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Microglial activation and recruitment, but not proliferation, suffice to mediate neurodegeneration

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Abstract

Microglial activation occurs during excitotoxin-induced neurodegeneration. We have reported that microglia can exhibit neurotoxic behaviors after injection of excitotoxins into the hippocampus. It is not known, however, whether microglial proliferation, which is part of the activation response, is required for neurodegeneration to be observed, or whether activation of the pre-existing resident microglia suffices. Using osteopetrotic (*op/op*) mice, in which injury-induced microglial proliferation does not take place, we demonstrate that only the microglia initially residing in the CNS are adequate to promote neurodegeneration. Our data suggest that there is a threshold at which a maximal microglial contribution to neurotoxicity is observed. This threshold appears to be sufficiently low, such that activation of just 40% of the microglia present in wild-type mice serves to trigger neurodegeneration. Furthermore, since the decrease in microglial numbers coincides with a decrease in tissue plasminogen activator's activity, we suggest that tissue plasminogen activator can be used as a marker for microglial proliferation.

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Abbreviations: tPA, tissue plasminogen activator; op, osteopetrotic; CSF-1, macrophage colony stimulating factor

Introduction

When an excitotoxin is introduced into the mammalian hippocampus, neuronal death soon follows. This neuronal injury is accompanied by activation of the resident microglial cells.[1] Onset of the microglial activation process is followed by their migration to the site of injury, local proliferation, changes in gene expression, presentation of class II major histocompatibility antigens, and phagocytosis. We and others have shown that microglia can exhibit neurotoxic properties when neuronal injuries are elicited by local injections of excitotoxins,[1,2] autoimmune inflammation,[3] or ischemia.[4,5] Cytokines and neurotoxins secreted by microglia may help regulate the responses to injury in the central nervous system.[6–8]

In mice deficient for tissue plasminogen activator (tPA), a secreted serine protease normally present in the brain, hippocampal neurons are resistant to excitotoxic glutamate analogs and the microglia display attenuated activation.[9] This attenuation is evident both as a decrease in the number of activated microglia present and as a decrease in the levels of expression of various microglial markers and cytokines.[10] Evidence in wild-type mice suggests that additional tPA is generated by microglia shortly after their activation and this tPA contributes to the ensuing neurotoxicity.[2] Accordingly, strategies to decrease the amount of tPA released for the purpose of achieving neuroprotection could include blocking microglial activation, and specifically microglial recruitment or proliferation. In this study, we describe experiments designed to evaluate if a decrease in the number of activated microglia reduces excitotoxic neural injury using the mutant osteopetrotic (*op/op*) mouse.

Op/op is a spontaneous mutation in C57Bl6 mice that results in a deficiency of the macrophage colony stimulating factor (CSF-1) gene product. A single base pair insertion within this gene causes a frameshift mutation and creates a truncated protein that is non-functional.[11] In cell culture, microglia isolated from *op/op* mice do not proliferate unless the medium is supplemented with CSF-1.[12] Furthermore, microglial proliferation is dramatically reduced in the nucleus of the transected facial nerve after axotomy in *op/op* mice.[13] However, the microglia that were present following axotomy in *op/op* mice displayed similar changes in morphology and gene expression as control *op* heterozygous (*op*/+) mice, indicating that they do respond to activation signals from injured neurons. Witmer-Pack *et al*. reported that microglial cells are not completely dependent on CSF-1, since they can be found in the brain.[14] Quantitatively, it has been reported that during cerebral cortical ischemic lesion the numbers of microglia per mm^2 on the contralateral side (which would also represent normal, uninjured numbers of microglia in that region) are similar between wild-type and *op/op* mice. However, around the injury side, the numbers of microglia remain unchanged in *op/op* mice, whereas those of wild-type animals increase by 2.9 fold.[15]

Accordingly, the *op/op* mouse offered us the opportunity to determine if the proliferation aspect of microglial activation plays a critical role in microglial-mediated excitotoxic neurodegeneration.

Results

Decreased proliferation but wild-type-like morphological activation of *op/op* microglia after kainic acid injection

After injection of kainic acid into the hippocampus of control (C57/Bl6) mice, microglial cells undergo activation. The kinetics of this activation process have been determined.[1] Although the process of microglial activation begins shortly after injury, maximum levels of microglial activation, as evidenced by dramatic morphological changes, are reached between 5 and 14 days after the injection.[1] Therefore we chose to examine the levels of microglial activation in *op/op* mice (identified by genotyping as described in Materials and Methods, Figure 1) 5 days after kainate injection when the morphological changes are most evident. To identify activated microglia, immunohistochemistry using the monoclonal antibodies F4/80 (Figure 2) and Mac-1 (data not shown) was performed on sections from control and *op/op* mice. Microglia in *op/op* mice acquire the morphological characteristics of activation after kainate injection (compare Figure 2E and F). However, only $\sim 30-40\%$ of the number of activated microglia are observed in the hippocampus of *op/op* mice in comparison to control mice (see Table 1), in agreement with the literature.[15] To further assess the relative quantity of activated microglia in *op/op* and control mice we evaluated the levels of TNF-α present in brain lysates from each genotype by Western blot analysis (data not shown). TNF-α is a cytokine that is produced by microglia as part of the activation process. Kainate-injected brain lysates from *op/op* mice contained 48% of the amount of TNF-α present in lysates from control mice. These quantitative data agree with the qualitative observation that smaller numbers of activated microglia are present in the injured hippocampus of *op/op* mice.

The migration of microglia in the *op/op* mice to the injury site was comparable to that of microglia in control mice (note the presence of activated microglia in the CA1 hippocampal subfield mainly in the injected sides for both genotypes, Figure 2A and B). Even at higher magnifications (Figure 2E

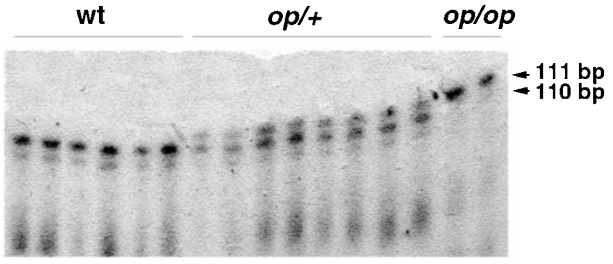

Figure 1 PCR-genotyping of *op/op* mice. Wild-type (C57/Bl6), *op*/+, and *op/op* mice were genotyped from tail DNA by a one-step PCR reaction as described in Materials and Methods. The PCR products were analyzed on a 6% polyacrylamide gel and visualized by autoradiography. C57/Bl6 mice amplify a single 110 base pair product (see arrow), *op*/+ mice amplify 110 and 111 base pair products, and the *op/op* mice amplify a single 111 base pair product (see arrow)

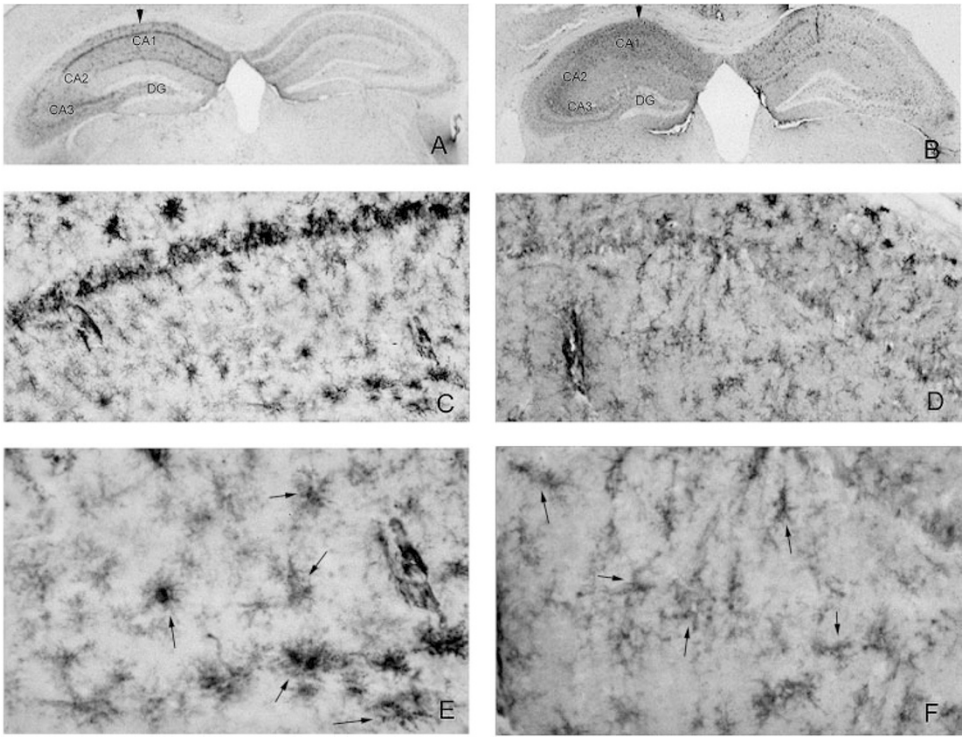

Figure 2 Microglial proliferation is deficient after excitotoxic injury, but microglial activation proceeds normally in *op/op* mice. Kainate was injected unilaterally into the hippocampi of control (*op*/+; *n*=11) and *op/op* mice (*n*=12) to induce microglial responses (arrows indicate sites of injection on **A** and **B**). Control mice respond with activation of their microglia on the injected side (**C** and **E**). Such activation also includes a strong microglial proliferative response (seen best in **C**). While the proliferation of microglia in the injected side of the hippocampus of the *op/op* mice is defective (seen best in **D**), a higher powered photomicrograph indicates that the microglia that migrate to the site of injury have the characteristics of activated microglia (**D** and **F**). The hippocampal subfields CA1, CA2 and CA3 as well as the dentate gyrus (DG) are labeled

and F), the microglia in the control and *op/op* mice appeared similar aside from minor morphological differences visualized using F4/80 staining, as reported previously.[16]

To evaluate the levels of microglial proliferation after excitotoxin injection, we used double immunofluorescence against F4/80 (detecting microglia) and BrdU (for proliferating cells). As shown in Figure 3, microglial proliferation was clearly evident in wild-type mice (wt), as well as in tPA$^{-/-}$ ones (even though the overall number of microglial cells was significantly reduced). Proliferation was not observed for F4/80^{+} microglia in *op/op* mice, as was earlier reported.[15] Therefore, in this excitotoxin-injection model, microglia in *op/op* mice display the phenotypic characteristics of microglial activation and are recruited to the sites of injury but do not mount a proliferative response (as evident by the presence of fewer activated F4/80^{+} cells and the observation that the F4/80^{+} cells are not BrdU labeled). This observation is in agreement with the data obtained in the facial nerve axotomy model.[13]

Pyramidal neurons in *op/op* mice undergo excitotoxic cell death

We previously showed that the microglial activation normally observed in wild-type mice following kainate injection is significantly attenuated in tPA-deficient mice.[9] Furthermore, the pyramidal neurons in tPA$^{-/-}$ mice, as well as in wild-type mice in which microglial activation has been delayed by macrophage-microglial inhibitory factor,[2] are resistant to excitotoxin-induced neuronal death. To evaluate whether eliminating the proliferative component of the microglial response was sufficient to confer protection against neuronal cell death, we assessed neuronal survival in control (*op*/+) and *op/op* mice after the unilateral injection of kainate. As seen in Figure 4, the pyramidal hippocampal neurons were sensitive to excitotoxin injection in both genotypes, suggesting that there were sufficient activated microglia present in *op/op* mice to mediate neuronal death.

Combined with the results above, these data suggest that although microglia in *op/op* mice are unable to respond to CSF-1 by proliferating, they can respond to the signals produced by injured neurons to cause their activation and ultimately promote neuronal death.

Table 1 Decreased numbers of activated microglia and lower levels tPA activity in the brains of *op/op* mice

Genotype	Activated microglia in CA1 $\pm$ s.d.*	tPA (ng/μg protein $\pm$ s.d.)**
Control	73 $\pm$ 7	4.63 $\pm$ 0.01
op/op	26 $\pm$ 10	1.99 $\pm$ 0.21

Activated microglia in the CA1 region of kainate-injected control (C57/B16) and *op/op* mice were counted as decribed in the text. Activated microglia were identified based on intensity of immunostaining by F4/80 and characteristic morphological changes. The data are presented as the average number of microglia present in four microscopic fields from cryostat coronal 14 μm sections of brain around the injection site. Few if any activated microglia were present in the non-injected side in both control and *op/op* mice. Brains from control (C57/B16) and *op/op* mice were homogenized, as listed in the Materials and Methods section, and the levels of tPA activity were measured by the amidolytic assay at 30 and 90 min, 12 and 24 h. tPA activity was calculated from initial rates in the amidolytic assay. Extracts from tPA$^{-/-}$ brains were used as negative controls. *$P<0.01$, value associated with Student's two-tailed *t*-test. **$P<0.05$, value associated with Student's two-tailed *t*-test

Decreased levels of tissue plasminogen activator in *op/op* mice

Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of the zymogen plasminogen to the active protease plasmin. Both neurons and microglia in the mouse brain express this protease. The synthesis of tPA is rapidly upregulated after microglial activation in cell culture. We have previously shown that tPA can promote excitotoxic cell death in the mouse brain and suggested that secreted microglial-derived tPA may contribute to the neurotoxic properties of these cells.[2] Given that the mutation that causes to the *op/op* phenotype results in defective proliferation of microglial cells only (but does not affect the numbers of neurons), we performed both zymographic and amidolytic assays to determine the levels of tPA. As shown in Table 1, there is a significant difference between the levels of tPA in the kainate-injected brains of control mice (4.63 ng of active tPA/μg of brain extract), and *op/op* mice (1.99 ng of active tPA/μg of brain extract) as demonstrated by the amidolytic assay. Similar differences in tPA levels were noted by zymographic analysis (data not shown). It is interesting to note in the *op/op* forebrain there is $\sim$43% tPA activity present compared to that of control mice, a number

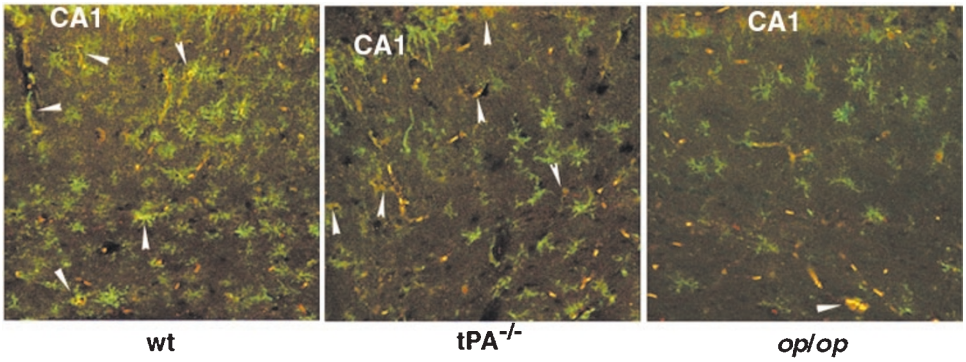

Figure 3 Microglial proliferation is nearly absent in *op/op* mice during excitotoxin-induced neuronal death. Microglial proliferation was evaluated by double immunofluorescence in wild-type, tPA-deficient and *op/op* mice on 14 μm cryostat coronal sections. Arrows indicate the presence of doubly labeled (proliferating) microglia

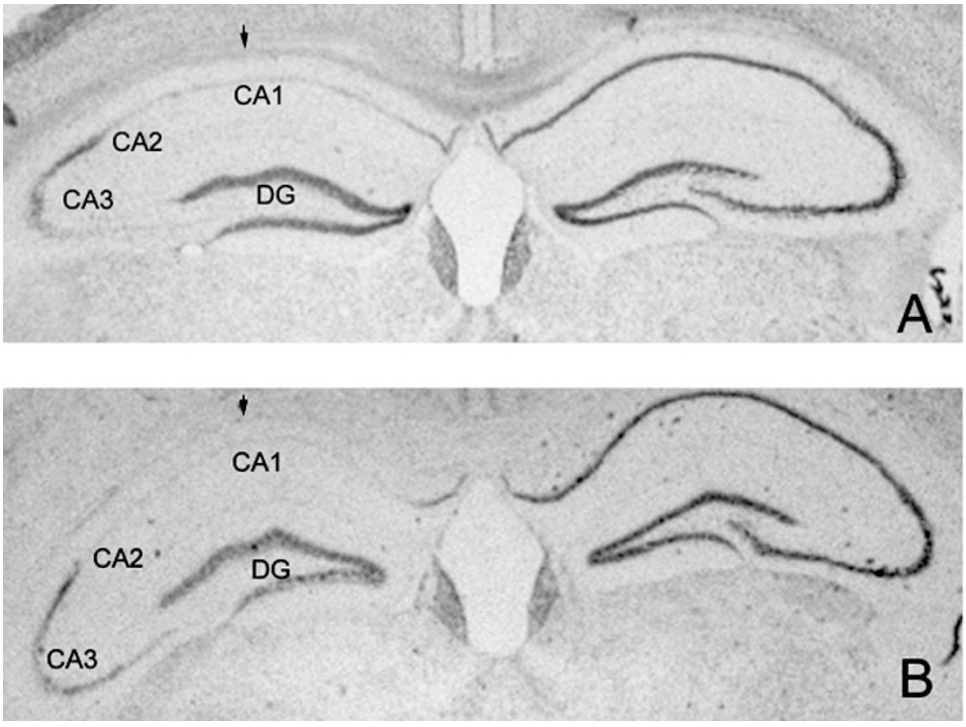

Figure 4 *Op/op* mice are sensitive to excitotoxin-induced neuronal death. The viability of pyramidal neurons in the hippocampus of injected control (*op*/+, *n*=11) and *op/op* mice (*n*=12) after unilateral intrahippocampal kainate injection was evaluated by cresyl violet staining. In kainate-injected *op/op* mice (**B**), the neurons at the injected side were eliminated to the same extent as those in control (**B**) mice. The arrows indicate the site of kainate injection. CA1, CA2 and CA3 indicate the hippocampal pyramidal subfields, and DG denotes the dentate gyrus of the hippocampal formation

close to the difference in activated microglia between the two genotypes (see Table 1). Since the amount of tPA correlated well with the numbers of microglia present in the injured hippocampus, and hippocampal neurons (the other producer of tPA in the mouse CNS parenchyma) were eliminated by the excitotoxin, we propose that tPA may be useful as a marker for microglial proliferation in the mouse forebrain.

Discussion

The *op/op* mouse carries a frameshift in the CSF-1 gene[11] that results in defective microglial proliferation both in cell culture and *in vivo*.[12,13] Previously, we reported that retarding microglial activation in response to excitotoxin injection could protect against neuronal death.[2] This suggested a neurotoxic role for microglia in the mouse brain during excitotoxicity. However, it was uncertain which component(s) of microglial activation is necessary for excitotoxin-mediated cell death. We set out therefore to ascertain whether impairment of microglial proliferation would confer protection from neuronal death, or if the initial number of activated microglia present would suffice to promote neuronal death. Intrahippocampal delivery of kainic acid into the brain parenchyma of the *op/op* mice resulted in neuronal cell death, a result consistent with the observed vulnerability of *op/op* cortical neurons to ischemic insult.[15] These results suggest that despite the diminished proliferative capability of microglia in *op/op* mice, the microglia that become activated are able to promote neurotoxicity and to phagocytose the debris of injured neuronal cells. In addition it is suggested that a threshold may exist for the number of activated microglial cells above which they cease to become a rate-limiting factor in this neurodegeneration pathway.

These neurotoxic properties of microglia may be the result of the up-regulation and secretion of several factors that these cells express, such as tPA and TNF-α.[10,17] Interestingly, at least TNF-α (and potentially tPA) lies at the beginning of potent signal cascades that can result in cell death.[18] This provides a rationale through which even a small number of activated microglia could provide the necessary downstream signals to effect neuronal cell death. We have already shown that the tPA/plasminogen proteolytic cascade promotes excitotoxic cell death.[19,20] Furthermore, microglial-derived tPA may initiate this cascade (C-J Siao, personal communication). The data presented here demonstrate that the number of activated microglia is diminished in the kainate-injected *op/op* mouse (36% of control), and these data correlate with the levels of decreased enzymatic activity of tPA in kainate-injected *op/op* mice (43% compared to those of control mice). This result further suggests that microglial tPA is a major source of tPA locally at the site of injury. The potential for signal amplification present in a proteolytic cascade initiated by tPA and plasmin may account for the strength of the neurotoxic effects of the few activated microglia in the *op/op* mouse. Moreover, we propose that tPA can be used as a marker for microglial proliferation following injury induced by kainate.

Additionally, TNF-α has been implicated to play a role in the neurotoxic properties which microglia possess.[21,22] TNF-α is secreted by activated microglia and can inhibit the re-uptake of glutamate by astrocytes, thereby allowing higher and potentially toxic concentrations of extracellular glutamate.[21] TNF-α lies at the beginning of a biochemical cascade that potentiates neuronal death following kainate injection.[18] In fact, we report here that there is approximately half the level of TNF-α present in the kainate-injected *op/op* mouse, yet excitotoxin-induced neurodegeneration proceeds normally. This may occur because TNF-α can stimulate the generation of IL-1β, IL-6 and other cytotoxic cytokines.[23] However, we have also previously shown that in tPA-deficient mice (where microglial activation is attenuated)[9] neurons are resistant to excitotoxicity and the microglia in these mice secrete 43% less TNF-α than wild-type mice in response to activation stimuli.[10] This amount of TNF-α is comparable to that secreted by *op/op* microglia. It is tempting to speculate that the different effects of kainate in tPA-deficient and *op/op* mice lie in the ability of activated microglia in *op/op* mice to produce sufficient tPA to cross a threshold and successfully initiate the biochemical cascades that lead to cell death.

Activated microglia have been implicated to play a role in several neuropathological conditions such as Alzheimer's disease, stroke and multiple sclerosis. Previously, Raivich *et al*.[13] reported that microglia in *op/op* mice underwent normal activation in the facial nerve axotomy model. In the present study we show that the activation of microglia, rather than their proliferation, is critical for effecting neuronal death. We and others previously demonstrated that microglia possess neurotoxic properties,[2,5,6,24] and that inhibition of microglial activation can be neuroprotective against kainate-induced neuronal injury and death. Taken together with the data presented here, we suggest that the toxic mediators, secreted by microglia after neuronal injury, are sufficiently potent that even decreased numbers of activated microglia are able to promote neuronal death. Therefore, any potential neuroprotective therapy involving suppression of microglial activation must take their potency into account.

Materials and Methods

Animal procedures

All experiments performed on mice were done in accordance to the NIH guide for the care and use of laboratory animals as well as the institutional guidelines set by the IACUC Committee and the Division of Laboratory Animal Research at Stony Brook. All efforts were made to minimize the use of animals and to ensure minimal suffering of those animals used.

Mice

The osteopetrosis (*op/op*) mouse arose form a spontaneous mutation in the CSF-1 gene in C57/Bl6 mice. This single base insertion within the coding region of CSF-1 leads to a truncated, non-functional protein.[24] Since homozygote *op/op* mice do not breed well, *op*/+ heterozygotes were mated to expand the *op/op* colony. The genotypes of the offspring were determined using a PCR-based assay. Primers that flanked the mutation were designed (primer 1: 5′-CAGCTGGATGATCCTGTTTGC-3′; primer 2: 5′-CTCGGTGGCGTTAGCATTGGG-3′) such that genomic DNA from a homozygote *op/op* mutant would yield a 111 base pair product whereas wild-type DNA would yield a 110 base pair product. Genomic DNA was prepared from the tails of mice using standard procedures. The genomic DNA was PCR-amplified using the above mentioned primers using the following protocol: 94°C 5 min followed by 30 cycles of 94°C 45 sec, 58°C 1 min, 72°C 1 min, with a final extension at 72°C for 7 min. ^{32}P-dATP was included in the PCR reactions. The products were separated on a 6% polyacrylamide gel and subjected to autoradiography to visualize the bands (Figure 1).

Intrahippocampal injection of kainate

Adult C57Bl6/J, *op*/+ and *op/op* male mice, between 20 and 25 grams, were injected intraperitoneally with atropine (0.6 mg/kg of body weight) and deeply anesthetized with 2.5% avertin (0.02 ml/gram of body weight). The mice were then injected with 1.5 nmol kainic acid (in 300 nl phosphate buffered saline) unilaterally into the hippocampus using stereotaxic coordinates (bregma -2.5 mm, medial/lateral 1.7 mm and dorsoventral 1.6 mm). The excitotoxin was delivered over 30 sec, and the injection needle remained in place for two additional min to prevent reflux of fluid. Five days after the injection, the brains of the injected mice were analyzed for neuronal survival and microglial activation.

BrdU labeling of proliferating microglial cells

At 48 h following kainate injection, BrdU (200 mg/kg) was delivered to the mice by intraperitoneal injection. These injections occurred once daily for 3 days. At day 5 post-kainate injection, the mice were sacrificed and subjected to immunohistochemistry as described below.

Immunohistochemistry

Coronal sections (30 μm) of the brains of the injected mice were cut at the level of the hippocampus. Non-specific immunoreactivity was blocked by incubation of the brain sections with 5% goat serum. The sections were incubated with antibodies either to the mature macrophage/microglia specific antigen F4/80 (1 : 100, Serotec) or Mac-1 (1 : 10, Roche Biochemicals). Biotinylated secondary antibodies were used (Vector Laboratories) and the avidin-biotin-peroxidase complex (ABC reaction) was visualized with diaminobenzidine and hydrogen peroxide (Vector Laboratories) as described previously.[20] For BrdU immunostaining the tissue sections were treated with 2N HCl prior to preincubation with goat serum. Fluorescent-labeled secondary antibodies (anti-rat FITC for F4/80 and anti-mouse TRITC for BrdU) were used to identify proliferating microglial cells.

Amidolytic assay for tPA activity

For quantitative determination of tPA activity, the amidolytic assay was performed as previously described.[25] Briefly, the tissue was lysed in 0.25% Triton X-100 and incubated at 25°C in a mix containing 0.3 mM S-2251 and 0.42 μM plasminogen in 0.1 M Tris, pH 8.1, 0.1% Tween-80). The change in absorbance (ΔA) at 405 nm was measured at different time-points. Known concentrations of recombinant tPA protein were used to generate a standard curve.

Quantitation of microglial cell numbers

F4/80$^+$ and Mac-1$^+$ microglia in the CA1 hippocampal subfield were counted in four successive cryostat coronal sections (cut at 14 μm)

around the injection site, and the numbers were averaged, as previously described.[26] Microglial cell counting occurred 5 days after the excitotoxic injury, when microglial activation (evident by F4/80 immunostaining) reaches peak levels.[1] Activated microglia were characterized as cells with a cell body larger than 10 μm in diameter, with short, thick processes and intense immunoreactivity. No resting microglia (characterized by small cell body, long processes and weak immunoreactivity) were observed at that time-point on the sections.

Quantification of microglial activation

Whole brain lysates were prepared by dounce homogenization in phosphate buffered saline from kainate-injected control and *op/op* mice. To estimate the relative amounts of activated microglia present in each brain, relative tumor necrosis factor alpha (TNF-α) levels were assessed by Western blot analysis. Briefly 20 μg of total lysate was separated through a 15% polyacrylamide gel and transferred to a PDVF membrane. TNF-α was detected using a rat-anti-mouse TNF-α antibody (clone MP6-XT3) at a dilution of 1 : 500 (Boehringer Mannheim) followed by biotinylated goat-anti-rat secondary antibody at a 1 : 3000 dilution (Vector Laboratories). Finally, the avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories) was visualized by chemiluminescence (LumiGLO, KPL). The relative amounts of TNF-α were determined using a Bio-Rad densitometer.

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