International Journal of Zoology Studies
ISSN: 2455-7269
Impact Factor: RJIF 5.14
www.zoologyjournals.com
Volume 3; Issue 2; March 2018; Page No. 166-170

Microglia in ageing rat brain: A quantitative study

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Abstract
Microglia are the cells of immune surveillance and performed physiological as well as pathophysiological functions in Central Nervous System (CNS). The aim of this study was to investigate age-related changes of microglia in striate cortex. Six age groups of female Wistar rats of 3, 6, 12, 18, 24 and 30 months were used for this purpose. The quantitative changes of microglia in ageing striate cortex were employed by immunohistochemistry with Iba-1 antibody. This study revealed that the number of microglia was increased as age advanced and showed activated phenotypic profile in the senile rat brain. Nevertheless, the area fraction of Iba-1 stained microglia did not show any significant difference between the age groups due to the proliferation and activation of these cells.

Keywords: striate cortex, microglia, ageing, Iba-1, immunohistochemistry

1. Introduction
Microglia are immunocompetent cells of CNS and represent about 5-12 percent of all glial cell types in the Central Nervous System [1]. They supposed to play a key role in physiological as well as pathophysiological function [2]. The origin of microglia has been a longstanding controversial issue. Some suggested that microglia are derived from blood-borne circulating monocytes [3-5] while others believed that microglia do not develop from circulating blood instead develop from hematopoietic precursors during embryonic development [6, 7]. Some hypothesized that microglia originate from glioblast cells [8, 9] or are of neurodermal in origin, deriving from either ventricular zone of the lateral ventricle [10] During postnatal development, amoeboid microglia proliferate and transformed into intermediate microglia and afterwards formed the resting microglia. Resting microglia involved in maintenance of homeostasis of the brain under the normal condition. Following CNS insult, these resting glia became activated and undergo phenotypic changes that include hypertrophy, hyperplasia, enhanced expression of immunologically relevant cell surface molecules, and changes in cytokine and growth factor production [11] If the degeneration proceeds, microglia further transformed into phagocytes and involved in the removal of cellular debris from CNS.
Although, studies based on the role of microglia in injury as well as age-related neurodegenerative diseases are available in a considerable amount while the changes in microglia in normally aging brain have been studied to a lesser extent. Thus the aim of this study is to reveal the changes in microglial population in striate cortex during the process of healthy ageing.

2. Material and methods
2.1 Experimental animals
To evaluate quantitative changes of microglia in ageing, female Wistar rats of 3, 6, 12, 18, 24 and 30 months of age (n=3) obtained from the animal house of the School of studies in Neuroscience, Jiwaji University, were used in this study. The animals were housed (three animals per cage) in a controlled environment (25±2°C, 50-55% humidity). Standard 12h light-dark cycle was strictly maintained and were allowed to standard rat pellet feed and water *ad libitum*. The experimental procedure was approved by the Institutional Animal Ethical Committee of Jiwaji University, Gwalior.

2.2 Tissue processing
Animals were deeply anesthetized with ether vapors and intracardially perfused with 0.01 M phosphate buffer saline (PBS) followed by 2% paraformaldehyde prepared in 0.01 M phosphate buffer (PB) solution. Brains were removed and placed in the same fixative for overnight at 4°C. Next day tissues were rinsed in PBS to remove excess fixative. Tissue were then cryoprotected in serial (10%, 20% and 30%) concentrations of sucrose solutions prepared in 0.01M PB at 4°C and then cryosectioned at 15µm thickness using Leica CM 1900 cryostat. Serial sections (two per slide) were collected on clean chromalum gelatin coated slides and stored at -20°C till further use.

2.3 Immunohistochemistry
For immunohistochemical studies, the brain tissue sections through the striate cortex region selected and immunolabelled with Iba-1antibody for the study of quantitative changes of microglia during ageing. Air dried sections were washed with three changes of PBS (pH 7.4) keeping 5 min in each. The sections were then treated with 0.5% Triton X-100 (SIGMA) in PBS to increase permeability, washed in PBS (three changes of 5 min each), followed by 30 min incubation with 1% hydrogen peroxide in PBS to block endogenous peroxidase reactivity. After a thorough wash in PBS (three changes of 5 min each), nonspecific protein binding was

blocked by incubating sections in blocking solution made up of 1% normal goat serum (NGS) in PBS at room temperature for 60 min. The sections were then incubated with primary antibody Iba-1 (1:300, Rabbit polyclonal, Wako, Japan) diluted with 1% BSA in PBS at 4°C for overnight. Next day, after washing in PBS (three changes of 5 minutes each), sections were incubated with secondary antibody anti-rabbit biotin labelled raised in goat (1: 100, Sigma) for 1 hr at room temperature. The sections were again washed in PBS and incubated in srteptavidin- biotin HRP complex (1: 200, Amersham) diluted in 1% BSA in PBS for 1 hr at room temperature and then washed in PBS (three changes of 5 min each). The chromagen reaction was employed using 0.025% 3-3' diaminobenzidin (DAB) and 0.06% hydrogen peroxide in PBS for 20 minutes. The sections were properly washed with water, dehydrated in absolute alcohol (two changes of 10 minutes each), cleared in xylene for 10 minutes and coverslipped with DPX. The sections were finally visualized with Leica Laborlux microscope fitted with DC200 Camera.

2.4 Quantitative analysis

Microglial cell population in str18, str17 and str18a areas of striate cortex was counted by interactive count method using Leica Qwin software. For the purpose of counting, four frames of 5981.7µm^2 were selected from every area/section of both sides of striate cortex. For the tissue mean of the microglial population in the areas constituted, the number of microglia in all the frames was summed up. The area fraction occupied by Iba-1 stained microglia was quantified by ImageJ software inspired by the National Institute of Health (NIH).

2.5 Statistical analysis

All values are expressed as the means ± SEM and statistical significance was evaluated by using one-way analysis of variance (ANOVA), followed by post-hoc analysis with Tukey test or Dunn's method with the help of Sigma stat 3.5 software.

3. Results

3.1 Microglial population in striate cortex

In the Striate18 area, cell number of microglia initially decreased till 12 months, thereafter increased continuously until 30 months (P=0.013). The increase was significant at 30 months as compared to 12 months (P<0.05). In Striate17 area, the population of microglia was decreased at 6 months, then almost stable till 18 months, thereafter steadily increased till 30 months (P=0.009). The difference was significant between 30 and 6 months of age (P<0.05). In striate18a area, the population decreased at 6 months, then stable till 12 months, thereafter increased till 30 months (P=0.012). The increase was significant at 30 months as compared to 6 and 12 months (P<0.05).

In general, striate cortex as whole, 3 months showed densely populated resting microglia, after that a slow decrease in cell numbers with less ramification of microglia was observed at 6 months of age. Afterwards, the almost stable population was found till 12 months and then it increased till 30 months (P=0.010). At 24 and 30 months, microglia showed activated phenotypic profile and cluster formation. The population was reached at its peak at 30 months of age and the increase was significant as compared to 6 and 12 months (P<0.05; fig. 1, 2).

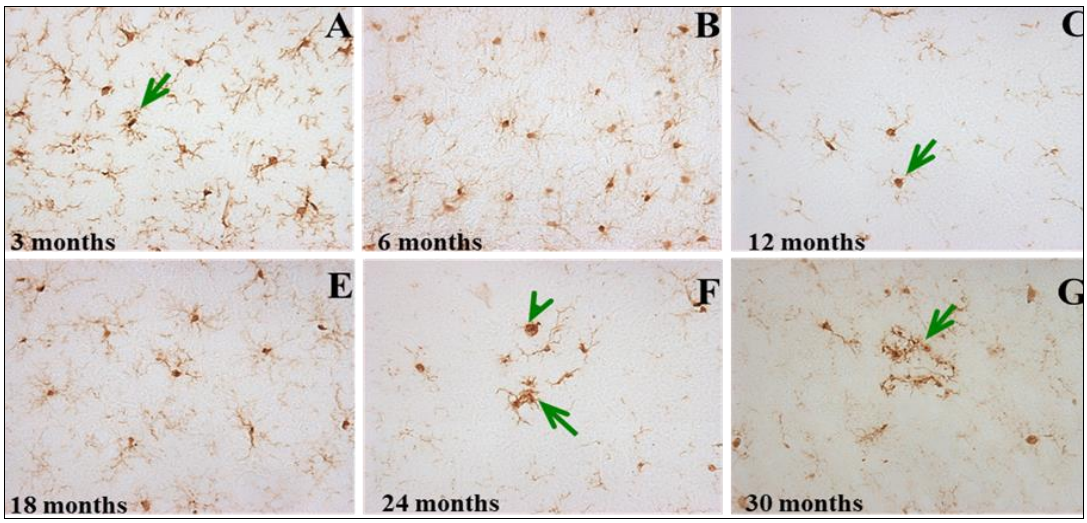

Fig. 1. Iba-1 immunostained microglia in striate cortex of various age groups

High density of resting microglia in 3 months (A; arrow). Decreased population of resting microglia in 6 months (B; arrow). Microglia with reduced arborization in 12 months (C; arrow). Rise in population density at 18 months (E; arrow). Amoeboid microglia (F; arrowhead) and cluster of activated microglia in 24 months (F; arrow). Microglial cluster at 30 months (G; arrow).

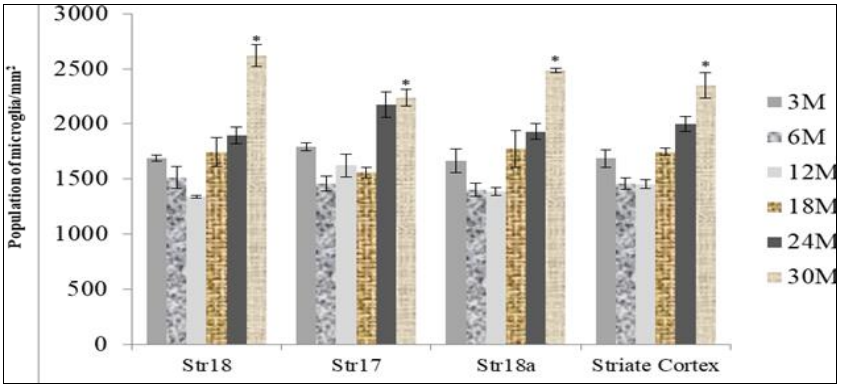

Fig 2: Microglial population in striate cortex of various age groups. Data is expressed as mean±SEM, *P<0.05

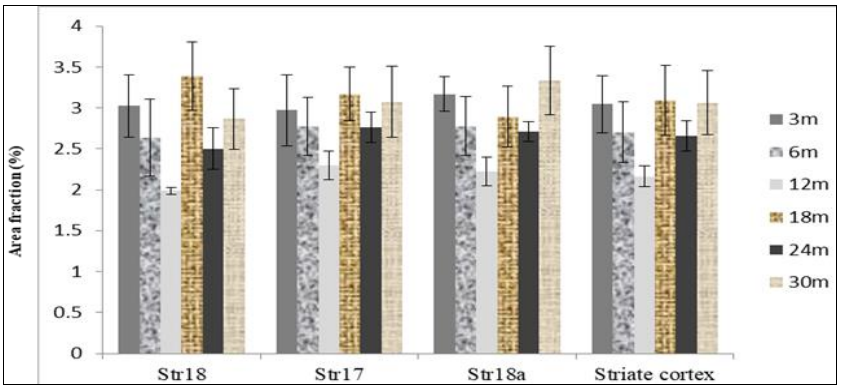

Fig 3: Percent area occupied by Iba-1 immunostained microglia in various age groups

3.2 Area fraction of microglia

Microglia were immunolabelled with anti Iba-1 antibody and the percent immunostaining per frame was measured by digital computer assisted method using ImageJ software. The percent area occupied by Iba-1 stained microglia found to be not a statistically significant difference due to their phenotypes and population change. Microglial hyperplasia leads to the higher occupation of the Iba-1 positive surface area. Similarly microglial hyperactivity leads to an initial increase in cellular area and subsequent to phagocytic activation, the area falls (fig. 1,3).

4. Discussion

The results of this study showed that microglial population highly increased in senile animals. Several investigations have been proved that the number of microglia increased as the function of age in visual cortex [12] as well as other brain regions [13-16]. The microglial cells maintain their population by self renewable and due to the presence of blood brain barrier, progenitor recruitment is not possible from the circulating blood [17]. Thus the microglial proliferation and apoptosis contributing in population maintenance in the adult CNS [18]. But with each mitosis, telomeres shorten progressively and finally reached at a critical length, which results in replicative senescence [19, 20]. Microglial potential as a mitotic cell, have a finite lifespan and due to the telomere shortening, microglia are subject to be replicative senescence [21, 22]. This mitotic capacity of microglia is also applicable to microglia in ageing brain, which calls attention to their senescence.

It has been well established that microglia get activated upon any pathological condition. Following CNS injury, microglia usually proliferate [23, 24]. and showed an inflammatory profile[25], which indicates the neuroprotective role of microglia. On the other hand, chronic microglial activation has been reported to be cytotoxic [21, 26]. and contribute to the pathogenesis of neurodegenerative disorders. In Alzheimer's disease, microglia put enough effort for efficient and effective removal of amyloid deposits. The failure of this process has been termed as "Frustrated Phagocytosis" [27-30]. Age-related activation of microglia has been reported in earlier studies in various CNS regions [12, 14, 31-37]. Our study has also observed the activated phenotypic profile of microglia in striate cortex during progression of ageing. It is well established that ageing brain undergoes neurodegenerative changes [38, 40] Thus microglial proliferation and their activation might be an effort to provide neuroprotection to CNS. After a critical time, due to telomere shortening and frustrated phagocytosis microglia may be become vulnerable to ageing associated changes and undergo the process of senescence by themselves. Various studies have demonstrated the neuroinflammatory response of microglia during ageing, which may be further involved in the progression of age-related neurodegenerative diseases like Alzheimer's [41] and Parkinson's disease [42]. This supports the idea that the microglial functions deteriorate with the progression of age, which may cause disability to provide neuroprotection and contribute to age-related neurodegenerative diseases. The other support being the total area occupied by microglia in the various reference frames presents no significant change, because as area fraction of

newer microglia with the results of proliferation almost equal to the microglia with reduced arborisation or amoeboid phagocytic phenotype.

5. Conclusions

Our findings of the present study suggest that the microglial cells undergo senescent changes in the senile brain. May be these senescent microglia become a subject of frustrated phagocytosis as well as telomere shortening and further studies could be contribute a better understanding of the role of microglia in age related brain dysfunction.

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