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AXONAL LESION-INDUCED MICROGLIAL PROLIFERATION AND MICROGLIAL CLUSTER FORMATION IN THE MOUSE

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Abstract—Microglia are innate immune cells and form the first line of defense of the CNS. Proliferation is a key event in the activation of microglia in acute pathology, and has been extensively characterized in rats, but not in mice. In this study we investigated axonal-lesion-induced microglial proliferation and surface antigen expression in C57BL/6 mice. Transection of the entorhino-dentate perforant path projection results in an anterograde axonal and a dense terminal degeneration that induces a region-specific activation of microglia in the dentate gyrus. Time-course analysis showed activation of microglial cells within the first week post-lesion and cell counting demonstrated a significant 1.6-fold increase in microglial numbers 24 h post-lesion reaching a maximal 3.8-fold increase 3 days post-lesion compared with controls. Double staining for the microglial macrophage antigen-1 and the proliferation marker bromodeoxyuridine, injected 1 h prior to perfusion, showed that lesion-reactive microglia accounted for the vast majority of proliferating cells. Microglia proliferated as soon as 24 h after lesion and 25% of all microglial cells were proliferating 3 days post-lesion. Immunofluorescence double staining showed that most activated, proliferating microglia occurred in multicellular clusters and co-expressed the intercellular adhesion molecule-1 and the hematopoietic stem cell marker cluster of differentiation 34. In conclusion, this study extends observations of axonal lesion-induced microglial proliferation in rats to mice, and provides new information on early microglial proliferation and microglial cluster formation and surface antigen expression in the mouse.

Key words: bromodeoxyuridine, CD34, hippocampus, ICAM-1, axotomy.

Activation of microglia plays a key role in the response of the CNS against infectious and inflammatory diseases, trauma, ischemia, and neurodegeneration (Streit et al., 1999; Nimmerjahn et al., 2005) and microglia are the first cells to respond to even minor pathological changes in the CNS (Kreutzberg, 1996). Microglial activation involves a stereotypic pattern of cellular responses, including morphological and immunophenotypic changes, the release of inflammatory mediators, and expansion of the cell population, predominantly because of proliferation of resident microglia (Hailer et al., 1999; Ladeby et al., 2005a,b). Neuronal injury results in microglial activation, which at the histological level is reflected in cellular hypertrophy, decrease in distal ramification, movement of cells or cellular processes into direct contact with the injured neurons, and cellular proliferation (Gehrmann et al., 1991; Jensen et al., 1994; Peterson et al., 1994).

For studying the activation and proliferation of microglial cells to neural injury the perforant pathway (PP) lesion model provides excellent features. It is based on the highly organized anatomical structure of the PP projection, which originates in the entorhinal cortex and terminates in the hippocampal dentate gyrus (Amaral and Witter, 1995). Transection of the PP projection leads to a distantly located and distinctly laminar anterograde axonal and dense terminal degeneration in the PP termination zone in the outer two thirds of the dentate molecular layer (Matthews et al., 1976; Finsen et al., 1999) resulting in a reproducible microglial activation. Pulse-labeling studies with injection of the proliferation marker bromodeoxyuridine (BrdU) 1 h prior to perfusion in PP-lesioned rats have shown that as many as 18–20% of the lesion-induced reactive microglial cells were actively proliferating 3 days post-lesion (dpl) (Hailer et al., 1999). In addition, although the PP lesion does not result in junctional breakdown of the blood–brain barrier (Jensen et al., 1997), studies of bone marrow-chimeric mice have shown a lesion-site specific recruitment of microglial precursors (Ladeby et al., 2005b; Wirenfeldt et al., 2005; Bechmann et al., 2005).

This study aimed to investigate PP lesion-induced microglial morphological transformation and proliferation in C57BL/6 mice, a mouse strain that is widely used in neurobiological research. In the same study we also analyzed microglial expression of the intercellular adhesion molecule-1 (ICAM-1), being expressed by axonal lesion-induced reactive microglial cells in mice (Werner et al., 1998; Kloss et al., 1999) and in rats (Hailer et al., 1997) and the hematopoietic stem cell marker cluster of differentiation (CD)34, that is expressed by proliferating resident microglial cells in SJL mice (Ladeby et al., 2005a; Wirenfeldt et al., 2005). We report that activated microglia constitute the vast majority of BrdU-incorporating cells within the first week after lesion, that 25% of all microglia are actively proliferating 3 dpl in C57BL/6 mice, and that most of these proliferating microglia are aggregating in multicellular clus-

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Abbreviations: ab, antibody; ANOVA, analysis of variance; AP, alkaline-phosphatase; BrdU, bromodeoxyuridine; CD, cluster of differentiation; DAPI, 4′,6′-diamidino-2-phenylindole; dpl, days post-lesion; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FJ, fluorojade; hpl, hours post-lesion; Iba1, ionized calcium binding adaptor molecule 1; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; Mac-1, macrophage antigen-1; o.n., overnight; PP, perforant pathway; SA, streptavidin; TBS, Tris-buffered saline; TBS+T, Triton diluted in Tris-buffered saline.

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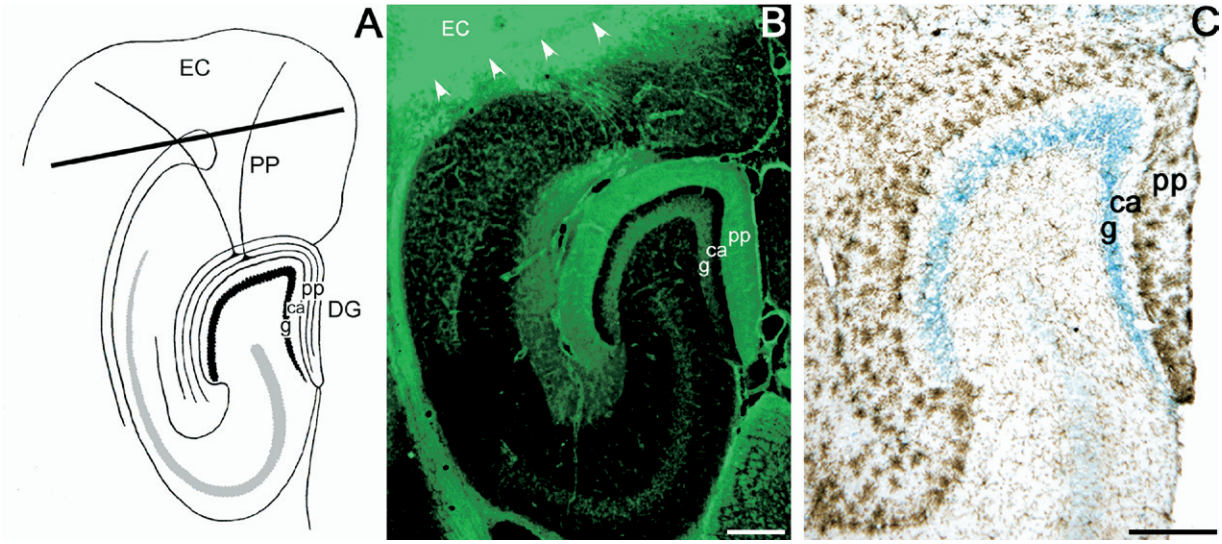

Fig. 1. The PP axonal lesion model. (A) Schematic illustration of the hippocampal formation. (B) FJ-stained section of mouse hippocampus 3 dpl. Transection of the PP projection result in an anterograde axonal and a dense terminal degeneration giving rise to an intense band of green fluorescence in the deafferented outer two thirds of the molecular layer of the dentate gyrus. Arrowheads indicate the location of the lesion. (C) Mac-1-stained section (counterstained with Toluidine Blue) of the murine dentate gyrus 3 dpl. The lesion-induced degeneration leads a region-specific microglial reaction in the PP termination zone of the dentate gyrus. ca, Commissural-associational zone; DG, dentate gyrus; EC, entorhinal cortex; g, granule cell layer; pp, perforant pathway termination zone. Scale bar=200 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

ters. In addition, we report on the up-regulation of ICAM-1 on the vascular endothelial cells in the deafferented areas, and on the expression of ICAM-1 and CD34 on reactive microglia in C57BL/6 mice.

EXPERIMENTAL PROCEDURES

Animals

The study was performed using male C57BL/6 mice, 6–8 weeks old, purchased from Bomholtgaard (Ry, Denmark). The animals were kept at the Biomedical Laboratory at University of Southern Denmark and were cared for in accordance to guidelines of the Danish National Animal Care Committee thereby meeting international guidelines on the ethical use of animals including all efforts to minimize the number of animals used and their suffering.

Surgery

The transection of the entorhino-dentate PP-projection (Fig. 1A) was made using a wire knife (David Kopf Instruments, USA) in a stereotactic frame (Stoelting, Wood Dale, USA) under a microscope. The mice were anesthetized by s.c. injections of 0.01 ml/g of a 1:1:2 solution of Hypnorm (fentanyl citrate 0.315 mg/ml, fluanisone 10 mg/ml, VetaPharma, Leeds, UK), stesolid (diazepamum 5 mg/ml, Dumex-Alpharma, Copenhagen, Denmark), and isotonic saline. The mice were fixated in the stereotactic device and the lambdoid fissure was exposed and a hole was drilled in the skull 0.7 mm caudal to the fissure and 2.0 mm lateral to the midline. A folded wire knife was inserted 3.2 mm at an angle of 10° from the vertical, reaching the entorhinal cortex where it was unfolded and retracted 3 mm dorsally transecting the PP (Fig. 1A). The knife was refolded and withdrawn from the brain. Sham-surgery was carried out following the same procedure but without insertion of the wire-knife into the brain. Finally, to generate T-cell-infiltrated tissue, mice with experimental autoimmune encephalomyelitis induced by passive transfer of T cells reactive to proteolipid protein (Toft-Hansen et al., 2004) had their PP transected. The mice were treated for post-surgical pain with temgesic (Shering-Plough, Brussels, Belgium) diluted in isotonic saline and placed to recover at 28 °C.

BrdU injection

BrdU, an analog of the naturally occurring nucleotide deoxythymidine which incorporates in the DNA of proliferating cells in the mitotic S-phase (Goz, 1977; Nowakowski et al., 1989), was injected intraperitoneally (50 mg/kg, Sigma Aldrich, St. Louis, MO, USA diluted in isotonic saline) 1 h prior to perfusion. DNA containing BrdU was subsequently visualized immunohistochemically.

Tissue processing

The mice were anesthetized with pentobarbital and killed by transcardial perfusion through the left ventricle with a solution of 5 ml 0.15 M phosphate buffer (8.2 mM KH_2PO_4, 1.8 mM $Na_2HPO_4 \cdot 2\ H_2O$, pH 7.4) followed by 20 ml of a similar buffer containing 4% paraformaldehyde. The brains and segments of the small intestine (for control) were collected and post-fixated in 4% paraformaldehyde for 1.5 h and cryoprotected in a 20% saccharose solution (VWR International, Leicestershire, UK) overnight (o.n.) at 4 °C. Finally, the brains were frozen in CO_2-snow and sectioned into eight parallel horizontal series of 20 μm thick sections for the time course study and into 10 parallel horizontal series of 16 μm thick sections for the proliferation study using a cryostat at −18 °C.

Validation of the lesion with fluoro-jade (FJ)

FJ C (Histo-Chem Inc., Jefferson, AK, USA) is known to stain all degenerating neurons (Schmued et al., 2005) and was used to validate the location of the lesion and the lesion-induced degeneration in the PP termination zone of the dentate molecular layer. One series of sections from all animal was immersed in 100% ethanol for 3 min, in 70% ethanol for 1 min, and rinsed in