Rapid Communication

Depth and time-dependent heterogeneity of microglia in mouse hippocampal slice cultures

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ABSTRACT

Microglia are the brain-resident immune cells with the phagocytic capacity to engulf dead and living neurons in health and disease. However, the mechanisms underlying the neuron-microglia interaction remain elusive partly because proper in vitro systems are lacking. Specifically, the highly activated status of microglia with amoeboid morphology in primary culture is different from the ‘resting’ microglia with multiple processes in vivo. Here, we performed a detailed investigation of microglial properties in mouse hippocampal slice cultures, focusing on the changes in morphology in the activated state, finding a depth and time-dependent localization of in vivo-like microglia in slice cultures.

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Microglia develop from erythromyeloid progenitors in the early embryonic yolk sac and migrate into the brain, where they reside for life. Microglia engulf dead cells and cell debris with their phagocytic capacity as immune cells (Hanisch and Kettenmann, 2007). In the healthy brain, microglia with ramified morphology survey their surroundings (Nimmerjahn et al., 2005) and contribute to the refinement of neural circuits by engulfing immature or weak synapses (Schafer et al., 2012; Paolicelli et al., 2011). In neurodegenerative diseases, including Alzheimer’s disease (Streit et al., 2009) and Parkinson’s disease (Wang et al., 2007), microglia are activated and exhibit amoeboid morphology. Activated microglia produce and release several pro-inflammatory mediators and cytokines (Hanisch et al., 2001) and could injure neurons, leading to the aggravation of neurodegenerative diseases (Perry et al., 2010). Thus, any advance in understanding the mechanisms impacting microglial properties and their role in health and disease is of great interest to neuroscientists. For this purpose, a reliable in vitro system to study microglial properties is necessary because in vitro systems possess several advantages compared with in vivo systems, including well-regulated pharmacological and genetic approaches and time-lapse imaging experiments, especially when the deep brain regions are targeted for study.

A series of previous studies using the primary culture of microglia on artificial plastic and glass surfaces have promoted our understanding of some aspects of microglial function (Suzumura et al., 1991; Fujita et al., 1996; Giuliani and Baker, 1986; Abd-El-Basset et al., 2004; Kloss et al., 2001). Microglia in primary culture, however, exhibit highly activated properties with amoeboid morphology, which is different from the ‘resting’ microglia with ramified morphology under physiological conditions in vivo (Nimmerjahn et al., 2005). Further, in the activated microglia, non-physiological levels of gene expression, especially those related to inflammation, are induced (Chhor et al., 2013).

An alternative to studying the physiological function of microglia in vitro is the use of organotypic slice cultures. Brain slice cultures preserve primary neural circuits and almost all types of cells, including microglia, astrocytes, oligodendrocytes, and neurons, allowing us to study neuron-glia interactions under environmental conditions, which are similar to in vivo conditions. Thus, organotypic slice cultures have been used to study the characteristics of microglia (Gahwiler, 1984; Zimmer and Gahwiler, 1984; Dailey et al., 1994; Haller et al., 1996) such as the motility of microglia (Stence et al., 2001), polarization and plasticity (Ajmone-Cat et al., 2013), gene expression (Girard et al., 2013), and microglia-neuron interaction (Wu et al., 2014). However, previous studies have not taken into account nor quantitatively analyzed and discussed the heterogeneity of microglia within a cultured slice, bracketing all types of microglia together for analyzing their

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properties. Here, we performed a detailed investigation of microglial properties in mouse hippocampal slice cultures, focusing on the changes in morphology and activated state, to explore depth-, time-, and region-dependent heterogeneity of microglia in cultured slices.

In the present study, animal experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo [approval number: 24-70] and according to the University of Tokyo’s guidelines for the care and use of laboratory animals. The animal experiment ethics committee at the University of Tokyo approved this study [approval number 24-70].

C57BL/6J mice (SLC, Shizuoka, Japan) and CX3CR1-GFP/GFP mice (Jackson Labs., Farmington, CT, USA) were housed in cages in standard laboratory conditions (a 12-h light/dark cycle, free access to food and water). All efforts were made to minimize the animals’ suffering and the number of animals used. For preparing hippocampal slice cultures, the mouse pups were decapitated after they were deeply anesthetized on ice, and their mothers were euthanized by isoflurane.

To prepare slice cultures, P6 mouse brains were sectioned into 400-μm-thick horizontal slices using a DTK-1500 vibratome (Doasaka, Kyoto, Japan) in aerated, ice-cold Gey’s balanced salt solution (GBSS) containing 36 mM glucose. The hippocampal regions of slices were dissected out and incubated for 30-90 min at 4 °C in incubation medium containing minimal essential medium (MEM), 9.0 mM Tris, 22.9 mM HEPES, and 63.1 mM glucose supplied with penicillin and streptomycin. Following this incubation, the slices were placed on Omnipore® membrane filters (JHWP02500; Merck Millipore, Billerica, MA) on the doughnut plates (Hazai-Ya, Tokyo, Japan; Koyama et al., 2007) in a solution containing 50% MEM, 25% horse serum (26050-088; HS, heat-inactivated and filter-sterilized, Gibco, Grand Island, NY), 25% HBSS, 6.6 mM Tris, 16.9 mM HEPES and 4.0 mM NaHCO3 supplemented with 29.8 mM glucose and 1% gentamicin sulfate solution (16672-04; nacalai, Kyoto, Japan).

Finally, the slices were cultured at 35 °C in a humidified incubator with 5% CO2 and 95% air. The culture medium was changed twice weekly. In some experiments (Fig. 4), lipopolysaccharides (LPS; 10 µg/ml; Sigma, St. Louis, MO) and minocycline (30 µM; Sigma) were applied to the culture medium.

For immunostaining, cultured slices were fixed in 4% PFA at 4 °C for 24 h. The fixed samples were rinsed 3 times with phosphate-buffered saline (PBS). Slices were then permeabilized and blocked for 1 h at room temperature in PBS + 0.3% Triton X-100 with 10% goat serum. The samples were subsequently incubated with primary antibodies in PBS + 0.3% Triton X-100 with 10% goat serum at 4 °C for 48 h with agitation. Samples were rinsed 3 times with PBS and then incubated with secondary antibodies in PBS + 0.05% Hoechst + 0.3% Triton X-100 with 10% goat serum at 4 °C overnight with agitation. After the rinse, samples were embedded in PermaFluoro (Thermo Fisher, Waltham, MA).

The following primary antibodies were used for immunostaining: chicken anti-GFP (1:500; ab13970; Abcam, Cambridge, UK), mouse anti-NeuN (1:1000; MAB377, Merck Millipore), rabbit anti-GAP (1:500; G9269, Wako, Osaka, Japan), and rat anti-CD68 (1:500; MCA1957GA, Serotec, Oxford, UK). The following secondary antibodies were used for immunostaining: Alexa Fluor 488-, 594-, or 647-conjugated secondary antibodies (1:500; Thermo Fisher).

Images for analyzing microglial morphology and representative images were acquired using an FV1200 scanning confocal microscope (Olympus, Tokyo, Japan) equipped with diode lasers (405, 473, 559 and 635 nm) and analyzed using ImageJ (NIH). Z-series images were collected with a 0.75 NA 20× objective at a voxel size of 1.224–1.224–2.0 μm (x–y–z) for microglial density and a 0.95 NA 40× objective at a voxel size of 0.306–0.306–0.5 μm (x–y–z) for representative images. Microglial density (cells/mm³) was analyzed in CA1, CA3, and DG and averaged.

Images for analyzing microglial morphology and CD68 expression were acquired using a BX61 WI microscope (Olympus) equipped with a two-photon laser-scanning system (Spectra-Physics, Mai Tai; a mode-locked Ti: sapphire laser with a 100-fs pulse width, 80-MHz pulse frequency, and 900 nm wavelength) and analyzed using ImageJ (NIH). Z-series images were collected with a 0.95 NA 40× objective at a voxel size of 0.306–0.306–0.5 μm (x–y–z). To determine % expression, the following calculation was used: Volume of CD68 (μm³)/Volume of microglial cell (μm³). All analyses were performed blinded to experimental condition.

Finally, the data are represented as the means ± standard error of the mean (SEM). Data were pooled from at least 3 independent experiments. Data were collected and statistically analyzed with the researcher blinded to experimental condition. Tukey’s test after one-factor analysis of variance (ANOVA) was used for statistical analysis.

We prepared horizontal entorhinal-hippocampal slices (400-μm-thick) from postnatal day 6 (P6) CX3CR1-GFP mice (Fig. 1A; a transgenic line in which all microglia express EGFP under the control of fractalkine receptor type1, CX3CR1) (Jung et al., 2000) and cultured them on membrane filters (Koyama et al., 2007). The immunostained images of cultured slices at 3 days in vitro (DIV) indicated that the overall distribution of GFP-positive microglia, glial fibrillary acidic protein (GFAP)-positive astrocytes, and neuronal nuclei (NeuN)-positive neurons was preserved (Fig. 1A).

Specifically, neuronal cell layers, such as the granule cell layer (GCL) and the pyramidal cell layer (PCL) (Fig. 1A, 3 DIV), resembled those cell layers in acute slices (Fig. 1A, P6).

Next, we investigated the vertical distribution of neuron and glia in cultured slices because the cultured slices maintain 3-dimensional structures, and the heterogeneous distribution of cells along the vertical axis of slices had been unclear. For this purpose, after immunostaining 3 and 21 DIV cultured slices with neuronal and glial markers, we cut slices vertically to include the dentate gyrus (DG) and the hippocampal CA3 field (Fig. 1B, schematic diagram). We found that the thickness of cultured slices decreased from ~300 μm to ~100 μm between 3 and 21 DIV. Both GCL and PCL were preserved at 3 and 21 DIV (Fig. 1B), though the NeuN-positive neurons localized in the superficial region at 3 DIV and gradually moved to middle-bottom regions between 7 and 21 DIV (Fig. 1C). GFAP-positive astrocytes covered the surface of cultured slices at both 3 and 21DIV (Fig. 1B). GFAP-positive astrocytes mainly localized to the superficial region of cultured slices from 3 to 21 DIV (Fig. 1C). At 21 DIV, however, GFAP-positive astrocytes also existed in the bottom region in the edge of cultured slices (Fig. 1C, 21 DIV, edge), which suggests that superficial astrocytes wrap the cultured slices from the edge to the center in a culture period-dependent manner. In contrast to GFAP-positive astrocytes, GFP-positive microglia existed both in the superficial and middle region of cultured slices as early as 3 DIV (Fig. 1C). At 21 DIV, microglia existed in all the superficial, middle, and bottom regions of cultured slices (Fig. 1C), but the morphology of microglia appeared to be heterogeneous along the vertical axis (Fig. 1C). It is also likely that microglial properties in each layer were influenced by surrounding neurons and astrocytes, especially because neurons and astrocytes were also shown to change their locations during the culture periods.

To examine the observed heterogeneity of microglia along the vertical axis in detail, we imaged the immunostained slice cultures using two-photon microscopy and categorized the vertical regions to three layers (Fig. 2A): the superficial layer, from the surface to 15 μm in depth; the middle layer, from 15 to 30 μm in depth; the bottom layer, 0–10 μm from a membrane filter. The superficial layer was covered with microglia with amoeboid morphology, which were categorized as Cell type 1. The middle layer was mainly occupied by microglia with ramified morphology, which is
similar to the resting microglia in vivo (Nimmerjahn et al., 2005). These microglia were categorized as Cell type 2. The bottom layer, which attaches a slice culture membrane, was densely packed by microglia with a small round-shaped soma and few processes, which were categorized as Cell type 3.

We also examined the expression of CD68, a lysosomal marker specific to microglia that increases during phagocytosis (Perry et al., 1985; Ransohoff and Cardona, 2010; Ransohoff and Perry, 2009), via immunohistochemistry in three types of microglia (Fig. 2B). We found that the CD68 expression is significantly higher in Cell type 1 than the other cell types, indicating that Cell type 1 microglia were highly activated amoeboid microglia (Fig. 2C). In contrast, Cell type 2 microglia with ramified morphology with multiple processes exhibited relatively low levels of CD68. These properties of Cell type 2 microglia resemble those of resting microglia in vivo (Kozlowski and Weiner, 2012) and will be appropriate to study microglia in vitro. To our knowledge, Cell type 3 microglia with small soma and few processes and low CD68 levels have not been reported from in vivo studies. Thus, Cell type 3 microglia may be slice culture-specific microglia that are likely necessary to attach slices to a membrane filter.

We examined the culture period-dependent changes in microglial density in all three cell types. Total microglial density in the superficial and middle layer decreased in a culture time-dependent manner (Fig. 3A). In contrast, total microglial density significantly increased in the bottom layer after 14 DIV (Fig. 3A). An increase of the total microglial density in the bottom layer likely reflected a significant increase in Cell type 3 microglia (Fig. 3B). The density of Cell type 1 microglia did not change in all three layers, while the density of Cell type 2 microglia decreased in the superficial and middle layer between 3 and 21 DIV (Fig. 3B). The observed trend of the changes in microglial density was similar between the hippocampal subfields CA1, CA3, and DG (Table 1).

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As for in vivo-like microglia, i.e., Cell type 2, they are rich in the superficial to middle layers throughout the culture periods (Fig. 3B). However, because the superficial layer is covered by highly activated Cell type 1 microglia, it is recommended to focus on the middle layer to study microglial properties in mouse hippocampal slice cultures. It is also recommended to use 7–14 DIV cultures because Cell type 3 microglia increased in the middle layer at 21 DIV (Fig. 3B).

Fig. 2. Microglial heterogeneity with the depth in cultured slices. (A) Horizontal images of the superficial, middle, and bottom layer of cultured slices prepared from P6 CX3CR1<sup>+</sup>GFP mice. The images of the superficial and middle layer were taken at 7 DIV, and the image of the bottom layer was taken at 21 DIV. (B) Classification of microglia in cultured slices. Cultured slices were immunostained for CD68 at 7 DIV. Cell type was determined by the morphology and CD68 expression levels: Cell type 1, amoeboid morphology with high CD68 expression; Cell type 2, ramified morphology with low CD68 expression; Cell type 3, rounded soma with low CD68 expression. (C) Quantification of % volume of microglia occupied by CD68-positive lysosomes in three cell types. Data represent the mean ± SEM. **P<0.01 versus Cell type 1, Tukey’s test, n = 20 cells.

Fig. 3. Comparison of microglial density at different time points. (A) Total density of GFP<sup>+</sup> microglia in superficial, middle and bottom layers at 7, 14, and 21 DIV. Data represent the mean ± SEM. *P<0.05 and **P<0.01 versus 7 DIV, Tukey’s test, n = 4 slices. (B) Density of each cell type in the superficial, middle and bottom layers at 7, 14, and 21 DIV. **P<0.01 versus the same cell type at 7 DIV, Tukey’s test, n = 4 slices.
Table 1

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Data represent mean ± SEM; n = 4 slices for three regions.

* P<0.05 vs DIV7 (Tukey's test).

** P<0.01 vs DIV7 (Tukey's test).

Fig. 4. Pharmacological manipulation of microglial activity in cultured slices. Cultured slices were treated with LPS (10 μg/ml) and minocycline (Mino; 30 μM) from 11 to 14 DIV, and were fixed at 14 DIV for immunohistochemistry. The bar graph indicates the quantification of % volume of microglia occupied by CD68-positive lysosomes in Cell type 2 microglia. Data represent the mean ± SEM. **P<0.01 versus control and **P<0.01 versus LPS, Tukey's test, n = 20 cells from 6 slice cultures.

Finally, to test the applicability of microglia in the slice cultures, we examined the changes in microglial activation by changes in CD68 expression as in Fig. 2. Thus, we performed pharmacological manipulations of microglial activation using LPS, a proinflammatory molecule that induces microglial activation (Hagland et al., 2015), and minocycline, an established inhibitor of microglial activation (Buller et al., 2009). When applied from 11 to 14 DIV, LPS (10 μg/ml) increased CD68 expression in Cell type 2 microglia at 14 DIV, which was blocked by simultaneously applying minocycline (30 μM), while minocycline alone did not affect the CD68 expression (Fig. 4). These results indicate that microglial activation state can be pharmacologically manipulated in the slice cultures.

Overall, the present study reveals the proper depth and culture periods of mouse hippocampal slice cultures to investigate microglial properties.

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References


