Occlusal Disharmony in Mice Transiently Activates Microglia in Hippocampal CA1 Region but Not in Dentate Gyrus

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Occlusal disharmony is induced by various conditions such as the loss of teeth and inappropriate vertical dimension of crowns, bridges, or dentures. Occlusal disharmony sometimes causes indefinite complaint syndromes, which may be associated with astrocytic hypertrophy and the reduction of numbers of neuronal somata and their dendritic spines in the hippocampus. Microglia monitors the condition of neurons and responds to their degeneration accompanying with astrocytes. However, the effect of occlusal disharmony on the microglia has not yet been investigated. We artificially increased the occlusal vertical dimension by placing dental resin on the upper molars in mice and immunohistochemically investigated the effects of the increase in the vertical dimension on microglia of the hippocampal formation using an antibody against ionized calcium-binding adaptor molecule 1 (Iba-1), a marker protein for microglia. We measured the area occupied by Iba-1-immunoreactive microglia in the hippocampal CA1 region and dentate gyrus 1, 3, and 5 days after increasing the vertical dimension, and compared it with that of control mice. The hippocampal CA1 region contains vulnerable neurons and the dentate gyrus durable neurons. We found that the areas occupied by microglia in the hippocampal CA1 region increased, with the peak on the third day after increasing the vertical dimension, and it gradually declined by the fifth post-operative day. However, such an increase of the area occupied by microglia was not seen in the dentate gyrus. In conclusion, abnormal mastication may activate microglia in the area harboring vulnerable neurons, but not in the area harboring durable neurons.

Keywords: Microglia; hippocampus; occlusal vertical dimension; immunohistochemistry; Iba-1


Occlusal disharmony is induced by various conditions such as the loss of teeth and inappropriate vertical dimension of crowns, bridges, or dentures (Bjertness 1991). These conditions sometimes cause indefinite complaint syndromes. Recent studies demonstrated that occlusal disharmony affects not only the peripheral nervous system but also the central nervous system (CNS) (Mushimoto et al. 2004; Hada and Mushimoto 2005). In general, oral dysfunction is common in the elderly with symptoms of senile dementia (Bjertness 1991; Nagao 1992; Jones et al. 1993). We previously reported that masticatory disorders reduce input activities in the hippocampus, thereby leading to deficits in learning and memory in aged senescence-accelerated prone 8 (SAMP8) mice (Onozuka et al. 1999, 2000, 2002; Kubo et al. 2005). It has been demonstrated that increasing the occlusal vertical dimension caused various pathological changes in the hippocampus that are involved in deficits of spatial learning and memory (Kubo et al. 2007a, 2007b, 2008; Ichihashi et al. 2008). However, the mechanism(s) by which abnormal mastication induces these neuropathological changes has been not yet elucidated.

Microglial cells are typically described as the resident macrophages occupying the parenchyma of the CNS (Gehmann et al. 1995). Microglia in the CNS can assume at least three clearly identifiable states: (i) resting or ramified microglia, as are present in the normal healthy CNS; (ii) activated or reactive microglia that are found in pathological states; and (iii) phagocytic microglia that represent full-fledged brain macrophages (Streit 2005). Resting microglia are characterized by their small somata and long, fine processes. Under various pathological conditions, these resting microglia alter their morphology into a typical activation state of enlarged cell somata with multiple short, thick processes. In the third category, microglia adopt the macro-
phage-like features of phagocytic microglia, that is, a round body with one to two processes (Streit et al. 1999; Sugama et al. 2007; Vannucchi et al. 2007). Microglial cells have been well investigated as possible mediators of inflammation-associated neuronal damage.

The hippocampal formation is composed of the hippocampus, dentate gyrus and subiculum and the hippocampus is further subdivided into cornu ammonis (CA) 1, 2, 3 and hilus. Among these regions, the CA1 region contains vulnerable neurons and the dentate gyrus contains durable neurons. In the present study, we investigated the effects of the increase in the occlusal vertical dimension on microglia of the CA1 region and dentate gyrus.

Materials and Methods

Animal handling

Male ddY mice (n = 32) were treated with the approval of the Ethics Committee of Kanagawa Dental College, employing guidelines established by the committee.

Mice were anesthetized with sodium pentobarbital (35 mg/kg; Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the vertical dimension of the bite was raised approximately 0.1 mm by placing ultraviolet ray–polymerized resin (UniFil LoFlo, GC Corporation, Tokyo, Japan) on the upper molars after treatment with a Single Bond Dental Adhesive System (3M Dental Products, Irvine, CA, USA). Control animals (n = 5) underwent the same anesthetic procedure but no resin was applied. After the vertical dimension had been increased, the mice were given free access to pellet chow and water (Kubo et al. 2007b).

After the operation (1, 3, 5 days later) and sham-operation (3, 5 days later), the animals (n = 5 each condition) were deeply anesthetized by pentobarbital sodium. They were then perfused with 0.9% NaCl and, subsequently, with 4% formaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 6.9). The brain was rapidly dissected out and further fixed for one or two days at 4°C in the same fixative. After washing in PB and immersing in 20% sucrose, samples were cut into 20-µm-thick transverse sections using a sliding microtome equipped with a frozen stage, and sections were immunostained by the free-floating method.

Immunohistochemistry

Immunohistochemistry was performed according to our routine method (Yamamoto et al. 2008). Briefly, the sections were washed overnight in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS), and incubated with rabbit anti-Iba-1 antibody (Wako Pure Chemical Industries, Ltd.) diluted to 1.25 µg/ml in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS-BSAT) for 2 days at 4°C. This antibody was raised against a synthetic peptide corresponding to the C-terminus of Iba-1, is specific to microglia and macrophages, and does not cross-react with neurons and astrocytes (manufacturer’s instructions). After washing in PBS, the sections were incubated with a secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in PBS-BSAT for 1 h at room temperature. The sections were then washed again in PBS and incubated with avidin-biotin-horseradish peroxidase complex (ABC; Vector Laboratories) diluted 1:200 in PBS-BSAT for 1 h at room temperature.

Results

The profiles of Iba-1-immunoreactive microglial cells in the CA1 and dentate gyrus of control animals were similar to those of resting microglial cells and characterized by their small somata and long, fine processes (Fig. 1A and 2A) as reported previously (Sugama et al. 2003; Cho et al. 2006). Similar microglial features were seen in the CA1 and dentate gyrus of the animals one day after the vertical dimension was increased (not shown). Three days after the vertical dimension was increased, Iba-1 immunoreactive microglial cells in the CA1 region showed morphological changes. Somata of immunoreactive cells seemed to be larger than those of control animals. Processes emanating from the somata seemed to be thicker and the processes per cell to be more abundant than those of control animals (Fig. 1B). However, the microglial changes in the dentate gyrus of the animals after three days were not as remarkable (Fig. 2B). At five days the vertical dimension was increased, the configurations of Iba-1 immunoreactive microglia in the
Fig. 1. Iba-1-like immunoreactivity in hippocampus. Shown is the distribution of Iba-1-like immunoreactivity in hippocampal CA1 (A, B and C) and preabsorption test (D). Iba-1 immunoreactive microglial somata (arrows) on the third day (B) after operation seem to be larger than those of the control (A) and on the fifth day after operation (C). Note the absence of these staining profiles due to the preabsorption of antibody with recombinant Iba-1 (D). Scale bars in A-D = 200 µm.

Fig. 2. Iba-1-like immunoreactivity in dentate gyrus. Note the similar appearance in Iba-1 immunoreactive microglial cells of the control (A), 3rd-day (B), and 5th-day (C) animals after operation. Scale bars in A-C = 200 µm.
CA1 region were similar to those at three days, but the number of changed cells seemed to have declined (Fig. 1C). The appearance of immunoreactive cells in the dentate gyrus of animals at five days was similar to that of control animals (Fig. 2C). Omitting the primary antiserum and pre-absorption with recombinant Iba-1 resulted in elimination of these staining profiles (Fig. 1D). Western blot analysis of hippocampus extracts showed this antiserum recognized a single protein band that migrated at the position of approximately 17.7 kDa (Fig. 3B). This band was not detected when the processes were performed from the step of the secondary antibody (Fig. 3C).

Morphological changes were assessed by measuring the area occupied by Iba-1 immunoreactive microglial cells (Table 1). The areas of microglial cells were significantly increased in the hippocampal CA1 region on the third day after increasing the vertical dimension (116.2 ± 3.6%, \( p < 0.05 \)) compared to the control (100.0 ± 3.5%) and the first day after operation (101.6 ± 3.8%) (Fig. 4). The increase on the third day was also statistically significant compared to that on the third day after sham-operation (Table 1). Although the differences between the third- and fifth-day animals were not statistically significant, the microglial areas in the CA1 region of fifth-day animals were smaller than those of third-day animals (Fig. 4). In the dentate gyrus, the areas occupied by microglia after the increase in the occlusal vertical dimension tended to be smaller than those of control animals, especially on the first day following the operation, but these differences were not statistically significant (Fig. 4).

**Discussion**

Antibody specificity was supported by the elimination of the staining profiles after Iba-1 antibody preabsorption with recombinant Iba-1, and there was no staining in sections when the procedures were performed with only the secondary antibody. Furthermore, Western blotting analysis indicated that this antibody recognized a single band that migrated to the position of approximately 17.7 kDa. This value of 17.7 kDa is slightly higher than that 17.0 kDa that was previously reported (Ito et al. 2001; Hwang et al. 2006). The difference may be owing to a difference in technique or to high concentrations of proteins in the supernatant. The results suggest that the antiserum used here recognized mouse Iba-1.

This study showed that increasing the occlusal vertical dimension caused a transient increase in microglial areas in the hippocampal CA1 region but not in the dentate gyrus. Although we did not thoroughly analyze them, the globus pallidus seemed to show a similar transient increase in
Table 1. Areas occupied by Iba-1 immunoreactive cells.

<table>
<thead>
<tr>
<th></th>
<th>CA1 (µm²)</th>
<th>DG (µm²)</th>
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<tbody>
<tr>
<td></td>
<td>mean ± s.e.</td>
<td>mean ± s.e.</td>
</tr>
<tr>
<td>Control</td>
<td>8,154 ± 284 b</td>
<td>8,670 ± 394</td>
</tr>
<tr>
<td>1 d after operation</td>
<td>8,283 ± 313 b</td>
<td>8,111 ± 406</td>
</tr>
<tr>
<td>3 d after operation</td>
<td>9,472 ± 291 a</td>
<td>8,383 ± 296</td>
</tr>
<tr>
<td>3 d after sham operation</td>
<td>8,123 ± 125 b</td>
<td>8,374 ± 159</td>
</tr>
<tr>
<td>5 d after operation</td>
<td>8,978 ± 283 ab</td>
<td>8,611 ± 278</td>
</tr>
<tr>
<td>5 d after sham operation</td>
<td>8,318 ± 160 b</td>
<td>8,227 ± 151</td>
</tr>
</tbody>
</table>

Sum of areas occupied by Iba-1 immunoreactive cells per unit area (250 x 250 µm²) in the hippocampal CA1 region (CA1) and dentate gyrus (DG) of the control and various survival times (n = 5 for each group). Data are presented as mean ± s.e. Identical letters at the right of each CA1 data point indicate that the difference has no statistical difference, and different letters indicate a statistical significance (p < 0.05). Between data points of the DG, no combination had statistical significance.

Microglial areas, like the CA1, but the central amygdala did not, like the dentate gyrus. Increasing the vertical dimension has several effects on the brain, such as increasing the catecholamine concentration and c-Fos mRNA in the hippocampus (Budts-Jørgensen 1981; Areso et al. 1999; Yoshihara et al. 2001; Kobayashi and Mushimoto 2004; Mushimoto et al. 2004). Furthermore, the increase in the vertical dimension decreases the number of dendritic spines in the hippocampal CA1 region and reduces learning ability (Kubo et al. 2008). In aged SAMP8 mice, an increase in the vertical dimension increased plasma corticosterone levels (a stress marker) and deficits in spatial memory (Kubo et al. 2007a, 2007b; Ichihashi et al. 2008) accompanied by various pathologic changes, such as degeneration of hippocampal CA3 pyramidal cells (Ichihashi et al. 2008), a decrease of c-Fos induction in the hippocampal CA1 region (Kubo et al. 2007b), and an increase of the number of hippocampal CA3 astrocytes (Kubo et al. 2007a). All these lines of evidence suggest that an increase in the occlusal vertical dimension is a form of stress. In the CNS, stress has been implicated in neurodegeneration (Uno et al. 1989) and, more recently, in neuronal plasticity (Sapolsky 2003).

Microglial cells respond to inflammation in the brain instead of macrophages in the peripheral organs, by releasing and sensing inflammatory markers such as interleukins (Hanisch 2002). Activation of microglia, in a response to these inflammatory markers, has been demonstrated to occur after traumatic brain injuries such as the axotomy of facial nerve (Raivich 2002). In addition, activation of microglia also occurs in pathological conditions such as Alzheimer’s and Parkinson’s disease (McGeer et al. 1989; Dickson et al. 1993). One of the most easily distinguishable features of the activated microglial cell is its morphological transformation, which in the initial stages of activation, from minutes to a few hours following injury, can be recognized as a partial retraction and a slight hypertrophy of the microglial cell processes (Ladeby et al. 2005). These early morphological changes appear to be unrelated to the type of neural injury, although the morphological transformation of the lesion-reactive microglia in the later stage of activation is dependent on the type of neuronal degeneration (Morioka et al. 1991; Finsen et al. 1993; Streit et al. 1999). Acute neuronal injury causes the highly characteristic microglial activation and reactive microgliosis, but expansion of the microglial cell population is usually transient (Jørgensen et al. 1993; Finsen et al. 1999; Kato et al. 2003; Ladeby et al. 2005). The transient increase of microglial areas in the CA1 after the increase in occlusal vertical dimension may belong to these acute reactions. Although the underlying mechanisms of the transient increase of microglial area in the CA1 are unclear, possible pathways, such as neuronal and humoral pathways from the oral cavity to the hippocampus, were recently reviewed (Ono et al. 2010). One possibility is that the increased plasma corticosterone after increasing the vertical dimension is detected by corticosterone receptors localized in neurons of the hippocampal formation (Han et al. 2005; Galeeva et al. 2006; Sarabdjitsingh et al. 2009) and that such neurons affect microglia. Alternatively, the increased corticosterone directly affects microglia, which also express corticosteroid receptors (Tanaka et al. 1997).

In contrast to the CA1 region, microglial areas in the dentate gyrus showed no significant difference. The dentate gyrus has several characteristic features compared with the hippocampal CA1 region. First, the dentate gyrus is one of the restricted areas where neurogenesis continues throughout adulthood (Ming and Song 2005; Lledo et al. 2006). Second, neurons in the dentate gyrus are more resistant to ischemia than those in the CA1 (Green et al. 1992). The dynamics of microglia may certainly depend on the surrounding neurons’ characteristics. Therefore, the lack of significant differences in the dentate gyrus might be associated with the above characteristics of brain regions. Similar differential responses of microglia were seen after transient
global cerebral ischemia in the rat. Microglia, as visualized by microglial response factor-1 (MRF-1) immunostaining, became activated and developed a more stout morphology showing enlarged cell bodies and contraction of their processes. However, only moderate morphological activation was seen in the CA3 region and dentate gyrus (Kato et al. 2003). It is worth noting that microglia are involved not only in neuronal degeneration, but also in neuronal survival (Nakajima and Kohsaka 2005). In conclusion, abnormal mastication, for example due to an increasing in the occlusal vertical dimension, activates microglia that may affect neurons and possibly lead to deficits of learning and memory ability.

Acknowledgment

This work was supported by an Open Research Center subsidy (2006) from MEXT.

References


Microglia after Increasing Occlusal Vertical Dimension


