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Differential plasticity of microglial cells in the rostrocaudal neuraxis of the accessory olfactory bulb of female mice following mating and stud male exposure

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ABSTRACT

The formation of an olfactory recognition memory by female mice for the stud male pheromones requires two fundamental conditions: incidence of mating and retention of the stud male with the female for a critical 6 h interval following mating. This biologically critical recognition memory results from plasticity of reciprocal dendrodendritic synapses in the accessory olfactory bulb (AOB). In this study, a microglia marker antibody (ionized calcium-binding adaptor protein, Iba1) was used to determine how mating and stud pheromones affect microglia in the AOB rostrocaudal axis in female mice. The results showed that compared with estrus and mating only, mating and pheromone exposure significantly increased Iba1 immunoreactivity in the AOB evidenced by increased complexity of ramified microglial processes characteristic of resting microglial morphological phenotype, particularly in the rostral AOB. The density of Iba1 staining after mating and stud pheromone exposure was higher in the rostral – compared to caudal – AOB and was most prevalent in the external plexiform layer, the site of reciprocal mitral-granule dendrodendritic synapses. While cells with activated phenotype were observed in caudal AOB during estrus, mating/pheromone exposure appeared to induce a morphological transformation to the resting microglia phenotype. Since previous evidence implicate the rostral AOB in processing pheromonal signals and microglial cells monitor active synapses, these observations have important functional implications for a potential role for microglia in processing pheromonal signals in the AOB during the formation of olfactory memory.

1. Introduction

Microglia cells make up about 5–15% of all female mouse cells in the mammalian brain [24] and exist in two functional morphological phenotypes. The first, activated microglia, have retracted processes and a large soma [23,26–28], function as scavenger cells but also constitute an extremely plastic and alert resident immune defense system within the brain [21] by responding to virtually any kind of disturbance in the nervous system [1]. Consequently, they exercise immune surveillance necessary for the maintenance of normal brain tissue homeostasis [19]. The second, the resting or ramified microglia, have a small cell body with numerous highly branched processes or ramifications [26,37]. These highly motile processes scan their microenvironment [8,19] and can transform to several grades of activated microglia phenotype depending on 'on' signals that promote activation and 'off' signals that repress it [2,10]. Information is scanty on the in vivo dynamics of microglia in functional plasticity within discrete neural systems. Also, unlike neurons and other glial cells which display regional differences, little is known about the functional implication of microglial cells heterogeneity [22]. For example, although a heterogeneous population of microglial cells exists within the accessory olfactory bulb (AOB) of female mice [33], the functional implication of the different microglia phenotypes is unknown.

Mating activates synaptic mechanisms in the female mouse AOB resulting in the formation of an olfactory memory for the stud male pheromones present immediately within 6 h of mating [4,17,18,20,31,32]. This biologically critical memory prevents the initiation of neuroendocrine mechanisms that would result in pregnancy block such as would be caused by strange male pheromones. This memory, formed within the critical period of 6 h following mating and stud male presence, lasts at least thirty days and involves the mitral-granule cells reciprocal dendrodendritic synapses in the external plexiform layer of the AOB [4,16,18]. Processing of pheromonal signals involves glutamate,

Abbreviations: AOB, accessory olfactory bulb; Iba1, microglia-specific ionized calcium binding adaptor binding protein-1; E, estrus; M-only, mating only; M + 1, mating plus 1 h; M + 2, mating plus 2 h; E_{caudal}, estrus caudal AOB; $E_{rostral}$, estrus rostral AOB; M-$only_{caudal}$, mating-only caudal AOB; M-$only_{rostral}$, mating-only rostral AOB; M + 1_{caudal}, caudal AOB mating plus 1 h; M + $1_{rostral}$, rostral AOB mating plus 1 h; M + 2_{caudal}, caudal AOB mating plus 2 h; M + $2_{rostral}$, rostral AOB mating plus 2 h.

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GABA [25,29], noradrenaline [19], nitric oxide synthase [31,32,34] and a metabotropic glutamate receptor subtype-2 which reduces GABA inhibitory transmission [17]. These synaptic interactions result in the formation of olfactory memory for male pheromones present during the critical 4–6 h period after mating. Although some evidence link microglia with memory [11,15], synaptic remodeling [38] or the formation and maintenance of certain synaptic structures [27], little is understood about how endogenous microglial cells become engaged during the processing of specific physiological stimuli. Hence, the heterogeneous distribution of microglial cells in the female mouse olfactory bulb [33] raised the possibility of microglia involvement in bulbar physiology. The AOB is a suitable model to examine the possible physiological role of microglial cells in signal processing. First, it is the first relay station in processing pheromonal inputs into the accessory olfactory system and is known to exhibit robust signal-induced plasticity. Second, resting and activated phenotypes of microglial cells have preferential distribution in the rostral and caudal AOB, respectively [33]. Third, the non-immune actions of microglia are regulatory and respond to environmental cues including sex steroids [24]. Finally, mating and pheromone exposure can induce two types of synaptic plasticity in the AOB [6]. Therefore, the aim of this study was to determine how mating and stud pheromone exposure, the two necessary and sufficient conditions for the formation of olfactory memory, affect microglial cells within distinct AOB subdivisions in female mice using the microglia marker (ionized calcium binding adaptor protein-1, Iba1).

2. Materials and methods

Adult males and virgin females (Balb/c strain, Jackson Lab., Maine, USA) housed at 12:12 reversed light cycles (lights on at 19.00 h), $23 \pm 1\,^{\circ}C$ room temperature and unlimited access to food and water were used. The estrous cycle was monitored daily by vaginal swabs and estrus mice were mated with conspecific males. The males were changed if within 15 min, mounting had not occurred and visual confirmation of a vaginal plug defined successful mating. The neurotransmitter changes responsible for olfactory memory occurs within 2 h after mating [5] and mating/pheromone exposure induce different AOB synaptic mechanisms [6]. In the basic olfactory memory model, the stud male is left with the female following mating for the formation of recognition memory for the stud pheromones, usually within maximum of 6 h. Hence, pheromone exposure is herein defined as presence of stud after mating and the female mice ($n = 5$–7/group) were grouped as follows: estrus (E), mating only (M-only), mating plus 1 h pheromone exposure (M + 1) and mating plus 2 h pheromone exposure (M + 2). At the end of the experiments, the mice were anesthetized with Avertin (2,2,2-tribromoethanol; 0.25–0.5 mg/g administered intraperitoneally) and processed for Iba1 immunohistochemistry using 20 μm thick cryostat sections through the AOB neuraxis [7] as described previously [33].

Photomicrographs through the AOB neuraxis were acquired using NIS-Elements Advanced Research (AR) 3.1 imaging software and a NIKON Eclipse 80i microscope fitted with a NIKON digital sight camera. Images for semi-quantization were taken at 40-fold objective magnification (12-bit, 1280×1024 pixels; 0.16 μm/pixels), saved as jpeg files and were converted by the camera's software to 8-bit images with 256 brightness levels. These images were analyzed with the imaging software using the same settings for intensity of staining, density of staining per unit area and circularity (a measure of the compactness of a shape with a circle being the most compact shape). The more compact a shape is, the more closely it resembles a circle (which has a circularity value of 1.0); this dimensionless index was applied for two-dimensional assessment of the processes in the resting and activated morphological phenotypes. Data were obtained from the different AOB regions, pooled for each animal and expressed as mean ± SEM values. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test and Bartlett's test for equal variance among the different groups and $p < 0.05$ was used to reject the null hypothesis.

3. Results

To minimize inter-staining variability, the sections were processed simultaneously. The entire AOB neuraxis was subdivided into rostral and caudal AOB based on the mouse brain atlas [7]. In estrus (Fig. 1A, E, and F), Iba1 immunoreactivity was present throughout the glomerular, external plexiform, granule and mitral cellular layers of the AOB and the adjacent main olfactory bulb. Particularly, numerous ramified/resting phenotype cells were in the external plexiform layer compared with the granule or mitral cell layers (Fig. 1E versus F). This is significant because recognition memory involves mitral-granule cell reciprocal dendrodendritic synapses [16], present in the external plexiform layer [4]. Elongated unipolar, bipolar and multiple processes but less ramifications than the ramified/resting morphological phenotype present were also in the external plexiform layer. Compared with estrus, mating and pheromone (M + 2) significantly increased Iba1 staining (Bonferroni's multiple comparison test) along the entire AOB (Fig. 1B–D vs. A; Fig. 2A). Further analysis with Bartlett's test for equal variances indicated that the variances differed significantly ($p < 0.0001$) suggesting intrabulbar region-specific differences. For instance, staining intensity in $M + 2_{rostral}$ was significantly higher than the corresponding $E_{rostral}$ and M-$only_{rostral}$ ($p < 0.05$, Bonferroni test; Fig. 2B). Also, staining was significantly higher in $M + 1_{rostral}$ and $M + 2_{rostral}$ compared with $M + 1_{caudal}$ and $M + 2_{caudal}$, respectively.

In addition, the staining area (1280×1024 pixels; 0.16 μm/pixel) was significantly different along the entire AOB (Fig. 2C). Subregional variances ($p = 0.0076$, Bartlett's test for equal variances; Fig. 2D) revealed that: (i) $E_{rostral}$ was significantly ($p < 0.05$; $t = 6.2$, df = 9) higher than the corresponding E_{caudal} suggesting predominant Iba1 staining in the rostral AOB; (ii) $M + 2_{rostral}$ was significantly higher than $E_{rostral}$, suggesting the importance of sustained pheromone exposure after mating to rostral AOB microglia plasticity; (iii) M-$only_{caudal}$ and $M + 2_{caudal}$ showed higher Iba1 staining than the corresponding E_{caudal}, suggesting that mating alone as well as sustained exposure to stud pheromones induce changes in the microglial cells. Taken together, these observations reveal activity-dependent (re)organization of the bulbar microglia domain. Hence, compared with estrus, mating plus stud pheromone exposure progressively increased the total immunoreactivity of Iba1 within the AOB subregions (Fig. 2B) with the highest staining observed in M + 2 animals compared to M + 1 and M-only mice.

Higher magnification photomicrographs (Fig. 1G–J) were semi-quantified for circularity (Fig. 2E and F), a two-dimensional geometric tolerance and a good index to compare the ramified arborizations of one microglia and another. Fig. 2E shows significantly different arborization in the AOB neuraxis of M-only, M + 1 and M + 2 compared with E. Within the AOB topography, there were significant differences (Fig. 2F) in $M + 2_{caudal}$ vs. $E_{rostral}$ ($p < 0.05$; $t = 3.5$); E_{caudal} vs. M-$only_{rostral}$ ($p < 0.001$; $t = 5.4$); M-$only_{rostral}$ vs. M-$only_{caudal}$ ($p < 0.05$; $t = 3.6$); M-$only_{rostral}$ vs. $M + 2_{rostral}$ ($p < 0.01$; $t = 4.3$) and M-$only_{rostral}$ vs. $M + 2_{caudal}$ ($p < 0.001$; $t = 5.4$). These observations underscore the idea of a topographical reorganization of the endogenous microglia following mating and pheromones. Interestingly, the predominant microglia in the caudal AOB was the