.** Outline of the experimental protocol for testing of neuroprotective effects of triflusal (100 μM) and corresponding effects of NFκB expression in 2-week-old hippocampal slice cultures, derived from 1-week-old mice. Photographic recordings of cellular uptake of PI marking neuronal cell death, were performed before and 24 h after OGD. Triflusal was added to the Neurobasal medium according to 4 different treatments schedules (OGD + Trif B-D-A; OGD + Trif B-D; OGD + Trif D-A; OGD + Trif D). The same abbreviations for triflusal treatment are also in subsequent figures.

OGD, the cell death marker PI was added to the medium to record spontaneous (basic) neurodegeneration. The potential neuroprotective effect of triflusal against OGD, was tested by adding triflusal (for doses, see below) to the mediums according to four different schedules: –a) 24 h before OGD + during OGD + 24 h after OGD; –b) 24 h before OGD + during OGD; –c) during OGD + 24 h after OGD, and –d) during OGD only. After a 24 h post-OGD (and recording of PI-uptake) period, cultures were fixed in 4% paraformaldehyde (PFA) phosphate buffer and cryoprotected in 20% sucrose overnight and frozen. Later, cultures from the different groups were cryostat sectioned in two parallel series of 20  $\mu\text{m}$ , one series was immunostained for Mac1 to evaluate microglial cells in the hippocampal slice cultures; the other was immunostained for the transcription factor NFkB.

#### Determination of dose–response for triflusal

In order to determine the optimal concentration of triflusal for use in the OGD experiments, hippocampal slices were exposed to increasing concentrations of triflusal (0, 100, 250, 500, 750, 1000  $\mu\text{M}$ ) during OGD and for the following 24 h until fixation. Quantification of CA1 pyramidal cell death by PI uptake revealed that 100  $\mu\text{M}$  triflusal was the most effective dose in reducing PI uptake. This concentration was therefore used for further experiments.

#### Microglia depletion of slice cultures

Treatment of slice cultures with the ribosome-inactivating protein saporin coupled to the microglial receptor Mac1 can selectively eliminate both Mac1 positive and tomato lectin binding microglial cells (see Fig. 2 for experimental protocol). Saporin is an immunotoxin that interferes and disrupts the translation process taking place in the ribosomes, resulting in the death of the targeted cell, in this case Mac1 positive cells.

Seven-day-old hippocampal slice cultures were exposed to the Mac1-saporin immunotoxin complex (1.3 nM) for the next 7 days prior to the OGD experiments. This treatment depletes the slice cultures of virtually all microglial cells (Montero et al., submitted for publication). Other cultures, serving as controls, were not microglia depleted.

#### Visualization of microglia

##### Mac1 immunohistochemistry

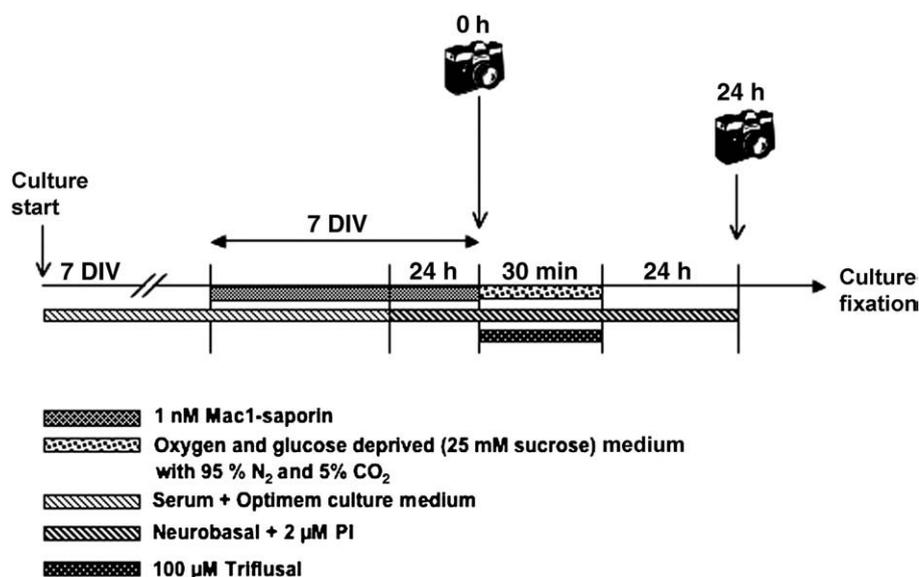
For visualization of Mac1 expressing microglia cells, cryostat sections of the slice cultures were washed in Tris buffered saline, pH 7.4 (TBS) and TBS+ 1% triton X-100 (Sigma) and blocked for unspecific staining with 5% goat serum (DAKO, X 0907) in TBS before incubated for 2 days at 4 °C with the primary rat-anti Mac1 antibody (Advanced Targeting Systems, AB-N05) diluted 1:600 in goat serum. After a wash in TBS+ 1% triton X-100 the sections were incubated with the secondary biotinylated goat anti-rat antibody (RNP1005V, Amersham Pharmacia Biotech) diluted 1:200 in goat serum, before being rinsed in TBS and TBS+ 1% triton X-100 after incubation with streptavidine HRP (P0397, Dako) diluted 1:200 in goat serum and a wash in TBS, in the HRP was visualized by 25 mg of diaminobenzidine (DAB) diluted in 50 ml of goat serum and 16.5  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$ . The stained sections were finally washed in TBS and distilled water, dehydrated in ethanol, cleared in xylene, and coverslipped with DePex mounting media.

##### Tomato lectin histochemistry

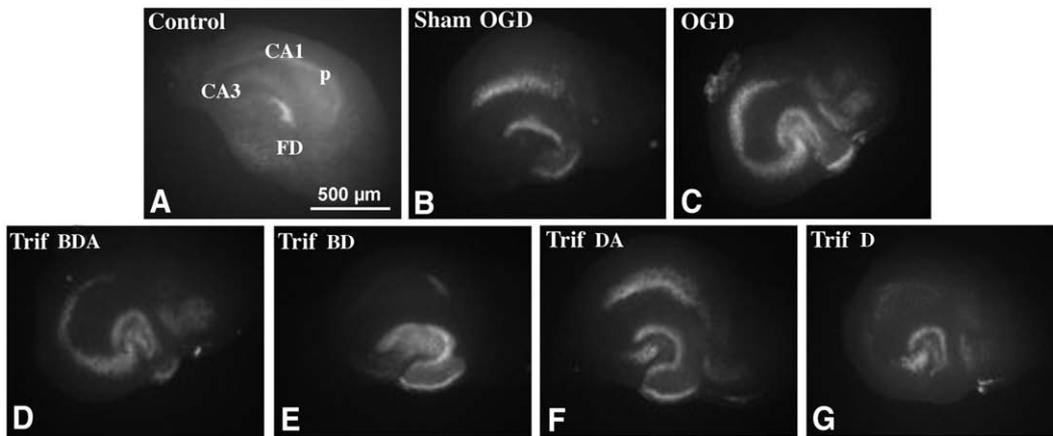
Besides expressing Mac1 immunohistochemistry, microglial cells also bind tomato lectin, which was used as an alternative visualization of microglial cells. For this, cryostat sections of slice cultures were washed in TBS+ 1% triton X-100 (Sigma) and incubated for 2 h at 37 °C with the biotinylated lectin from *Lycopersicon esculentum* (tomato) (6  $\mu\text{g}/\text{ml}$ ) (Sigma, L-9389). The sections were then washed in TBS and incubated in avidin peroxidase 1:200 (Dakopatts, P-0364) for 1 h at RT before the location of peroxidase was visualized by incubation in 100 ml TBS with 50 mg DAB and 33  $\mu\text{l}$  of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 15 min. Finally, the sections were washed in TBS, rinsed in distilled water, dehydrated in 70, 96 and 99% ethanol, cleared in xylene and coverslipped with DePex mounting media.

##### Labeling of dividing microglia

For double immunohistochemical staining of microglial cells for Mac1 expression and cell division, detected by BrdU (bromodeoxyuridine)



**Fig. 2.** Schematic illustration of the experimental protocol used for testing of the effect of microglia depletion on the neuroprotective effects of triflusal (100  $\mu\text{M}$ ). Hippocampal slice cultures, derived from 7-day-old mice were first pretreated with the toxin saporin coupled to an antibody against the Mac1 receptor for 7 days, starting 1 week after culture set up, and thereafter, when 2 weeks old, exposed to 30 min OGD with triflusal present during OGD. Photographic recordings of OGD-induced PI uptake in the CA1 pyramidal cell layer were performed before the insult (basic uptake) and 24 h after OGD.



**Fig. 3.** Fluorescent microphotographs of mouse hippocampal slice cultures recorded PI-uptake in regular (control and sham-OGD cultures) 24 h after 30 min of OGD, showing neuroprotective effects of triflusal. Maximal PI uptake in the CA1 pyramidal cell layer (p) was found in OGD cultures not treated with triflusal (C). Among the different triflusal treatments (D–G), the presence of triflusal during OGD only, was the most effective (G). Scale bar in (A) applies to all figures. For abbreviations, see Fig. 1; for quantitation and statistics, see Fig. 4.

incorporated in the DNA, freeze-stored sections were first stained for Mac1 expression, according to the above protocol, and up to the washes after development in diaminobenzidine (DAB). Instead of dehydration and mounting, the sections were then further washed in saline–sodium citrate (SSC) at room temperature, and at 60 °C before exposed to 49% formamide in SSC 2 h at 60 °C. After washing in SCC, the sections were treated with 2 N hydrochloric acid (HCl) in TBS for 30 min at 37 °C, and rinsed in 0.1 M Borat buffer (pH 8.5) for 10 min and thereafter TBS and TBS+ 1% triton X-100. After blocking unspecific staining by 10% FBS for 30 min, the sections were incubated overnight with mouse anti-BrdU 1:600 (DAKO M0744) at 4 °C, followed by rinse in TBS+ 1% X-triton and incubation in alkaline-phosphatase conjugated secondary anti-mouse antibody 1:200 for 1 h at room temperature. The sections were then washed in TBS and TBS+ HCl (pH 9.5) before the presence of alkaline-phosphatase, and hence BrdU containing all nuclei, was visualized by incubation in freshly prepared NBT/BCIP (Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine Salt) in the presence of levamisole for 30 min at room temperature. Finally, the sections were rinsed in distilled water and dehydrated in 50%, 70%, 90% and 99% acetone, cleared in xylene, and coverslipped with DePex mounting media.

#### NFκB immunohistochemistry

For immunohistochemical staining for the inflammation regulating transcription factor NFκB slice culture sections were rinsed in TBS+ 1% X-triton followed by blocking of unspecific staining in 10% Fetal calf serum (FCS) and then incubated with the primary antibody anti-NFκB (p65) 1:100 (Santa Cruz Biotechnology, sc-109) at 4 °C overnight. Thereafter the sections were rinsed in TBS+ 1% X-triton and incubated with the secondary biotinylated anti-rabbit 1:200 (Amersham, RPN 1004) for 1 h at RT, followed by a rinse in TBS+ 1% X-triton and incubated with avidin peroxidase HRP (1:200; Dakopatts, P-0364) at RT for 1 h before HRP-development in 50 mg of diaminobenzidine (DAB) diluted in 100 ml of FCS and 33 μl of H<sub>2</sub>O<sub>2</sub> for 15 min. The sections were finally washed in TBS and distilled water, before dehydrated in 70%, 96% and 99% ethanol, cleared in xylene, and coverslipped with DePex mounting media.

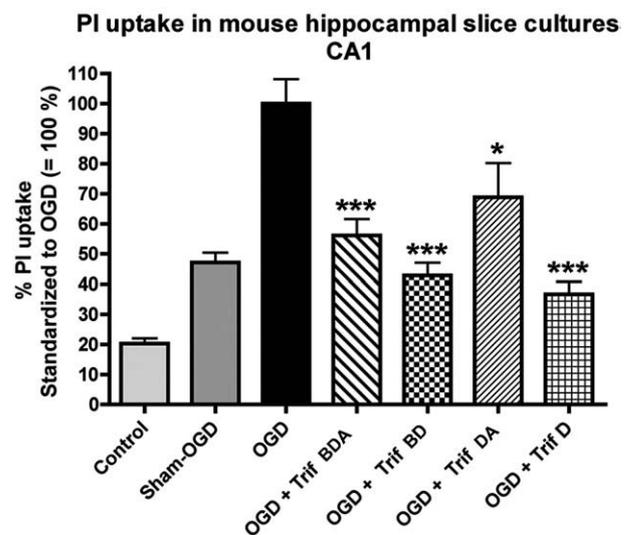
#### Stereological cell counts by the optical dissector method

Cells single labeled by Mac1 or BrdU and double-labeled by Mac1-BrdU were counted stereologically using the fractionator method.

Only sections with complete hippocampal subfields were used for the counting which was performed with experimental and control groups blinded to the observer. The CA1 pyramidal layer and the so-called inner (CA1-close) blade of the dentate granule cell layer, including the subgranular zone, were outlined in the immunostained sections, using Olympus Castgrid Stereology equipment. Estimates of the total number of Mac1, BrdU and Mac1-BrdU immunoreactive cells per culture ( $N$ ) were calculated using the formula:

$$N = \Sigma Q^- \cdot (1 / \text{ssf}) \cdot (1 / \text{asf})$$

$Q^-$ : number of cells counted.  
ssf: sections sampling fraction.  
asf: area sampling fraction.



**Fig. 4.** Densitometric measurements of PI uptake in control and experimental mouse hippocampal slice cultures, including regular controls (control) and sham-OGD control cultures as well as exposed to 30 min OGD and treated with triflusal as presented in Fig. 1. For comparison OGD-induced PI uptake in the CA1 pyramidal cell layer of regular, otherwise untreated cultures was set to 100% ( $n=20-28$ ) (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ). For abbreviations see Fig. 1. Cultures exposed to triflusal only during the insult displayed the highest numerical decrease in PI-uptake compared to OGD-lesioned cultures, although there was no statistically significant difference between the 4 different protocols of triflusal application used.

### Statistical analysis

Densitometric measurements of PI uptake were expressed as mean  $\pm$  SEM. Statistical significance was determined by using ANOVA followed by Bonferroni's post-hoc analysis of relevant experimental groups. Differences of  $p < 0.05$  were considered significant.

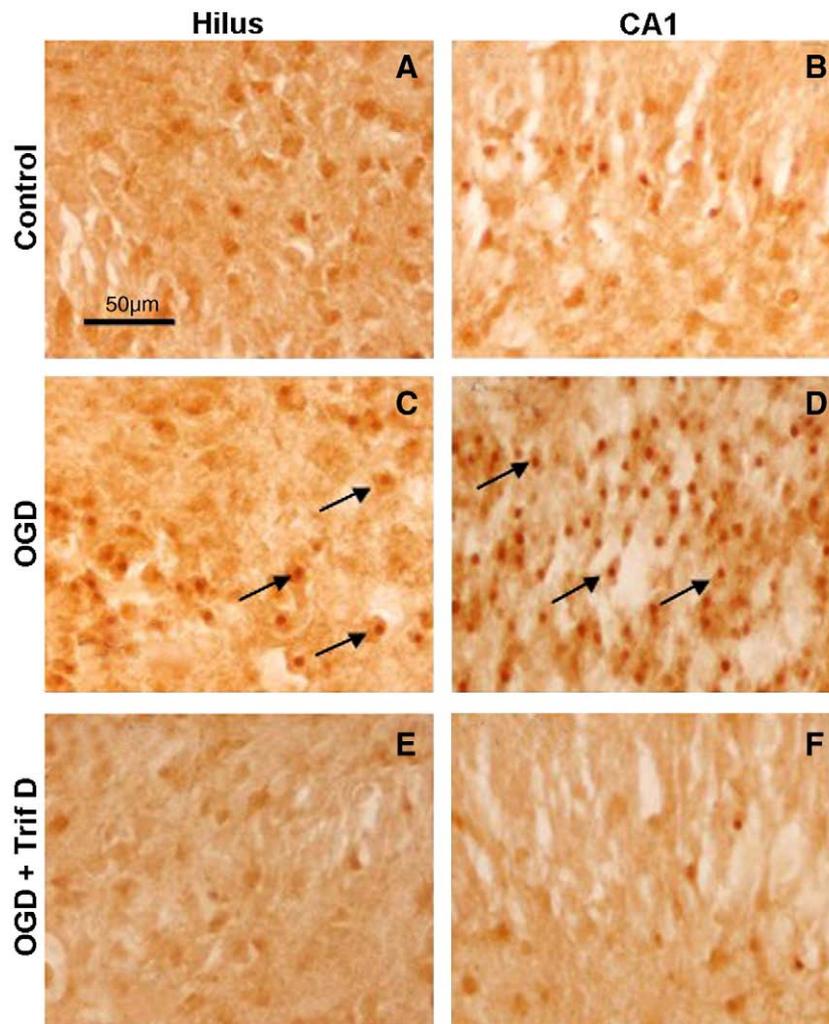
### Results

#### Determination of $ET_{50}$ value for OGD

To assess and evaluate viability of the cultures, only hippocampal slices with a well-structured organization of the hippocampal subfields and displaying basal levels of PI uptake were chosen for the following experiments. In order to determine the time in OGD medium needed to induce 50% neuronal cell death of CA1 pyramidal cells (the  $ET_{50}$  value), two-week old hippocampal slice cultures were exposed to OGD for 10, 20, 30, 45, 60, 90 and 120 min and the extent of cell death assessed by densitometric measurements of the cellular uptake of PI at 24 h after OGD. The  $ET_{50}$  value was found to be 30 min (data not shown).

#### Timing of triflusal treatment for neuroprotective effects

To test for neuroprotective effects of triflusal and how this might depend on the timing of the application in relation to OGD, triflusal was applied according to the following schedules: –1) 24 h before + during + 24 h after OGD, –2) 24 h before + during OGD, –3) during + 24 h after OGD, and –4) during OGD only (Fig. 1). Setting the OGD-induced uptake of PI in the CA1 pyramidal cell layer of otherwise untreated cultures to 100% (absolute values in arbitrary units:  $575 \pm 47$ ), the basal PI uptake in non-OGD control cultures was  $20 \pm 2\%$  ( $n = 21$ ) (absolute values in arbitrary units:  $116 \pm 11$ ;  $p < 0.001$ ), raising to  $47 \pm 3\%$  ( $n = 20$ ) (absolute values in arbitrary units:  $271 \pm 19$ ;  $p < 0.001$ ) in submersed sham-OGD cultures. In cultures exposed to triflusal 24 h before, during and after OGD PI uptake was reduced to  $56 \pm 6\%$  (absolute values in arbitrary units:  $323 \pm 32$ ;  $p < 0.001$ ). Treatment with Triflusal 24 h before and during OGD reduced the PI uptake to  $43 \pm 4\%$  (absolute values in arbitrary units:  $247 \pm 24$ ;  $p < 0.001$ ), while cultures treated with triflusal during and for 24 h after OGD displayed a PI uptake of  $69 \pm 11\%$  (absolute values in arbitrary units:  $396 \pm 65$ ;  $p < 0.01$ ). The most efficient triflusal-induced reduction of PI-uptake to  $37 \pm 4\%$  (absolute values



**Fig. 5.** Immunohistochemical expression of NFkB in the dentate hilar region (CA4) and in the CA1 pyramidal cell layer of mouse hippocampal slice cultures exposed to 30 min OGD without (OGD) and with triflusal present during OGD (OGD + Trif D). Control cultures (A, B) with few scattered NFkB-immunoreactive cells in both the dentate hilus (A) and the CA1 pyramidal cell layer (B). In OGD-lesioned cultures a significant increase in both number and staining density of NFkB-immunoreactive cells (arrows) occurred in both the dentate hilus (C) and the CA1 pyramidal cell layer (D). In OGD-exposed cultures treated with triflusal during OGD (OGD + Trif D), the number of NFkB immunoreactive cells remained at control levels in both the dentate hilus (E) and the CA1 pyramidal cell layer and (F). Scale bar in (A) applies to the entire figure.

in arbitrary units:  $211 \pm 24$ ;  $p < 0.001$ ) was found in cultures exposed to triflusal during OGD only. Based on these results (Figs. 3 and 4) we chose to only add triflusal during OGD in further experiments, as it was numerically the most efficient. The neuroprotective action of triflusal observed in CA1 also included the CA3 pyramidal cell layer and the dentate granule cell layer (data not shown).

#### Effects of OGD and triflusal treatment on cellular expression of nuclear transcription factor NFκB

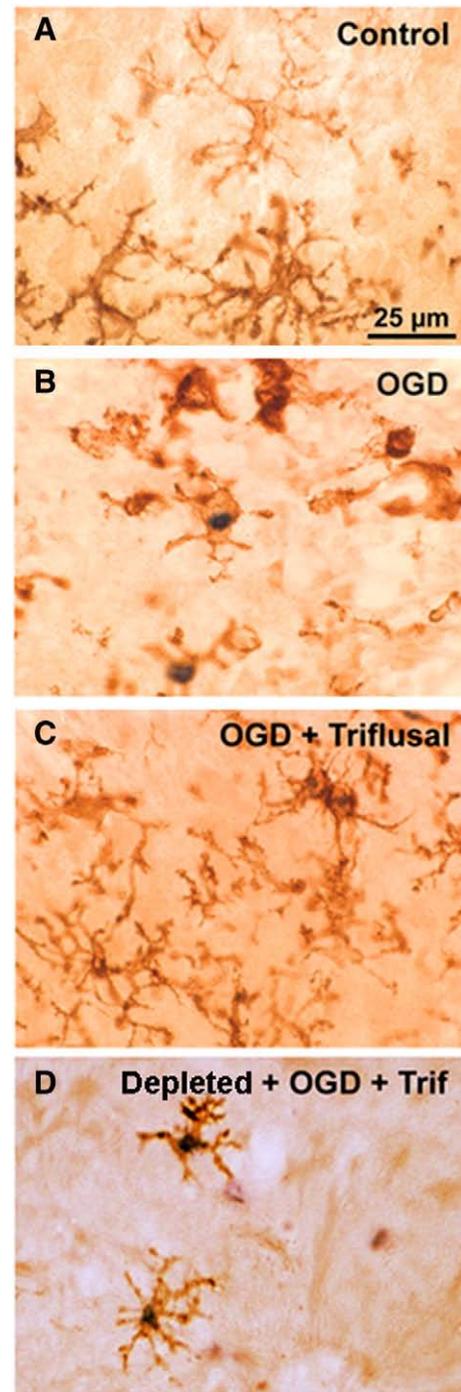
In non-lesioned regular hippocampal slice cultures, no NFκB-immunoreactive cells were present neither within nor outside of the neuronal cell layers (Figs. 5A and B). Some light NFκB immunoreactivity, mainly located to the neuronal cell layers, was, however, observed in the sham-OGD group, possibly corresponding to the slight increase in PI-uptake (cell death) seen in this group (data not shown). Cultures subjected to 30 min OGD without presence of triflusal, displayed strong NFκB expression in CA1, when observed 24 h after OGD, with densely stained pyramidal cell nuclei (Figs. 5C and D). Presence of triflusal during OGD reduced the neuronal NFκB expression to scattered, moderately stained cell nuclei in the neuronal layers, in accordance with the reduced PI uptake in these cultures (Figs. 5F and E). What appeared as an even stronger reduction of OGD-induced NFκB expression was seen when triflusal was present 24 h before and during OGD, with almost no NFκB immunoreactive cells being present. In contrast to that, NFκB expression was not clearly reduced in cultures exposed to triflusal during OGD and for 24 h thereafter, when compared to OGD-exposed cultures not treated with triflusal, contrasting a slight, but statistically significant reduction of PI uptake in this group (Fig. 4, OGD + TrifDA).

#### Effects of OGD and triflusal treatment on microglial cell reactions

Microglial cells react to central nervous system (CNS) damage by morphological changes, cell proliferation and expression and release of trophic factors and chemokines. Here the OGD-induced morphological reactions of microglial cells and the possible modulations induced by triflusal were visualized by Mac1 immunohistochemistry. Control cultures displayed highly ramified microglia, corresponding to a resting (non-activated) state (Fig. 6A). In cultures surviving for 24 h after OGD, microglial cells had increased in numbers, stained more densely and displayed an activated amoeboid-like morphology with enlarged cell bodies and fewer and shorter processes (Fig. 6B). In cultures exposed to OGD with triflusal present in the medium during OGD, the inflammatory microglial activation was severely reduced, with most microglial cells displaying a resting phenotype similar to microglial cells in control cultures (Fig. 6C). In cultures, depleted of microglia before exposed to OGD, triflusal treatment also reduced the OGD-induced microglial activation (Fig. 6D).

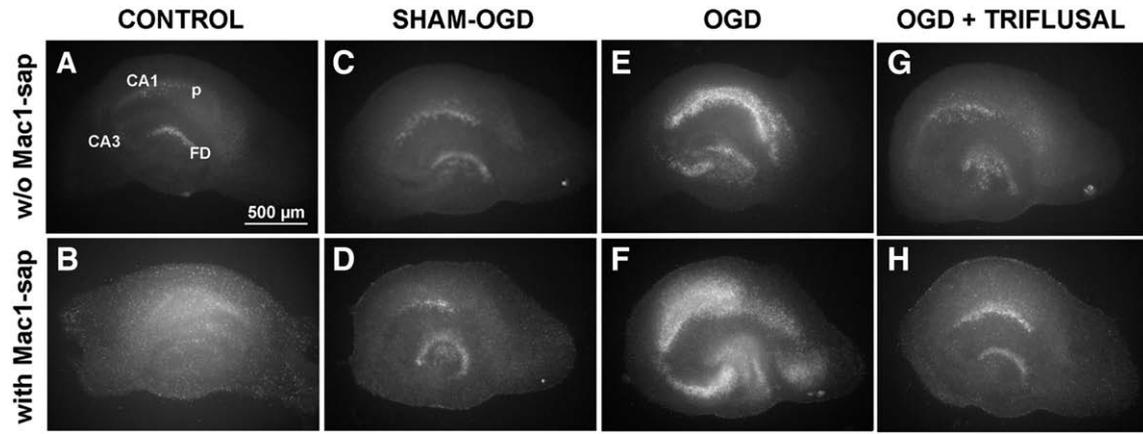
#### Effects of microglial depletion on OGD-induced neurodegeneration

The role of microglial cells in reducing or enhancing the degenerative outcome of CNS insults is still unsettled. Depletion of microglial cells from hippocampal slice cultures before OGD is one approach to elucidate the role of microglia in cerebral ischemia, and as shown separately (Montero et al., submitted for publication). In the present study we examined to what extent the presence of microglia was needed for triflusal to exert its neuroprotective effects. Setting the OGD-induced PI uptake in otherwise untreated cultures to 100%, the PI uptake in control cultures and sham-OGD cultures was found to be  $34 \pm 2\%$  and  $34 \pm 3\%$ , respectively. The presence of triflusal during the 30 min OGD of such regular cultures decreased PI uptake to  $55 \pm 10\%$  ( $p < 0.001$ ). Microglia-depleted control cultures, not subjected to OGD, displayed a PI uptake of  $35 \pm 2\%$ , and corresponding microglia-depleted sham-OGD cultures a PI uptake of  $45 \pm 4\%$ , as compared to



**Fig. 6.** Microglial cells from CA1 pyramidal cell layer of mouse hippocampal slice cultures visualized by immunostaining for the microglial receptor Mac1 (or CD11b). — A: in 2-week-old control cultures, microglial cells displayed a highly ramified phenotype, corresponding to a resting, non-activated state *in vivo*. — B: 24 h after OGD, the microglial cells displayed an amoeboid, activated morphology with increase in cell body size and shorter and thicker ramifications. — C: in OGD-lesioned cultures treated with triflusal during OGD, the OGD-induced activation (see B) was prevented, leaving the microglial cells in an apparent non-activated state (cp. C and A). — D: in cultures immunotoxically depleted of microglia by pretreatment with Mac1-sap (see text) before subjected to OGD with triflusal present, very few microglial cells were present and those remaining, were in a non-activated or semi-activated state like in the microglia-depleted cultures not subjected to OGD (see Figs. 9C and 10F). Scale bar in (A) applies to the entire figure.

regular OGD-lesioned cultures. In OGD-lesioned microglia-depleted cultures the PI uptake rose significantly to  $177 \pm 9\%$ , but was reduced to only  $40 \pm 3\%$  ( $p < 0.001$ ) in the presence of triflusal (Figs. 7 and 8). In



**Fig. 7.** Fluorescent microphotographs of PI uptake in regular (upper panels; A, C, E, G) and microglia-depleted (lower panels; B, D, F, H) non-lesioned (control, sham-OGD) and OGD-lesioned mouse hippocampal slice cultures. The PI uptake was recorded 24 h after 30 min of OGD in cultures without and with addition of triflusal during OGD (G, H). The highest PI uptake occurred in the CA1 pyramidal cell layer (p) and CA3 pyramidal cell and dentate granule cell layers) in microglial-depleted cultures exposed to OGD (F) without presence of triflusal. The presence of triflusal effectively reduced the OGD-induced PI uptake in both the regular (cp. E and G) and microglia-depleted cultures (cp. F and H). Abbreviations: CA3, hippocampal subfield CA3; CA1, hippocampal subfield CA1; FD, fascia dentata; p, CA1 pyramidal cell layer. Scale bar in (A) applies to the entire figure.

accordance with this, the low number of microglial cells present in these cultures primarily displayed a non-activated morphology.

#### Microglial migration towards the site of injury

After focal CNS lesions microglial cells typically migrate from adjacent areas into the lesion site (Heppner et al., 1998). For demonstration of such migratory activity in the present lesion-paradigm, fluorescent tomato lectin was used to visualize microglial cells. From microscopical analysis and fluorescence microphotographs taken of the CA1 pyramidal cell layer of control and OGD-lesioned cultures, control cultures not subjected to immunotoxic microglia depletion, were found to display a homogeneously distribu-

tion of ramified non-activated microglial cells (Fig. 9A). After OGD, the microglial cells adopted an activated, amoeboid morphology with an increase in cell density primarily in the lesioned CA1 pyramidal cell layer (Fig. 9B). In non-lesioned microglia-depleted cultures only a few tomato lectin positive cells were found scattered in the hippocampal slice cultures with amoeboid cell bodies and short processes (Fig. 9C). 1 day after OGD, these cultures also displayed an increase in amoeboid cells primarily in the CA1 pyramidal cell layer (Fig. 9D).

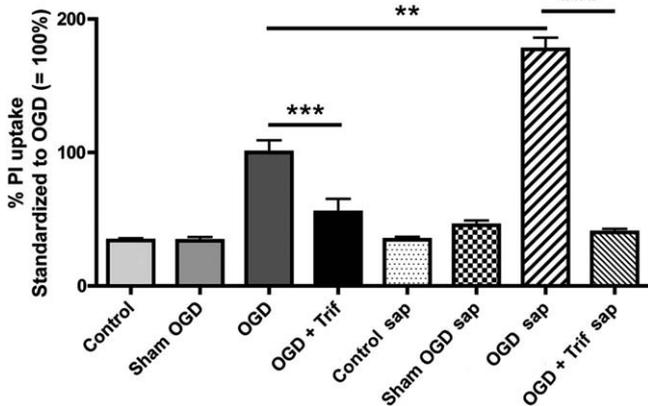
#### Effects of OGD, microglial depletion and triflusal treatment on cell proliferation in the CA1 pyramidal layer

Microglial proliferation was examined by use of Mac1-BrdU double immunohistochemical staining (Fig. 10), followed by stereological cell counts of Mac1 and BrdU single labeled cells and Mac1-BrdU double labeled cells in the CA1 pyramidal cell layer (Fig. 11).

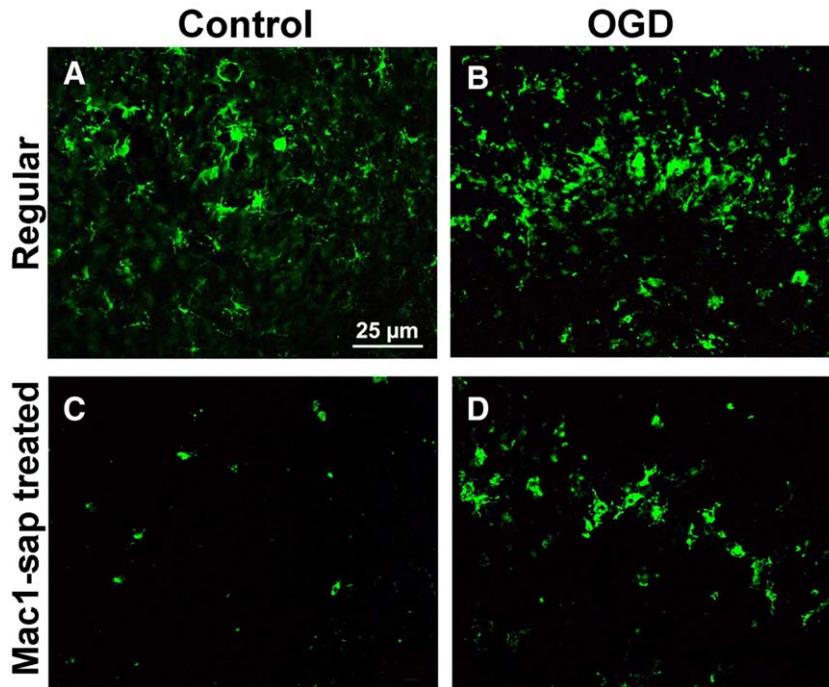
In the OGD-lesioned CA1 pyramidal cell layer of regular hippocampal slice cultures the number of *Mac1* positive microglial cells was significantly increased at 24 h postlesion ( $850 \pm 122$  cells/culture) (Figs. 10C and D) as compared to both control ( $375 \pm 60$  cells/culture) (Figs. 10A and B) and sham-OGD cultures ( $444 \pm 38$  cells/culture) (Fig. 11). In accordance with its neuroprotective effects,  $100 \mu\text{M}$  triflusal applied during OGD effectively reduced the increase in Mac1 immunoreactive cells ( $412 \pm 39$  cells/culture,  $p < 0.001$ ) compared to OGD-lesioned cultures. In non-lesioned (control) microglia-depleted cultures, the number of microglial cells in CA1 was significantly reduced (Figs. 10E and F) compared to non-depleted control cultures (Fig. 11). The microglia depleted cultures, thus displayed an average of  $111 \pm 15$  cells/culture compared to  $375 \pm 60$  cells/culture in non-depleted controls. Microglia-depleted sham-OGD cultures had  $158 \pm 41$  cells/culture. After OGD the number of Mac1-positive cells in CA1 of microglia-depleted cultures was only  $176 \pm 21$  cells/culture (Figs. 10G and H), which was not significantly different from neither microglia-depleted control nor sham-OGD cultures. When triflusal was added to the microglia depleted cultures during OGD, the average number of microglial cells in CA1 was  $119 \pm 17$  cells/culture, but again this number was not statistically different from any of the low number of microglial cells counted in the OGD non-triflusal treated cultures.

When counting *BrdU*-labeled, dividing cells in the CA1 pyramidal layer these were  $234 \pm 38$  cells/culture in OGD cultures, compared to  $54 \pm 10$  cells/culture in control cultures and  $47 \pm 8$  cells/culture in sham-OGD cultures. Addition of triflusal during OGD effectively blocked the OGD-induced increase in BrdU-labeled cells ( $53 \pm 10$

#### PI uptake in mouse hippocampal slice cultures CA1



**Fig. 8.** Densitometric measurements of PI uptake in CA1 pyramidal cell layer of regular and microglia-depleted mouse hippocampal slice cultures including non-lesioned control and sham-OGD cultures, and cultures exposed to 30 min OGD with presence of  $100 \mu\text{M}$  triflusal during OGD (Fig. 6). PI-uptake was measured in the CA1 pyramidal cell layer ( $n = 25\text{--}30$  for all groups). For comparison the OGD-induced PI-uptake in otherwise non-treated cultures was set to 100%. Triflusal significantly reduced OGD-induced neuronal cell death in regular microglia-containing slice cultures (OGD + Trif;  $***p < 0.001$ ). PI-uptake in the microglia-depleted control cultures (control-sap, sham-OGD-sap) was not different from uptake in regular controls, but microglia-depleted cultures exposed to OGD displayed a significantly higher PI uptake than corresponding OGD-lesioned regular cultures (cp. OGD-sap and OGD;  $**p < 0.01$ ). In microglia-depleted cultures subjected to OGD, triflusal prevented any increase in PI-uptake above control levels (cp. OGD + Trif sap and OGD sap;  $***p < 0.001$ ).



**Fig. 9.** Microglial cells in the CA1 pyramidal cell layer and adjacent parts of strata oriens and radiatum of mouse hippocampal slice cultures visualized by fluorescent tomato lectin histochemistry. 24 h after 30 min of OGD, microglial cells accumulated in the CA1 pyramidal cell layer (B) as compared to control cultures (A), where the cells were more evenly dispersed in all layers. In Mac1-sap treated cultures, almost no tomato lectin positive cells were left after the depletion treatment (C), but 24 h after OGD these cultures also displayed an accumulation of tomato lectin positive cells in the OGD-lesioned CA1 pyramidal cell layer. Scale bar in (A) applies to the entire figure.

cells/culture,  $p < 0.001$ ). In microglia-depleted control and sham-OGD cultures the numbers of BrdU-labeled cells present in the CA1 pyramidal cell layer were  $26 \pm 5$  cells/culture and  $40 \pm 8$  cells/culture, respectively. In microglia-depleted cultures subjected to OGD there were  $50 \pm 13$  dividing cells/culture, but this number was not significantly different from the numbers in microglia-depleted control and sham-OGD cultures. Addition of triflusal during OGD did not significantly alter cell proliferation compared to non-triflusal treated OGD cultures ( $27 \pm 6$  cells/culture).

Counts of *Mac1-BrdU double-labeled cells* were performed to determine the fraction of microglial cells dividing as well as the fraction of dividing cells being microglia. The number of double-labeled Mac1-BrdU positive cells was significantly increased in OGD lesioned ( $94 \pm 15$  cells/culture) compared to control ( $36 \pm 7$  cells/culture) and sham-OGD cultures ( $33 \pm 6$  cells/culture). Triflusal treatment during OGD reverted the number of Mac1-BrdU positive cells in the CA1 from OGD-lesioned to normal levels ( $41 \pm 8$  cells/culture,  $p < 0.001$ ).

In microglia-depleted control and sham-OGD cultures the numbers of Mac1-BrdU double labeled cells were  $18 \pm 4$  cells/culture and  $22 \pm 5$  cells/culture, respectively. This value rose to  $37 \pm 8$  cells/culture after OGD, but remained at control level ( $18 \pm 4$  cells/culture) when triflusal was added during OGD. However, differences between these values were not statistically significant.

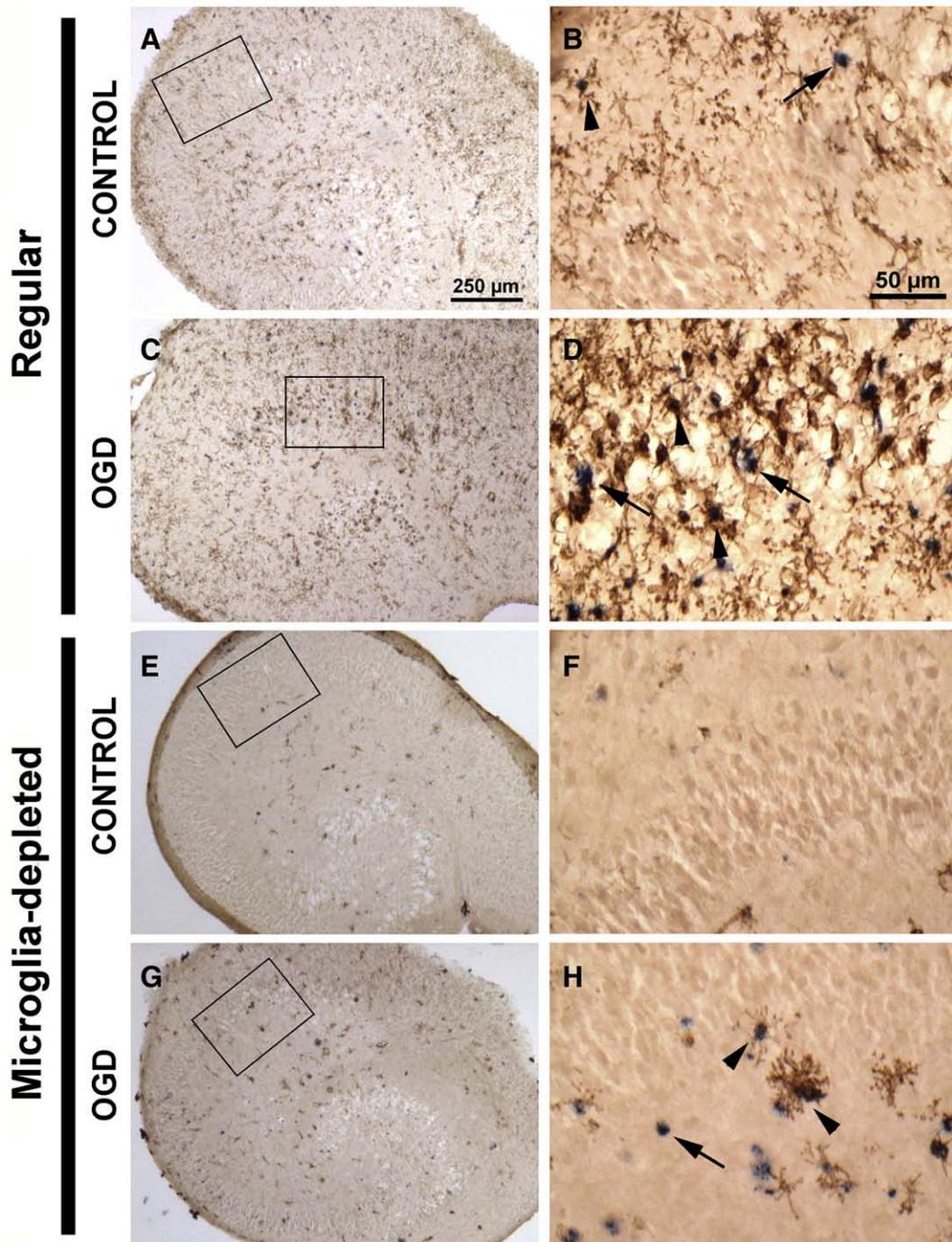
#### *Effects of OGD, microglial depletion and triflusal on cell proliferation in the dentate granule cell layer and subgranular zone*

The fascia dentata is one of the neurogenic regions of the adult brain. Since neuroinflammation affects neurogenesis (Monje et al., 2003) we included the dentate granule cell layer and the adjacent neural progenitor cell-containing subgranular zone in the analysis of microglial reactivity, with cell counts of Mac1, BrdU and Mac1-BrdU positive cells. *Mac1-positive microglial cells* in the inner blade of the dentate granule cell layer were significantly increased in number in OGD-lesioned cultures ( $443 \pm 71$  cells/culture,  $p < 0.001$ ) compared to

control cultures ( $232 \pm 34$  cells/culture). Counts for sham-OGD cultures were  $305 \pm 30$  cells/culture. Treatment with  $100 \mu\text{M}$  triflusal during OGD effectively decreased the number of Mac1 immunoreactive cells in the dentate granular and subgranular layers to  $281 \pm 25$  cells/culture ( $p < 0.001$ ). In microglia-depleted cultures the number of Mac1 positive cells were only  $112 \pm 24$  cells/culture in control and  $117 \pm 36$  cells/culture in sham-OGD cultures. OGD-lesioned microglia-depleted cultures contained an average number of  $111 \pm 14$  cells/culture. For cultures treated with triflusal during OGD the number of microglial cells was  $82 \pm 12$  cells/culture, which was not statistically significant from OGD-lesioned cultures.

As described for the CA1 pyramidal cell layer, OGD also induced an increase in *BrdU positive dividing cells* in the dentate granule cell layer and subgranular zone ( $87 \pm 21$  cells/culture) as compared to control cultures ( $24 \pm 5$  cells/culture). Sham-OGD cultures had an average of  $20 \pm 8$  cells/culture. Triflusal ( $100 \mu\text{M}$ ) applied during OGD effectively reduced OGD-induced cell proliferation to counts of only  $15 \pm 3$  BrdU-labeled cells/culture ( $p < 0.001$ ). Regarding BrdU positive cells in the dentate granule and subgranular cell layers of microglia-depleted cultures, control and sham-OGD cultures contained an average of  $9 \pm 3$  cells/culture and  $15 \pm 3$  cells/culture, respectively. When subjected to OGD the number of BrdU positive cells was  $16 \pm 3$  cells/culture, which was not significantly higher than control and sham-OGD cultures. Addition of triflusal during OGD of microglia-depleted cultures did not significantly reduce the number of BrdU labeled cells ( $9 \pm 3$  cells/culture).

Counts of *Mac1-BrdU double labeled cells* in the dentate granule and subgranular cell layers resulted in  $13 \pm 3$  cells/culture in normal (non microglia-depleted) control and  $9 \pm 2$  cells/culture in sham-OGD cultures. In OGD-lesioned cultures the number of Mac1-BrdU positive cells rose to  $29 \pm 8$  cells/culture ( $p < 0.001$ ). When triflusal was added during OGD, this lesion-induced increase was abolished ( $11 \pm 2$  cells/culture), although this decrease was not statistically significant. In microglia-depleted cultures, numbers of double-labeled Mac1-BrdU positive cells for control and sham-OGD cultures were  $6 \pm 2$  cells/culture and  $9 \pm 2$  cells/culture, respectively. OGD of these



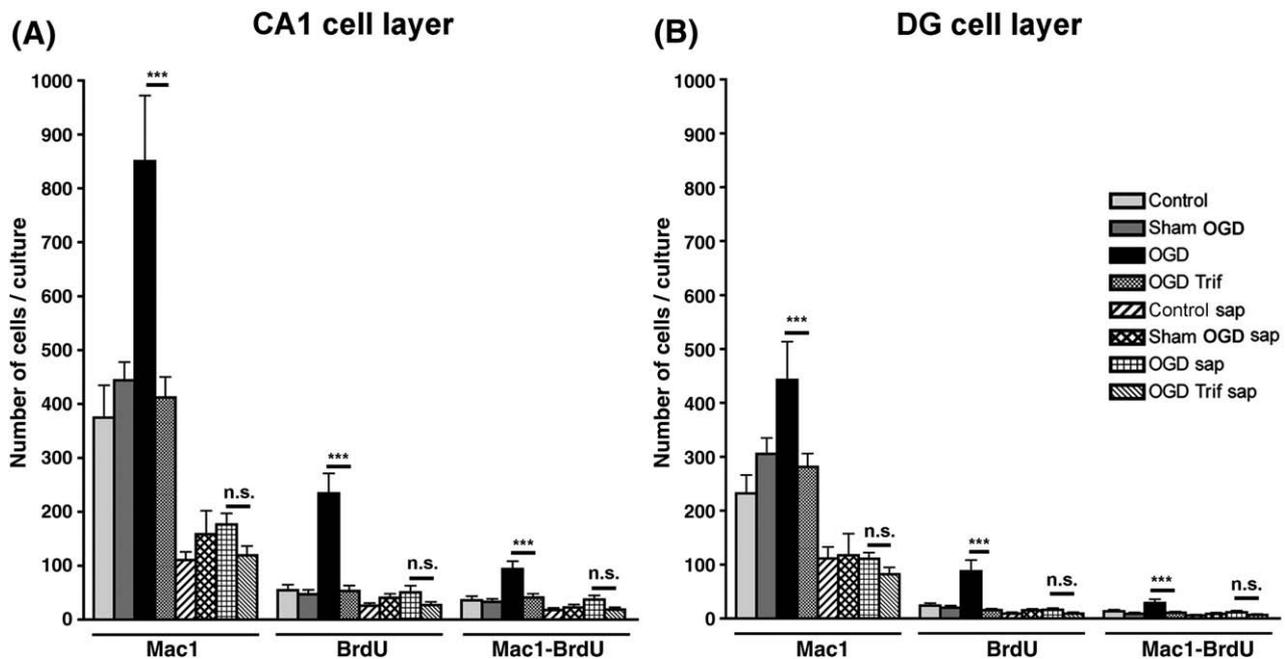
**Fig. 10.** Double immunohistochemical staining for microglial cells (Mac1) and proliferating cells (BrdU) in regular mouse hippocampal slice cultures not depleted of microglia (A–D) and corresponding cultures with microglia depletion (E–H). Framed areas in (A), (C), (E) and (G), including CA1 pyramidal cell layer, are shown at higher magnification in (B), (D), (F) and (H), respectively. —(A, B): slice cultures displaying normal, almost homogeneous distribution of microglial cells with very few single labeled BrdU (arrow, B) and double labeled Mac1-BrdU positive cells (arrowhead, B). —(C, D): 24 h after OGD, the number of Mac1-positive microglial cells and BrdU-positive proliferating cells have increased significantly. —(E, F): microglia-depleted cultures not exposed to OGD contained very few Mac1 positive cells, mostly with short processes. —(G, H): 24 h after OGD an increase or reappearance of Mac1 immunoreactive cells in the microglia-depleted cultures occurred. Some of these Mac1-positive cells were newly formed with coexpression of the proliferation marker BrdU (arrowhead, H). Other cells only stained for BrdU (arrow, H). Scale bar in (A) applies to (A), (C), (E) and (G). Scale bar in (B) applied to (B), (D), (F) and (H). Arrows: BrdU-positive cells. Arrowheads: Mac1-BrdU-positive cells.

cultures induced a numerical, but non-significant increase to  $12 \pm 3$  cells/culture. Addition of triflusal prevented this numerical increase, resulting in  $6 \pm 2$  cells/culture.

## Discussion

The purpose of this study was to test the potential neuroprotective role of the anti-inflammatory compound triflusal in hippocampal

slice cultures subjected to oxygen-glucose deprivation and to whether such effect required presence of microglial cells. Experimental slice cultures models of cerebral ischemia are widely used for studies of neurodegenerative processes and neurotoxicity because they are more accessible for inspection and manipulation and the extracellular environment more easily controlled than *in vivo* models. Deprivation of hippocampal slice cultures from oxygen and glucose in a standardized setting for a fixed period of time like



**Fig. 11.** Stereological counts of microglial cells (Mac1), newly divided cells (BrdU) and newly divided microglial cells (Mac1-BrdU) cells in the CA1 pyramidal cell layer (A) and the dentate granule cell layer (B) of regular and microglia-depleted mouse hippocampal slice cultures and corresponding experimental cultures exposed to 30 min of OGD with and without the presence of 100  $\mu$ M triflusal during OGD. OGD induced a significant increase in the number of Mac1, BrdU and Mac1-BrdU immunoreactive cells in both the CA1 pyramidal cell layer and the dentate granule cell layer. The presence of triflusal prevented this increase, keeping the respective cell numbers in both CA1 and the dentate granule cell layers at normal levels. In cultures immunotoxically depleted of microglial cells no significant differences in cell counts were found between control cultures, OGD-lesioned cultures and OGD-lesioned cultures treated with the anti-inflammatory triflusal. Data are expressed as the average number of cells per culture  $\pm$  SEM; \*\*\* $p$  < 0.001 (using ANOVA with Bonferroni's correction). Control: regular control cultures; Sham-OGD: regular control cultures submerged in non-OGD medium; OGD: regular cultures exposed to OGD; OGD Trif: regular cultures exposed to OGD with 100  $\mu$ M triflusal present during OGD; Control sap, Sham-OGD sap, OGD sap, OGD Trif sap: groups of control and experimental microglia-depleted cultures corresponding to groups of regular slice cultures.

30 min is one such model (Bonde et al., 2002b, Montero et al., 2007a, 2007b).

#### Triflusal

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin or sodium salicylates, are widely used to treat inflammation (Weissmann, 1991) and have been demonstrated to be neuroprotective through inhibition of NF $\kappa$ B (Kopp and Ghosh, 1994). The use of aspirin for secondary prevention of ischemic stroke has been considered a golden standard compared to other antiplatelet compounds. Low doses of aspirin (50–325 mg/day) are recommended to avoid the risk of death or non-fatal ischemic injuries in patients that previously have had transient ischemic attacks (Costa et al., 2006) or stroke (Albers et al., 2001). The risk of salicylate-facilitated hemorrhagic events is, however, a major concern in the use of aspirin, prompting the search for compounds with the same efficacy but fewer side effects. In a randomized stroke study Culebras et al. (2004) found no significant differences between triflusal and aspirin in the prevention of vascular complications after TIAs or ischemic stroke, although triflusal seemed to be associated with a lower risk of hemorrhagic complications. In a larger European study the incidence of hemorrhagic complications in triflusal treated patients was, however, lower than in those treated with aspirin (Matias-Guiu et al., 2003).

For use in the present study, it was determined that triflusal was most effective present during OGD. Employing excitotoxic NMDA lesions of the cerebral cortex of early postnatal rats, Acarin et al. (2001) had earlier found that triflusal exerted maximal neuroprotection when injected 8 h after the application of NMDA. In the hippocampal slice culture model of cerebral ischemia triflusal was, however, not neuroprotective when only applied right after or up to 8 h after OGD (data not shown), contrasting its effects in our various

protocols where it was applied during the actual OGD (Fig. 4). Further experiments are required to investigate and compare the neuroprotective effects of triflusal *in vivo* and *in vitro*. Based on our *in vitro* results shown in Fig. 4, we chose application of triflusal during the 30 min of OGD only as the standard application in the present study, demonstrating that 100  $\mu$ M triflusal was highly neuroprotective against OGD in mouse hippocampal slice cultures. Regarding the PI uptake used for quantifying neuronal cell death, we did observe that in sham-OGD cultures PI uptake was higher than in non-manipulated control cultures. We interpret this difference to be due to a mild trauma induced to the sham cultures by adding the OGD medium on top of the cultures.

#### Neuroprotection by triflusal and NF $\kappa$ B expression

Regarding mechanisms of action, Acarin et al. (2000, 2001) showed that triflusal effectively downregulated both NF $\kappa$ B expression and astro- and microglial reactions after excitotoxic NMDA lesions of the postnatal rat neocortex. A similar correlation between triflusal-induced neuroprotection and reduced NF $\kappa$ B expression was found in this study (Fig. 6), which demonstrated a close linkage between reduced PI-uptake and reduced NF $\kappa$ B immunoreactivity. The transcription factor NF $\kappa$ B is normally expressed at low levels in neurons (Kaltschmidt et al., 1994) but is greatly increased in relation to seizure activity (Prasad et al., 1994, Grilli et al., 1996, Rong and Baudry, 1996, Matsuoka et al., 1999). Also ischemia has been shown to stimulate neuronal NF $\kappa$ B expression (Salminen et al., 1995, Clemens et al., 1997, Carroll et al., 1998, Zhang et al., 1998). Regarding other molecules, Acarin et al. (2002) also showed that the neuroprotective effect of triflusal was accompanied by a decrease in proinflammatory enzymes like inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and cytokines like interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). This is in

accordance with observations of neuronal NF $\kappa$ B activation being related to plasticity, development and survival (Kaltschmidt et al., 1994, Mattson et al., 2000), while NF $\kappa$ B in glial cells has been related to inflammatory responses (Mattson et al., 2000) inducing the expression of proinflammatory genes such as IL-1 $\beta$ , COX-2 or TNF- $\alpha$  (Baldwin, 1996, Grilli and Memo, 1999).

The transcription factor NF $\kappa$ B has been proposed as a neuroprotective element in the tumor necrosis factor (TNF) signaling pathway (Mattson, 1998, Mattson et al., 2000), but also expressed in relation to neurodegeneration (Nakai et al., 2000). In our OGD model NF $\kappa$ B immunoreactivity was upregulated in the CA1 and CA3 pyramidal cell and dentate granule cell layers 24 h after the insult, with a clear location in neurons (Fig. 5).

In cerebral ischemia, intracellular release of calcium (Choi, 1995), leads to activation of proteases like calpains (Saido et al., 1994), known to have a pivotal role in ischemic cell death and cytoskeletal integrity (Wang and Yuen, 1994). One substrate of activated calpain is the non-erythroid cytoskeletal protein  $\alpha$ -II-spectrin, also known as fodrin (Martin et al., 1995), which is split into  $\alpha$ -II-spectrin breakdown products (SBDPs) of 150 kDa and 145 kDa. Given that excitotoxic glutamate has been shown to activate neuronal expression of NF $\kappa$ B through calpain by cleaving I $\kappa$ B $\alpha$  (Scholzke et al., 2003), the present results thereby relate to our previous observations, that 30 min OGD significantly increased the formation of SBDPs as recorded 24 h after OGD (Montero et al., 2007b).

#### *Microglial activation and effects of depletion*

Non-activated microglial cells in control hippocampal slice cultures, visualized by Mac1 immunohistochemistry, had small cell bodies and slender highly ramified processes. In response to OGD microglial cells in the area of neuronal degeneration transformed into an activated phenotype, with enlarged cell bodies with amoeboid shape and shorter and coarser processes. Such microglial activation was effectively reduced in cultures treated with triflusal during OGD, resulting in amelioration of neurodegeneration.

In the present study, we also included slice cultures immunotoxically deprived of microglial cells to experimentally test the effect of triflusal with and without depletion of microglial cells. In microglia-depleted cultures, the PI uptake in control and sham-OGD cultures was similar to that in control cultures with normal content of microglia, showing that the microglia depletion was by itself not toxic to neurons (Montero et al., submitted for publication). Microglia-depleted cultures subjected to OGD did, however, almost double the increase in PI uptake compared to regular OGD-lesioned cultures, suggesting that normal presence of microglia in part protects against OGD. Corresponding conclusions were drawn by Neumann et al. (2006), showing that addition of exogenous cells derived from the microglial cell line BV2 reduced OGD-induced neuronal cell death in hippocampal slice cultures. Others have successfully depleted microglial cells from rat hippocampal slice cultures (Kohl et al., 2003) by addition of clodronate. Clodronate is a bisphosphonate used in the treatment of severe hypercalcemia in hyperparathyroidism, and in the treatment of osteoporosis and Paget's disease (Russell and Rogers, 1999). In agreement with our results they showed that NMDA excitotoxically lesioned hippocampal slice cultures treated with clodronate, displayed higher number of PI positive cells when depleted of microglia. In contrast to our results, Marin-Teva et al. (2004) showed that apoptosis of Purkinje cells in organotypic cerebellar slice cultures was strongly reduced by selectively eliminating microglial cells from the slices by incubating the cerebellar sections with liposomes containing clodronate diluted in phosphate buffered saline. The explanation for the difference in effects of microglia depletion observed in this study compared to Kohl et al. and ours, might be due to several factors, such as the use of a different model (hippocampal slice cultures vs. cerebellar slice cultures), the

different types and methods of inducing neuronal cell death, as well as the method employed to deplete microglial cells (Mac1-saporin vs. clodronate).

The hypothesis that microglial cells are the primary targets for the neuroprotective effect of triflusal could not be confirmed, as triflusal exerted its neuroprotective effect equally well in OGD-lesioned microglia-depleted cultures (Figs. 3 and 4). Triflusal, must accordingly, in this respect, act directly on neurons or astroglial cells. In their study of the NMDA-lesioned neocortex Acarin et al. (2000) showed that triflusal reduced the expression of proinflammatory cytokines by means of reduced NF $\kappa$ B activation in neurons and astroglial cells. Baichwal and Baeuerle (1997) also found that NF $\kappa$ B, once activated, regulated the expression of proinflammatory cytokines, major histocompatibility complex and COX-2, as well as the transcription of various antiapoptotic genes. With access to microglia-depleted cultures, it is now possible to extend these studies of the microglia-independent neuroprotective mechanisms of triflusal.

#### *Microglia dynamics*

Using fluorescence-labeled tomato lectin as a cellular marker, microglial cells were observed to migrate towards and accumulate in the OGD-lesioned CA1 pyramidal cell layer. This is in line with Heppner et al. (1998), who also reported the migration of microglial cells towards excitotoxic lesion sites in hippocampal slice cultures. In microglia-depleted cultures, only very few Mac1 and tomato lectin positive cells were left, but 24 h after OGD scattered microglial cells "reappeared", with a preferential location along the degenerating CA1 pyramidal cell layer. The Mac1 and tomato lectin positive microglial cells left after the Mac1-sap immunotoxic microglial depletion do accordingly retain the capacity to respond to insults by cell proliferation, phenotypic changes and migration towards the lesion site, as in non-depleted cultures.

The effect of triflusal on lesion-induced microglial dynamics and proliferation was assessed in both regular and microglia depleted hippocampal slice cultures. In OGD lesioned cultures there was a significant increase in Mac1 positive cells in both the CA1 (126%) and the dentate cell layers (90%) compared to control and sham-OGD cultures, demonstrating the well-known increase in microglial cell proliferation after injury (Dempsey et al., 2003, Eskes et al., 2003). Expressed as cells/culture, the percent increase in microglial cell numbers was larger in the CA1 than in the dentate, but this might simply reflect a more dense OGD-induced degeneration in CA1. In both areas, the OGD-induced microglial activation in terms of adopting an amoeboid appearance and accumulation in the degenerating cell layer was ameliorated by triflusal. Our explanation for this would be that by exerting its neuroprotective effect directly on neurons, triflusal indirectly prevents the intracellular signaling from affected neurons to microglia, that otherwise would activate these and stimulate their migration towards the CA1 pyramidal cell layer. In microglia-depleted cultures, residual microglial cells reacted to OGD, although the numerical increase was not statistically significant compared to control or triflusal treated cultures.

Regarding cell proliferation, an increase in dividing cells is known to occur after cerebral ischemia *in vivo* (Bingham et al., 2005). By counting BrdU immunoreactive cells we also found a significant increase in proliferating cells in the CA1 pyramidal cell layer (329%) of regular hippocampal slice cultures exposed to OGD, as compared to control or sham-OGD cultures. Addition of triflusal efficiently reduced the increase in cell proliferation to control levels, most likely by preventing neuronal cell death. In microglia-depleted cultures we were unable to statistically detect an increase in OGD-induced cell proliferation and an effect of triflusal on cell proliferation after OGD. The same pattern for general cell proliferation and effects of triflusal was observed in the dentate granule cell layer, with an increase in cell proliferation of 126% compared to control cultures.

When Mac1-BrdU double-labeled cells were counted in the CA1 pyramidal cell layer of otherwise untreated OGD-lesioned cultures, there was an increase of 163% in Mac1-BrdU immunoreactive, proliferating microglial cells compared to controls. Triflusal abolished this increase in proliferating Mac1-positive cells, as it prevented the increase in Mac1-positive cells in general (see above). In the dentate granule cell layer of the same cultures, we observed a similar pattern with significant increase in Mac1-BrdU positive proliferating microglial cells after OGD (118%) and prevention of this with triflusal present during the OGD. Here we made no attempt to distinguish proliferating microglia from dividing dentate neural progenitor cells. Although there was an increase in proliferating microglia in both the CA1 pyramidal cell layer and the dentate granule cell layer after OGD, most of the increase in Mac1-positive microglial cells at these sites is most likely due to migration from nearby areas, since the number of Mac1-BrdU double-labeled cells were significantly lower than the actual number of microglial cells present.

### Concluding remarks

This study has demonstrated that triflusal effectively can protect against ischemia-like neuronal cell loss in organotypic mouse hippocampal slice cultures, and that this neuroprotective effect coincides with reduced activation of the transcription factor NF $\kappa$ B. We moreover found that the neuroprotective effects of triflusal in the present experimental setting were most pronounced with triflusal being present during the actual OGD insult. Cultures depleted of microglial cells displayed an increase in OGD-induced degeneration of CA1 pyramidal cells (and dentate granule cells). Since triflusal prevented the increased, OGD-induced neuronal cell death in the microglia-depleted cultures, we moreover conclude that the neuroprotective effect of triflusal to a large extent is independent of microglia.

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