Microglial Cell Density and Morphology Classification in the Hippocampus of Early-Postnatal Female and Male Mice

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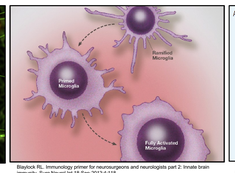
ABSTRACT

Microglia maintain normal brain function and support the brain's response to disease and injury. The hippocampus is an area of focus for microglial study due to its central role in numerous behavioral and cognitive functions. Interestingly, microglia and related cells in the hippocampus and throughout the brain are distinct in male vs. female rodents, even in early life. Indeed, postnatal day (P)-dependent sex differences in number, density, and morphology of microglia-like cells have been reported in certain hippocampal subregions. For example, P3 female mice have more phagocytic microglia in dentate gyrus (DG) molecular layer (Mol) and CA1-3 stratum oriens (SO) regions vs. male mice, while P8 — but not P15 — male rats have more volume immunoreactive for markers of microglia-like cells (Iba1 and CD68) in the CA1 stratum radiatum (SR) vs. female rats. In the mouse, P10 is roughly equivalent to human term gestation, making it a common timepoint to study for many translationally-relevant neurobiological processes. However, sex differences in hippocampal microglia have not been examined in the P10 mouse hippocampus. In addition, key subregions of the hippocampus — CA3 SR, DG hilus — have not yet been assessed for sex differences in microglia. To address these knowledge gaps, we quantified Iba1+ cell densities and classified Iba1+ cell morphology in P10 male and female C57BL/6J mice. Four subregions in the bilateral anterior hippocampus were analyzed in 40-micrometer coronal sections: DG Mol (Mol), DG Hilus, CA1 SR and stratum lacunosum moleculare (CA1), and CA3 SR and stratum lucidum (CA3). Light microscope images (40x) were analyzed offline for Iba1+ cell density and morphology by an observer blind to sex. The morphology of each Iba1+ cell was used to place cells into one of four previously-published categories: Round or ameboid (round-ish soma, no processes), Stout (round-ish soma, short process), Thick (irregular soma with few, thick processes), or Thin (irregular soma with multiple thin processes). Analysis of Iba1+ cell density shows no difference between male and female mice in Mol, Hilus, CA3, or CA1 (male n=6, female n=7). However, morphology classification shows a sex-dependent difference in the Mol and Hilus, with female mice having a greater percentage of Thick Iba1+ cells vs. male mice (Mol, Hilus), and a lower percentage of Thin Iba1+ cells vs. male mice (Mol). With our analysis, it is unclear whether this greater percentage of thick and lower percentage of thin Iba1+ cells in the female vs. male hippocampus means Iba1+ microglia in female mice are 'younger' or 'more active' than those in male mice. However, these data are important as they reveal sex differences in Iba1+ microglia in the P10 mouse hippocampus. We discuss these results in the context of the large literature on sex differences in rodent microglia in the early postnatal period.

BACKGROUND

- **Microglia** are the **brain's resident immune cells**. Microglia play an important role in both the maintenance of **normal brain function**, and in the brain's **response to injury and disease**.[1,2]
- Microglia support normal brain function by **clearing debris**, **pruning synapses**, modulating **neurogenesis**, and even regulating **cognition** and **mood-relevant behaviors.**[3,4]
- The **hippocampus** is one brain region of focus for **microglial study** due to its critical role in **mediating cognitive functions** including **learning** and **memory**, and its implications in **mood regulation.**[5]
- Interestingly, microglia are **sexually-dimorphic** cells. Previous studies have reported that microglia are **distinct** in **male vs. female rodents**, even in **early life**.[6,7]
- Specifically in the **hippocampus**, which continues developing well into the postnatal period, there are **sex-**, **postnatal day-**, and **subregion-dependent differences** with regards to **microglial number, density,** and **morphology.**[8,9]
- However, sex differences in hippocampal microglia have not been examined in the **P10** mouse hippocampus, a crucial timepoint given its **approximate neuroanatomical alignment with human full term gestation.**[10]
- Moreover, sex differences have not yet been assessed in key subregions of the hippocampus including **CA3** and the **DG hilus.**

https://neuro.med.ufl.edu/files/2013/10/Microglia.jpg



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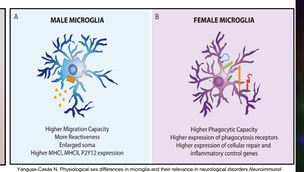

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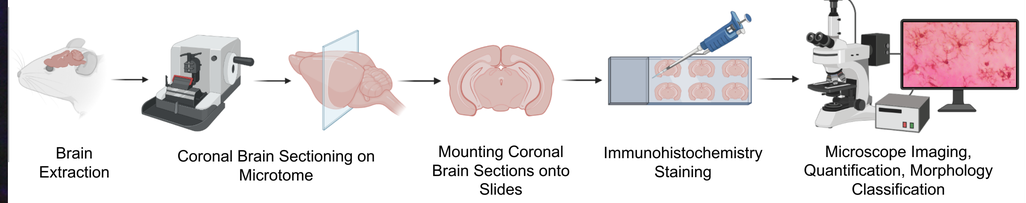
OBJECTIVES

- **Quantify microglial (Iba1+) cell densities** in four hippocampal subregions (DG molecular layer, DG hilus, CA1, and CA3) in P10 male and female C57BL/6J mice
- **Classify microglial (Iba1+) cell morphologies** in four hippocampal subregions (DG molecular layer, DG hilus, CA1, and CA3) in P10 male and female C57BL/6J mice
- Overarching goal: Assess for **baseline sex-dependent differences** in **microglial cell density** and **morphology** in the **naïve mouse hippocampus** at the **P10 timepoint**

METHODS OVERVIEW



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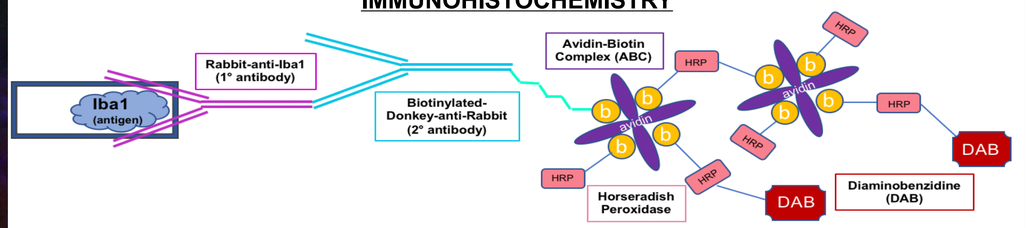


Figure 1. Immunohistochemistry was performed to label Iba1, a calcium-binding protein expressed in microglia. Citric acid pretreatment was used for antigen retrieval. Non-specific protein binding was blocked via incubation with 3% Normal Donkey Serum (NDS) and 0.3% TritonX-100 in PBS. Following pretreatment and blocking steps, sections were incubated overnight with the primary antibody rabbit-anti-Iba1 (1:500) in a carrier of 3% NDS and 0.3% Tween-20 in PBS. The following day, sections were incubated with the secondary antibody, biotinylated donkey-anti-rabbit (1:200). Endogenous peroxidase activity was inhibited via incubation with 0.3% hydrogen peroxide. An avidin-biotin complex was used for amplification. Immunoreactive cells were visualized via incubation with metal-enhanced diaminobenzidine. Slides were counterstained via Fast Red, dehydrated in a series of increasing ethanol concentrations and Citrosolv, and coverslipped with DPX.

RESULTS

Iba1+ cell densities in four hippocampal subregions were not different between male and female C57BL/6J mice at postnatal day 10 (P10)

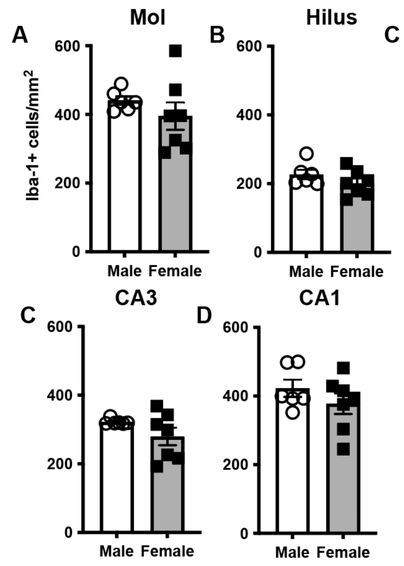


Figure 4. Iba1+ cell densities in four hippocampal subregions are not different between male and female C57BL/6J mice at P10.
Iba1+ cells were evident in all four subregions (Mol, Hilus, CA3, CA1) of all male (n=6) and female (n=7) mice examined. Following the guidelines for selecting subregion areas to consistently sample across mice **(Fig. 2)**, Iba1+ cell densities were quantified **(Fig. 4)**. Iba1+ cell density was similar in male and female mice in the Mol, Hilus, CA3, and CA1 (unpaired t-test, p's>0.05).

Classification of Iba1+ cell morphology in mouse P10 hippocampal subregions shows a sex difference

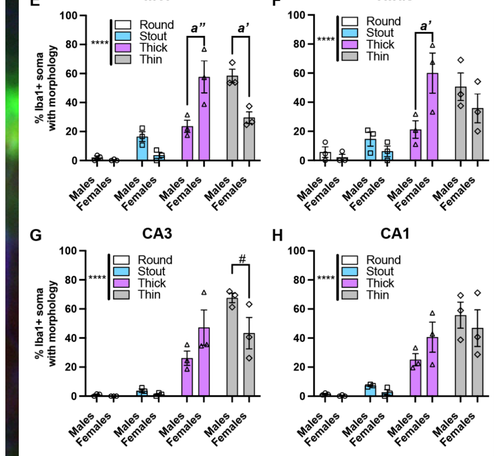


Figure 5. Iba1+ cell morphology (%) in male and female mice in each of four subregions of the anterior P10 hippocampus: molecular layer (Mol, **E**), hilus **(F)**, CA3 **(G)**, and CA1 **(H)**. In each subregion, Iba1+ Thick and Thin cells were evident, while Iba1+ Round and Stout cells were far more rare, and Round cells were sometimes absent. Analysis of percentage of Iba1+ cells in each morphology category revealed a sex-dependent effect. In the Mol **(Fig. 5E)**, female mice had 34% more Thick Iba1+ cells vs. male mice, but 29% fewer Thin Iba1+ cells vs. male mice. In the Hilus **(Fig. 5F)**, female mice had 39% more Thick Iba1+ cells vs. male mice. In CA3 **(Fig. 5G)**, female mice had borderline (p=0.052) 24% fewer Thin Iba1+ cells vs. male mice. In contrast to Mol, Hilus, and CA3, in CA1 there was no difference by sex in regard to Iba1+ cell morphology classification **(Fig. 5H)**.

IMAGE ANALYSIS & MORPHOLOGY CLASSIFICATION

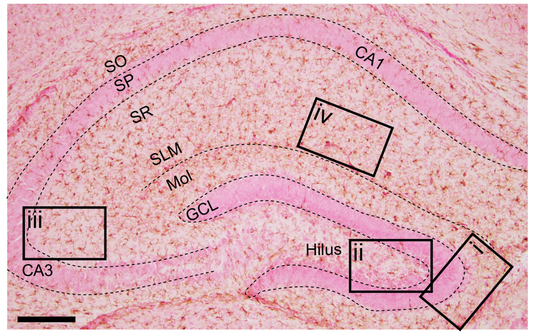


Figure 2. Hippocampal subregions analyzed for Iba1+ cell density and morphology.
This photomicrograph displays a P10 mouse hippocampal coronal brain section (-2.05mm from bregma) immunostained with an anti-Iba1 antibody and counterstained with FastRed. Image was taken at 400x (objective: 40x, 0.9 NA). Principal cell layers of the dentate gyrus (DG; granule cell layer, GCL) and Ammon's horn (pyramidal cell layer or stratum pyramidal [SP] in CA1 and CA3) are darker pink and outlined with dashed lines. The hippocampal fissure that separates the DG molecular layer (Mol) from the CA1 stratum lacunosum moleculare (SLM) is shown as a single dashed line. The four outlined rectangles show the precise placement used to collect the data in this study: (i) DG Mol, (ii) DG Hilus, (iii) CA3 stratum radiatum (SR) and stratum lucidum (not shown), and (iv) CA1 SR and SLM. SO, stratum oriens. Scale bar=200um.

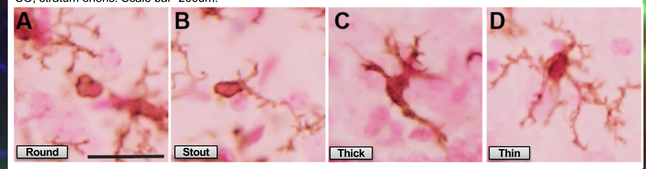


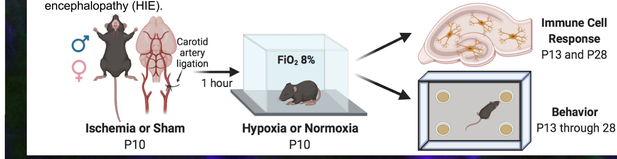
Figure 3. Microglia Cell Morphology Classification Photomicrographs from the P10 hippocampus of Iba1+ cells that are representative of one of four morphological classifications: **(A)** Round or amboid-like (no processes), **(B)** Stout (short, thick process), **(C)** Thick (soma with several thick processes), **(D)** Thin (soma with several thin processes). Scale bar = 25um and applies to **(A-D)**.

CONCLUSIONS

- While **no sex difference** was found in **microglial density** between male and female mice in the four hippocampal subregions at P10, the analyzed data does show a **sex difference** in **microglial morphology** in the **Mol** and **Hilus**.
- **Female mice** had **more Thick microglial cells** in the **Mol** and **Hilus**, and **fewer Thin microglial cells** in the **Mol** and **CA3** in comparison to males.
- **Additional analyses** are needed to **evaluate the implications of morphological differences on microglial function**, as the meanings of morphological categories are still debated.
  - In the **adult rodent**, similar microglia morphology categories have been used as a **gradient** to reflect the **degree of "activation"** of microglia; **Round microglia** (ameboid, no processes) are considered to be relatively **more active than the ramified and presumably surveilling Thin microglia**.
  - In contrast, in the **early postnatal rodent**, **microglia morphology** categories are proposed to reflect the **degree of "age"** or **maturation** of the microglia; **Round microglia** are considered relatively **younger** and **less mature** than the **ramified Thin microglia**.
- With our analysis, it is **not possible to know** whether the **greater percentage of Thick** and **lower percentage of Thin microglial cells** in the **female vs. male Mol** means **microglia in female mice** are **"younger"** or **"more active"** than those in **male mice**.

ONGOING & FUTURE DIRECTIONS

- **Quantifying microglial cell densities** and **classifying microglial morphologies** in **P10 intermediate** and **posterior hippocampal** sections, and in **additional timepoints**: **P3** and **P7** to **compare** the **developmental trajectory of microglia** between male and female mice.
- **Assessing** whether there are **sex differences** in **microglial cell density** and **morphology** following **injury**, using a **mouse model** of **perinatal hypoxic-ischemic brain injury** to model the human condition of hypoxic-ischemic encephalopathy (HIE).



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