Research Report

Immunotoxic depletion of microglia in mouse hippocampal slice cultures enhances ischemia-like neurodegeneration

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ABSTRACT

Microglial cells have been attributed both neuroprotective and neurodegenerative roles in cerebral ischemia. This study presents an immunotoxic method for depletion of microglia from mouse hippocampal slice cultures and the effect of this on ischemia-like oxygen-glucose deprivation (OGD). For microglial depletion hippocampal slice cultures were exposed for 7 days to saporin coupled to an antibody against the microglial receptor Mac1 (Mac1-sap). When subjected to OGD immediately thereafter, resulting neurodegeneration was quantified as cellular uptake of propidium iodide (PI). Cultures were processed 1, 7 or 14 days after OGD for general cell staining and immunohistochemistry for neuronal, astroglial and microglial markers. Following Mac1-sap treatment there was a near total loss of microglia, these microglia-depleted cultures displayed a significant increase in PI uptake and astrogliosis 1 day after OGD compared to non-depleted cultures. In cultures surviving 7 and 14 days after OGD there was a decrease in PI uptake compared to 1 day after OGD. At 7 and 14 days after OGD the differences in Mac1-sap treated or non-treated cultures were still noticeable in terms of more neuron loss in cultures deprived of microglia, while the astrogial reactivity seemed to equalize. Based on the finding that depletion of microglia significantly increased OGD-induced CA1 pyramidal cell degeneration, we conclude that microglia at least in the initial phase of injury exert a neuroprotective role in mouse hippocampal slice cultures.

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1. Introduction

Microglial cells, now known to contribute about 12% of the cells in the brain, were first described by del Río-Hortega (1932). Acting as "biosensors" for homeostatic regulation in normal and pathological conditions, microglial cells play a key role in the central nervous reactions to injury, including immune surveillance, extracellular fluid cleansing and cellular debris removal (Dheen et al., 2007; Schloendorn et al., 2007).

Resting microglial cells in the adult brain have fine ramified processes, but transform into a non-phagocytic activated phenotype, or a phagocytic activated phenotype when activated (Streit et al., 1999). The normal thin processes then transform into shorter coarse processes (Jensen et al., 1997; Streit et al., 1999), in parallel with the upregulation of normal markers like CD11b (or Mac1) (Perry et al., 1985), MHC class I and II molecules (Kato et al., 1995) and secretion of cytokines like interleukin-1beta (IL-1β) (Buttini et al., 1994), transforming...
growth factor beta-1 (TGF-β1) (Lehrmann et al., 1998) and tumor necrosis factors-alpha (TNF-α) (Buttini et al., 1996). The reactions to injury also include a drastic increase in the number of microglial cells at the lesion site, due to migration and local proliferation (Neumann et al., 2006).

In relation to microglial activation, it is still not clear to what extent or under which conditions activated forms of microglia exert positive or negative effects on neuronal survival (Li et al., 2007). Following activation microglial cells have thus been reported both to increase neuronal cell death through release of neurotoxic substances like glutamate, toxic cytokines and nitric oxide among others (Chao et al., 1992; Piani et al., 1992; Viviani et al., 1998), and to secrete trophic and local proliferation (Neumann et al., 2006). Microglial cells might help elucidate the role of microglial cells. For this purpose, microglial cells were successfully targeted and eliminated in mouse hippocampal slice cultures by exposure to the ribosome inactivating protein saporin coupled to an antibody towards the microglialy expressed Mac1 receptor (Mac1-sap). When normal and microglia-depleted hippocampal slice cultures thereafter were subjected to 30 min of oxygen–glucose deprivation (OGD), the microglia-depleted cultures displayed an enhanced neuronal degeneration.

2. Results

2.1. Microglia depletion by Mac1-sap treatment

Microglial cells are well represented and display an in vivo-like appearance and distribution in mouse hippocampal slice cultures (Fig. 1A). In order to define parameters for Mac1-sap induced microglia depletion, mouse hippocampal slice cultures grown for 7 days, were exposed to 1.3 nM Mac1–sap, added to the culture medium for 3 or 7 days, thereafter the cultures were immunostained for the microglial marker Mac1 or stained histochemically for microglial tomato lectin (TL) binding. Cultures exposed to Mac1-sap for 3 DIV displayed partial depletion of microglial cells (Fig. 1B), while cultures exposed for 7 DIV displayed an almost total loss of microglia (Fig. 1C). The remaining cells staining for Mac1 or tomato lectin displayed an activated amoeboid morphology with few and coarse processes (see inserts in higher magnification in Figs. 6F and N), while microglial cells in non-depleted control cultures, displayed the usual highly ramified morphology (Fig. 2), signifying a non-activated state. Cells visualized by tomato lectin histochemistry displayed the same cell morphology and appeared in equal numbers as the Mac1-positive cells in both the normal and the microglia-depleted cultures (Figs. 2C and D).

For evaluation of the possible effect of the microglial deprivation on slice culture neurons, both non-depleted (control) and microglia-depleted cultures were immunohistochemically stained for neuronal nuclear protein, NeuN, and MAP2 as markers for neuronal nuclei and dendrites, respectively. As in regular cell staining (Figs. 6A and E) both control and microglia-depleted cultures displayed a well-preserved CA1 pyramidal layer with healthy looking nuclear morphology (NeuN; Figs. 7A and E) and normal density, and appearance of dendritic processes (MAP2; Figs. 7J and M). Treatment with Mac1-sap for 7 days did, accordingly not appear to induce degeneration of CA1 pyramidal neurons judged from cultures analyzed 2 weeks after the immunotoxic treatment (Figs. 7E and M), even that some MAP2-immunoreactive CA1 pyramidal cell bodies were observed (Fig. 7M).

Immunohistochemical staining of 2-week-old mouse hippocampal slice cultures for the astroglial marker glial fibrillary acidic protein (GFAP) revealed healthy looking, star-shaped astroglial cells in and around the CA1 pyramidal cell layer (Fig. 8A). In cultures exposed to Mac1-sap for 7 days and then grown for additional 7 days the cellular organization of astroglial cells appeared slightly disturbed, in particular with regard to the orientation of process (Fig. 8E).

2.2. OGD-induced PI uptake

Focusing on OGD-induced degeneration of CA1 pyramidal cells, regular non-microglia-depleted cultures subjected to 30 min OGD displayed a significant increased PI uptake (set to 100%) when compared to non-OGD control cultures (Fig. 3). Basic PI uptake in normal (Control, Fig. 3) and microglia-depleted cultures (Control Mac1-sap, Fig. 3) was about one third (34±2% and 35±2%, respectively) of that induced by OGD in non-depleted cultures (OGD, Fig. 3). Microglia-depleted cultures exposed to OGD did however show a 77% increase of PI uptake (177±8%) (p<0.001) compared to regular cultures exposed to OGD (100%). In non-depleted cultures, allowed to survive for 7 days after OGD, the PI uptake in CA1 was reduced
Fig. 2 – Immunotoxically depletion of microglial cells in mouse hippocampal slice cultures treated with Mac1-saporin for 7 days. A and C: Microglial cells are well represented in normal mouse hippocampal slice cultures, assessed by Mac1 immunostaining (A) and tomato lectin histochemistry (C). B and D: Treatment for 7 DIV with Mac1-sap, just before histological processing after 2 weeks in culture, has eliminated almost all microglial cells. The few microglial cells remaining display an abnormal morphology as observed by Mac1 staining (B) and TL histochemistry (D).

Fig. 3 – Densitometric measurements of PI uptake in CA1 pyramidal layer of non-submersed, regular (control) and Mac1-sap treated cultures (control Mac1-sap) as well as corresponding cultures 24 h, 7 days and 14 days after OGD. OGD-induced neuronal cell death in regular cultures (OGD) was standardized to 100%. Cultures treated with Mac1-sap before exposure to OGD (OGD-sap) displayed a significant 77% increase in PI uptake compared to regular OGD cultures (OGD). At 7 days after OGD the cellular uptake of PI continuously present in the medium, was reduced from 100% to 70% in regular cultures and 177% to 87% in microglia-depleted cultures, but the levels at this stage and 14 days after OGD were not significantly different between regular and depleted cultures. Data are shown as a percentage of PI uptake standardized to OGD (n=11–18) (**p<0.001; n.s.: not significant).
to 70±4% of the uptake recorded 1 day after OGD, while it fell from 177% to 88±5% in corresponding microglia-depleted cultures. Fourteen days after OGD, PI uptake values were 57±5% for regular cultures and 73±6% for microglia-depleted cultures (Figs. 3 and 4).

2.3. Neuronal degeneration in toluidine blue cell staining

The OGD-induced neurodegeneration was confirmed by microscopical analysis of toluidine blue stained sections of control and OGD-lesioned cultures. In regular non-OGD cultures without (Fig. 5A) and with microglia depletion (Fig. 5E) the structural integrity of the CA1 pyramidal cell layer was well preserved, displaying the usual widening of the layer compared to the in vivo appearance. One day after OGD both microglial depleted and non-depleted cultures displayed a marked increase in number of pyknotic CA1 pyramidal cell nuclei (Figs. 5B and F), which gradually disappeared during the 1 (Figs. 5C and G) and two weeks (Figs. 5D and H) after OGD. Although not quantified over time, pyknotic nuclei appear to persist longer after OGD in the microglia-depleted than in the regular cultures, corresponding to the persistence of PI in these cultures (see above).

Fig. 4 – Fluorescent micrographs of PI uptake in control and OGD-lesioned mouse slice cultures with normal microglial content (A–D) and prelesional depletion of microglia (E–H). Comparison of panels A and E revealed a slight increase in PI uptake in the microglia-depleted cultures, represented by small dots corresponding to dying microglial cells (E). Most extensive PI uptake (cell death) after OGD was observed in the CA1.

Fig. 5 – Toluidine blue cell staining of CA1 pyramidal cell layer in normal and Mac1–sap microglia-depleted mouse hippocampal slice cultures with and without OGD-induced neurodegeneration. Control cultures without (A) and with microglia depletion (E) displayed a healthy pyramidal cell morphology. OGD of corresponding sets of cultures induced an increase in pyknotic nuclei in the CA1 pyramidal cell layer 1 day after OGD in microglial non-depleted (B) and depleted cultures (F). Some pyknotic cells were still present 7 (C, G) and 14 days (D, H) after OGD.
2.4. **OGD-induced neurodegeneration demonstrated by NeuN and MAP2 immunostaining**

Microscopical analysis of the structural integrity of neurons after immunohistochemical staining for NeuN and MAP2 confirmed the patterns of OGD-induced neurodegeneration revealed by PI uptake (Fig. 4) and general cell staining (Fig. 5). Regular hippocampal slice cultures exposed to 30 min of OGD and histologically processed 24 h later thus showed clear signs of neuronal degeneration in the CA1 pyramidal layer in terms of darkly stained, highly condensed NeuN-immunoreactive cell nuclei (Fig. 7B), as well as almost a total loss MAP2 stained dendrites in stratum radiatum (Fig. 7J). The same was observed in corresponding microglia-depleted and OGD-lesioned cultures (Figs. 7F and N). In both regular and microglia-depleted cultures analyzed 1 week after OGD (Figs. 7C and G) the loss of CA1 pyramidal cells had progressed from 24 h after OGD with stabilization of the picture and normalization of the NeuN immunoreactivity of the surviving cells after 14 days (Figs. 7D and H). In accordance with the PI uptake, the general loss of CA1 pyramidal cells was, however, more severe in the microglia-depleted than the regular cultures (cp. Figs. 7A–D and E–H). Regarding dendritic MAP2 staining, both regular and microglia-depleted, OGD-lesioned cultures maintained a loss at 7 and 14 days after OGD. Even with some reappearance of structured dendritic staining at 7 and 14 days...
after OGD, the loss was more severe in the microglia-depleted cultures (cp. Figs. 7K–O and L–P).

2.5. Microglia reactions to OGD in regular and microglia-depleted cultures

As shown in Figs. 1 and 2, Mac1-sap treatment for 7 days very effectively deprived the cultures of microglial cells.

One day after OGD of regular cultures the CA1 region and the CA1 pyramidal cell layer in particular, displayed a significant increase in reactive, densely stained and amoeboid microglial cells compared to controls both when visualized by Mac1 immunostaining (cp. Figs. 6A and B) and tomato lectin histochemistry (cp. Figs. 6I and J). Corresponding cultures depleted of microglia during the week before OGD also displayed an increase in Mac1- and TL-positive microglial cells in CA1 compared to the corresponding controls (cp. Figs. 6E and F, and N and M). Virtually all of these newly appearing, but still few microglial cells displayed an activated amoeboid morphology (Figs. 6F and N). One week after OGD, the CA1 pyramidal cell layer of regular, OGD-lesioned cultures, still contained an above normal number of microglial cells with amoeboid morphology, but most microglial cells had reverted towards a non-activated phenotype (Figs. 6C and K). In microglia-depleted cultures processed 1 week after OGD lesioning slightly more stained microglial cells had appeared compared to 1 day after OGD. Most of the cells now displayed a ramified morphology (Figs. 6G and O), providing a picture similar to the one observed in the regular cultures 7 days after OGD, except that the number of cells was smaller (cp. Figs. 6C and G). Two weeks after OGD, both regular and microglia-depleted cultures showed a general increase in ramified microglia (Figs. 6D, H, L and P), the only difference being the lower number of cells in the microglia-depleted cultures (Fig. 7).

Fig. 7 – NeuN (A–H) and MAP2 (I–P) immunohistochemistry of mouse hippocampal slice cultures, subjected to OGD with 1, 7 and 14 days postlesion survival. One day after OGD there was a significant increase in pyknotic nuclei (B) in the CA1 pyramidal cell layers (B, F) with corresponding loss of MAP2 staining in stratum radiatum (N) of CA1 (J, N). Both NeuN staining of neuronal nuclei and MAP2 staining of primarily neuronal dendrites confirmed the more severe loss of CA1 pyramidal cells in microglia-depleted cultures, both 24 hours after, and 1 and 2 weeks after OGD.
2.6. Astroglial reactions to OGD in regular and microglia-depleted cultures

Twenty-four hours after OGD, regular hippocampal slice cultures displayed a typical reactive astrogliosis in CA1 with increased GFAP-reactivity and enlargement of astroglial processes (Fig. 8B). Corresponding reactions were observed in microglia-depleted cultures, where the slightly disturbed astroglial organization turned into an almost normal looking astrogliosis in CA1 with the only noticeable abnormality being more irregularly oriented and slender processes (Fig. 8F). One week after OGD, both regular and microglia-depleted cultures maintained the significant increase in GFAP-positive astroglial cells with prominent processes and increased soma size, including the astroglial cells in the CA1 pyramidal layer (Figs. 8C and G). Two weeks after OGD, both types of cultures still displayed strong astrogliosis in CA1 (Figs. 8D and H), leaving the impression that the regular cultures displayed the most dense gliosis, while the astroglial cells in the microglia-depleted cultures displayed a less regular organization in terms of orientation of their processes.

3. Discussion

By this study we have established an easy and efficient way to deplete microglial cells from mouse hippocampal slice cultures.
cultures, and shown that such microglial deprivation enhanced OGD-induced, CA1 pyramidal cell degeneration.

3.1. Slice cultures

Mouse hippocampal slice cultures grown by the interface membrane method were used in all experiments. With this method, slices of developing brain tissue can be grown for weeks with preservation of its basic organotypic cellular and connective organization and preservation of normal electrophysiological properties (Gahwiler, 1984; Stoppini et al., 1991; Noraberg et al., 1999). Such cultures, are widely used for studies of neurodegeneration and toxicity in the brain, due to easy inspection and precise control of the extracellular environment (see Noraberg et al., 2005). Further advantages include preservation of a 3-dimensional structure where basic neuron–neuron and neuron–glia interactions are present (Zimmer and Gahwiler, 1984; Gahwiler, 1988; Frotscher et al., 1990; Gahwiler et al., 1997; Blaabjerg et al., 2003).

Here mouse hippocampal slice cultures were used to establish a method for immunotoxic depletion of microglia from the cultures by addition of a Mac1 antibody–saporin complex to the culture medium, followed by investigation of the effects of such microglial depletion on ischemia-like neurodegeneration induced by controlled oxygen–glucose deprivation.

3.2. Microglia depletion

Following a series of pilot experiments it was found that the immunotoxic Mac1 antibody–saporin complex (Mac1–sap) effectively removed microglia from mouse hippocampal slice cultures when they applied via the culture medium during the second week in culture. The depletion was confirmed by visualization of microglial cells by both Mac1 immunohistochemistry and tomato lectin histochemistry. Validation by means of Tomato lectin binding was important, as this method of visualization is independent of the Mac1 antigen–antibody immune reaction. Comparison of regular and microglia-depleted cultures (a) for neuronal cell death, as monitored by cellular uptake of PI, and (b) for general neuronal and astroglial content and organization by cell specific stains, provided no evidence that the depletion procedure was directly toxic to other cells, although some stress-like reactions were observed in terms of MAP2 immunostaining of CA1 pyramidal cell bodies (Noraberg et al., 1998) and slightly increased in astroglial GFAP staining together with some disturbances of orientation of astroglial processes.

3.3. OGD lesions

By subjecting 2-week-old, regular and microglia-depleted hippocampal slice cultures to standardized OGD, CA1 pyramidal cell death, as observed 1 day after OGD, was found to be significantly increased in the microglia-depleted cultures, suggesting a neuroprotective role of the normal content of microglia. The cellular PI uptake at 7 and 14 days after OGD, was clearly reduced as compared to 1 day after OGD in both regular and microglia-depleted cultures. Changes in PI uptake recorded at various time points after OGD are likely to include both removal and dissolution of already dead, PI-labeled cells and addition of newly degenerated cells through secondary or delayed degeneration. Occurrence of newly degenerated, PI-labeled cells within an area with existing PI-labeled cells is difficult to demonstrate, and has to be proven by detailed cell counts and with reference to estimates of total cell numbers present before degeneration, or quantification of living and dead cells present at the different postlesional stages. Overall decrease in PI-labeled cells can, on the other hand, be taken as evidence of clearance within the tissue of once dead and thereby PI-labeled cells. Seen in this perspective, the current data demonstrate that the capacity for clearance of dead, PI-labeled cells after OGD is at least as efficient, if not more, during the first week after OGD in the microglia-depleted cultures as in cultures with a regular content of microglia. For the second week the rates of clearance appeared very similar.

3.4. Roles of microglia in neurodegeneration

It is still debated whether and how microglia may exert beneficial or detrimental roles in relation to brain injury, including acute neurodegeneration. One argument supporting that microglia are actively involved in neurodegeneration is the lesion-induced release from microglia of cytotoxic agents capable of inducing cell death (Yenari et al., 2006). Dommergues et al. (2003) thus found that microglial activation enhanced excitotoxic lesioning in a mouse neonatal model of excitotoxicity. Also other in vivo observations support that microglia contribute to neuronal excitotoxicity (Campuzano et al., 2008). In vivo studies including ischemic and traumatic injuries have also shown that microglial activation actively contributes to neuronal cell death (Hailer, 2008). In contrast to these findings other studies have provided evidence for a beneficial (neuroprotective) role of microglial cells in acute brain injuries (Nakajima et al., 2001; Lu et al., 2005; Neumann et al., 2006) through release of factors known to promote cell survival and tissue repair (Polazzi and Contestabile, 2002; Streit, 2002). This includes TGF-β1 (Wesolowska et al., 2008) which may attenuate neuroinflammatory responses by inhibiting glial proliferating and expression of major histocompatibility class II (Suzumura et al., 1993). Depleting hippocampal slice cultures of microglial cells by the bisphosphonate clodronate, Kohl et al. (2003) reported an increase in NMDA-induced excitotoxic neurodegeneration, just as application of exogenous cell-line derived microglia has been reported to reduce the susceptibility of regular rat hippocampal slice cultures to OGD (Neumann et al., 2006).

Although hippocampal slice cultures are well suited for this kind of experiments due to easy access and preserved basic 3-dimensional organization and cell–cell interactions, they do, however, lack external immune and cell-mediated, inflammatory responses (Neumann et al., 2008), which may play a key role in pathological processes such as stroke or trauma. Having said that, in vivo systems do pose, however, also a problem for differentiating between and studying specific single elements, including potential differences in the roles and action of resident microglia and blood bone macrophages and neutrophils. Organotypic slice cultures might be of advantage here as only resident microglia, and some potentially persisting perivascular cells are present.
3.5. **Microglia and outcome of OGD lesions**

Mac1 immunohistochemistry and tomato lectin histochemistry were used to visualize microglial cells and evaluate their responses to OGD-induced neuronal cell death. In regular mouse hippocampal slice cultures grown for 2 weeks, ramified resting microglial cells could be observed both by Mac1 immunohistochemistry and tomato lectin staining, in agreement with the findings by Hailer et al. (1997). Correspondingly, as shown here, immunotoxic treatment of 1-week-old hippocampal slice cultures for 7 days with Mac1-sap effectively depleted the slice cultures of microglial cells. One day after OGD, the microglial cells in regular cultures both increased in number and staining density and underwent morphological changes from resting to activated microglia in the lesioned CA1 area. The increased number of microglial cells observed in the CA1 pyramidal layer might indicate that microglial cells besides of local cell division (Schloendorf et al., 2007) also migrated from neighboring areas into the lesion site in response to cytokines and other chemoattractant components, being released by the injured neurons (see Heppner et al., 1998). In microglia-depleted cultures, OGD induced a reoccurrence of Mac1- and TL-positive cells in CA1 24 h after OGD, meaning that the few visible Mac1- and TL-positive cells (and possibly some cells with staining below detection level) remaining after the immunotoxic treatment were still able to respond, possibly proliferate, and migrate to the CA1 pyramidal cell layer. Once activated and visible for analysis, the Mac1- and TL-positive microglial cells in the OGD-lesioned microglia-depleted cultures behaved like their counterparts in the regular OGD-lesioned cultures for the next 2 weeks. The increase in microglial cell numbers in the OGD-lesioned CA1 resulted in a virtual normalization of the microglial content, indicative of cell proliferation, and cellular appearance at 2 weeks after OGD, where most microglial cells displayed a resting state or almost resting state appearance.

For comparison of the lesion-induced and spontaneous reappearance of Mac1- and TL-positive microglial cells in Mac1-sap treated cultures, cultures subjected to microglial depletion only or depletion plus OGD were analyzed for microglial content and cellular appearance at corresponding time points after microglial depletion. Regarding OGD-induced CA1 pyramidal cell loss, microglial deprivation clearly enhanced neuronal degeneration, as shown by PI uptake and general and neuron-specific cell status like NeuN and MAP2. The difference between regular and microglial depleted cultures, determined by PI uptake, was largest (77%) at 24 h after OGD, being reduced to 20% and 28% at 7 and 14 days, respectively, after OGD (Fig. 3). In general terms it is reasonable to believe that ongoing neuronal degeneration and continuous presence within the tissue of dead or damaged neurons cannot be beneficial for recovery and regeneration. In this scenario elimination of such cells that have come to a point of no-return by microglia or astroglia might have a beneficial effect (Streit, 2002). As already mentioned above, there was however, no evidence of impaired clearance of dead (PI-labeled neurons) in the microglia-depleted cultures in this study.

Another explanation for the neuroprotective actions of microglia against CNS injuries is that in the lesion brain microglia gradually lose their functionality and their capacity to support neurons decreases, leading to a slow neuronal degeneration. This idea is reasonable since microglial cells are known producers of trophic factors like nerve growth factor (NGF) (Mallat et al., 1989) or basic fibroblast growth factor (bFGF) (Araujo and Cotman, 1992) as well as extracellular matrix molecules that are necessary for sustaining neuronal viability (Rabchevsky and Streit, 1997). This would be an explanation for the increased neuronal cell death observed after OGD in our microglia-depleted cultures.

As a response to brain insults, astroglial cells hypertrophy and proliferate, and this term is known as giving rise to reactive astrogliosis (Ridet et al., 1997). The precise interactions between astroglia and microglia in such conditions are, however, not well understood. Microglial release of IL1 has been shown to have an effect on astroglial cell proliferation (Kreutzberg, 1996). Also, microglia express extracellular matrix components such as laminin, the expression of which might be controlled by astroglia (Rabchevsky and Streit, 1997). In this study, OGD-induced CA1 pyramidal cell loss, induced a clear astroglial response, which at 1 day after OGD seemed more pronounced in the cultures deprived of microglia (Fig. 8), where the neuronal cell death was, on the other hand, enhanced. One week after OGD astroglial hypertrophy and increased GFAP immunoreactivity was still prominent in both regular and microglia-depleted cultures, while the astroglial reaction and cellular appearance in the microglia-depleted cultures 2 weeks after OGD showed signs of deviation in terms of more disorganized organization than in corresponding OGD-lesioned regular cultures.

3.6. **Concluding remarks**

This study has demonstrated that exposure for 1 week of mouse hippocampal slice cultures to a complex of the ribosome inactivating protein saporin and an antibody raised against the microglial receptor Mac1 efficiently can deplete cultures of microglia. By subjecting such microglia-depleted cultures to “experimental ischemia” by controlled oxygen-glucose deprivation, it was further demonstrated that microglia depletion significantly enhanced OGD-induced CA1 pyramidal degeneration. Since regular and microglia-depleted brain slice cultures are both easily accessible for addition of compounds and widely use as experimental models of CNS injury and disease, it is foreseen that microglia-depleted slice cultures can become a valuable tool in studies of microglial interactions with other cell types in the CNS, and microglia-mediated actions of for example anti-inflammatory compounds (Montero et al., 2009).

4. **Experimental procedures**

4.1. **Mouse hippocampal slice cultures**

Seven-day-old C57BL/6j mice were killed by instant decapitation, and the two hippocampi were isolated from the brain and cut in transverse slices at 350 μm by a McIlwain tissue chopper. The hippocampal slices were transferred to chilled...
concentration of 25 mM. After an initial 3049) with 25 mMD-glucose and 1 mML-glutamine (Sigma, Cat.
serum-free Neurobasal medium (Gibco BRL, Cat. No. 21103-
the medium was replaced by 1 ml of chemically defined,
time.
17504-010) and the cultures transferred to an incubator at
Corning, NY, USA) with 1 ml of culture medium in each well,
semiporous (0.4
hippocampal and meningeal tissues and placed randomly on
and placed in an incubator with 5% CO2 and 95% atmospheric
term effects of microglial depletion on other cell types. After
other control cultures were not manipulated at
medium was bubbled with a gas mixture containing 5% CO2
(2002), was used. The OGD medium consisted of 120 mM
NaCl, 5 KCl, 1.25 mM NaH2PO4·H2O, 2 mM MgSO4·7H2O, 2 mM
Mac1 (Advanced Targeting Systems) for 3 or 7 days, and then fixed for histology
(see below). Other cultures were grown for additional 7 or 14
days after the Mac1-sap exposure to examine possible long-
term effects of microglial depletion on other cell types. After
fixation in phosphate buffered 4% paraformaldehyde and
placement in 20% sucrose overnight, immunohistochemical
stainings for microglial cells, neurons and astroglial cells were
performed (see below).

4.3. Tomato lectin (TL) histochemistry for microglia

For visualization of microglial cells by tomato lectin binding
cryostat sections were thawed at room temperature and
washed in Tris buffered saline, pH 7.4 (TBS) and TBS with
triton X-100 (Sigma) before incubated for 1 day at 4 °C with
biontynilated tomato lectin (Lycopersicon esculentum; Sigma, L-
9389) diluted 1:100 in TBS. Following wash in TBS with
triton X-100, the sections were incubated with streptavidin
HRP (P0397, DakoCytomation), diluted 1:200 in TBS for 1 h at
room temperature, washed again in TBS with triton X-100,
TBS and in TB before diaminobenzidine (DAB)-based visual-
zation of HRP. After final washes in TBS and TB, and
rinsing in distilled water, the sections were dehydrated in
increasing concentrations of ethanol, cleared in xylene, and
coverslipped in DePex mounting medium.

4.4. Immunohistochemical staining for neurons, astroglia and microglia

Immunohistochemical staining for NeuN and microtubule-
associated protein 2 (MAP2) was used to visualize neurons,
glial fibrillary acidic protein (GFAP) for astroglial cells and
Mac1 for microglial cells. After thawing of sections at room
temperature, and wash in TBS with triton X-100 unspecific
staining was blocked with 5% goat serum (Dako, X 0907) for
Mac1, 10% sheep serum (Sigma S2263) for NeuN and MAP 2,
and 10% donkey serum (Chemicon S30) for GFAP all diluted
in TBS. The sections were then incubated for 2 days at 4 °C
with the primary antibodies, including mouse-anti NeuN
(Chemicon, MAB 377, diluted 1:500), mouse-anti MAP2
(Sigma, M 4403, diluted 1:1000), rabbit-anti GFAP (Dako, Z
0334, diluted 1:4000), and rat-anti Mac1 (Advanced Targeting
Systems, AB-N05, diluted 1:600) and then washed in TBS
with triton X-100 before incubated for 1 h at room
temperature with the secondary biontynilated antibodies
(goot anti-rat antibody (RNP1005V, Amersham Pharmacia
Biotech) for Mac1; sheep anti-mouse antibody (RNP1001,
Amersham) for NeuN and MAP2, and donkey anti-rabbit
antibody (RNP1004, Amersham) for GFAP; all diluted 1:200).
Following wash in TBS and TBS with triton X-100, sections
were incubated for 1 h with streptavidin HRP (P0397,
DakoCytomation) diluted 1:200 in goat serum for Mac1,
sheep serum for NeuN and MAP 2, and donkey serum for
GFAP, and washed again in TBS before diaminobenzidine
(DAB)-based visualization of HRP. After final wash in TBS
and distilled water followed by dehydration in increasing
concentrations of ethanol, sections were cleared in xylene
and coverslipped in DePex mounting medium.

4.5. Induction of ischemia-like oxygen–glucose deprivation (OGD)

For oxygen–glucose deprivation (OGD) the submersion pro-
tocol of Frantseva et al. (1999), as modified by Bondé et al.
(2002), was used. The OGD medium consisted of 120 mM
NaCl, 5 KCl, 1.25 mM NaH2PO4·H2O, 2 mM MgSO4·7H2O, 2 mM
CaCl2, 25 mM NaHCO3, 20 mM HEPES and 25 mM sucrose. For
depletion of O2 to a pressure measured to be close to 0 the
medium was bubbled with a gas mixture containing 5% CO2
and 95% N2. After removal of the normal medium and a
single wash of the culture well with the OGD medium, 1 ml of
this was placed below the semiporous membranes and 1 ml on top of the membrane to cover the hippocampal
culture slices. The culture trays were then wrapped in
airtight plastic foil and placed in the incubator at 36 °C for
30 min, a time period known from previous experiments to
induce 50% cell death in hippocampal CA1 pyramidal cells
(Montero et al., 2007). Cultures serving as sham-OGD
controls were handled and submersed in medium in the
same way as OGD cultures, using a non-oxygen deprived
"OGD medium" containing 25 mM glucose instead of
sucrose. Other control cultures were not manipulated at
any time, except for transfer to Neurobasal medium and
placement at 36 °C (controls). Following submersion, OGD
and sham-OGD cultures were washed once with Neurobasal,
transferred to culture trays with Neurobasal medium, and
returned to the CO2 incubator at 36 °C, where they remained
for 1, 7 or 14 days. The post-OGD medium, containing
propidium iodide for monitoring cell death (see below), was
changed twice a week until fixation in 4% paraformaldehyde
(PFA) and histological processing.
4.6. Quantification of cell death by propidium iodide (PI) uptake

Spontaneous (basic) and OGD-induced cell death was determined by densitometric measurement of the cellular uptake of the fluorescent dye propidium iodide (PI, Sigma). PI was added to the medium of control and experimental cultures 24 h before OGD to yield a 2 μM concentration. Fluorescence microscopy recordings of basic, pre-OGD uptake of PI was done immediately before submersion of cultures for OGD and at 24 h and 7 or 14 days after OGD, using a tetramethyl rhodamine isothiocyanate filter (510–560/590 nm) and a Sensys KAF 1400 G2 (Photometrics, Tucson, AZ) digital camera (Noraberg et al., 1999). Corresponding recordings were done from respective series of control cultures. The combined density and extent of PI uptake in the CA1 and CA3 pyramidal cell layers, the dentate granule cell layer and the sum of these were quantified densitometrically, using NIH Image software (version 1.64).

4.7. Determination of ET_{50} for OGD

In initial studies 2-week old mouse hippocampal slice cultures were exposed to OGD for 10, 20, 30, 45, 60, 90 and 120 min, followed by the assessment of the induced neuronal degeneration after 24 h (see below), in order to determine the duration of OGD needed to induce a 50% loss of CA1 pyramidal cells. This ET_{50} value was found to be 30 min (data not shown).

4.8. Protocol for OGD of normal and microglia-depleted cultures

Four-week-old normal and microglia-depleted hippocampal slice cultures, derived from newborn (P-7) C57BL/6J mice were treated according to the experimental protocol shown in Fig. 9. Regular hippocampal slice cultures were grown in medium-containing serum for 13 days, thereafter the cell death marker PI was added to Neurobasal medium for 24 h to detect basic neurodegeneration, before subjection of cultures to OGD. Cultures immunotoxically depleted of microglia by Mac1-sap treatment during the second week in culture were tested for basic PI uptake just before OGD and 24 h, 7 days, or 14 days after OGD. For histology, cultures were fixed in phosphate buffered 4% paraformaldehyde and frozen after placement in 20% sucrose overnight for cryoprotection. The different groups of cultures were then cryostat sectioned in three parallel series at 20 μm. One series was stained with toluidine blue for cell staining to evaluate the general morphology of the slice cultures, while sections from the other series were immunostained for neuronal and glial markers (see above).

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