**Highlights**

- The microglial landscape is rearranged daily in the healthy brain.
- The microglial landscape can be altered by manipulating brain activity.
- Daily microglial landscape rearrangement occurs predominantly by translocation.
- Rearrangement of the microglial landscape is controlled by the P2Y12 receptor.

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**In Brief**

Using a chronic *in vivo* imaging approach, Eyo et al. show that the physical positions of brain microglia change daily and that these changes increase following certain experimental manipulations. The mechanism underlying these changes involves cell translocation controlled by microglial-specific P2Y12 receptors.

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P2Y12R-Dependent Translocation Mechanisms Gate the Changing Microglial Landscape

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SUMMARY

Microglia are an exquisitely tiled and self-contained population in the CNS that do not receive contributions from circulating monocytes in the periphery. While microglia are long-lived cells, the extent to which their cell bodies are fixed and the molecular mechanisms by which the microglial landscape is regulated have not been determined. Using chronic in vivo two-photon imaging to follow the microglial population in young adult mice, we document a daily rearrangement of the microglial landscape. Furthermore, we show that the microglial landscape can be modulated by severe seizures, acute injury, and sensory deprivation. Finally, we demonstrate a critical role for microglial P2Y12Rs in regulating the microglial landscape through cellular translocation independent of proliferation. These findings suggest that microglial patrol the CNS through both process motility and soma translocation.

INTRODUCTION

Microglia, the tissue-resident macrophages of the CNS, are extremely dynamic and exhibit elaborate remodeling of their processes (Davalos et al., 2005; Nimmerjahn et al., 2005), through which they make repeated contacts with other CNS elements (Tremblay et al., 2010; Wake et al., 2009). Roles are now recognized for microglia in early CNS development where they clear apoptotic cells (Marín-Teva et al., 2004), facilitate brain wiring (Squarzoni et al., 2014), and regulate synaptic development (Paolicelli et al., 2011; Schafer et al., 2012). In adults, microglia participate in mechanisms underlying learning and behavior (Chen et al., 2010; Parkhurst et al., 2013).

Interest in understanding microglial homeostatic maintenance has revealed that they are an autonomous self-renewing population (Ajami et al., 2011) lacking long-term contributions from peripheral cells. In both the rodent and human brain, microglial numbers are maintained throughout adult life (Askew et al., 2017; Davies et al., 2017). Indeed, it is assumed that, as somatically stationary but motile cells, the microglial landscape is largely fixed. However, the mechanisms by which the microglial landscape is organized and maintained are not entirely clear. To address this, we used chronic two-photon in vivo imaging in the intact brain of double-transgenic CX3CR1GFP/+:Thy1YFP/+ mice to visualize microglia (CX3CR1-GFP) and dendrites (Thy1-yellow fluorescent protein [YFP]), which were used as landmarks for repeated positional imaging (Eyo et al., 2014; Feng et al., 2000; Jung et al., 2000). Specifically, we studied the daily changes in the microglial landscape, modulated these changes experimentally, and determined the mechanistic processes as well as a molecular factor underlying such changes.

RESULTS

Daily Microglial Landscape Rearrangement Occurs via Translocation in the Healthy Brain

Two to four weeks after window implantation, microglia underwent a round of activation and returned to a ramified morphology (Figure S1A). To re-locate specific fields of view (FOVs), blood vessels and dendrites were used as gross and fine landmarks, respectively (Figure S1B). In the limb/trunk region of the primary somatosensory cortex, microglia exhibited a rearrangement of their landscape: some cells emerged ("gained") into the FOV at a position where no cells resided previously, while others were missing ("lost") from the FOV at positions where they resided previously (Figure 1A). Approximately 10%–15% of microglia were rearranged (Figure S2A), regardless of the frequency of daily observation (Figure S2B) or gender (Figure S2C). Similar degrees of rearrangement occurred in both the superficial layer I (depth, 50–120 μm) and deeper layer II/III (depth, 180–240 μm) of the cortex (Figure 1B), though rearrangement differed between the limb/trunk and whisker barrel areas (Figure 1C).

Since coupled proliferation and death (Askew et al., 2017) or microglial translocation could explain these findings, we assessed proliferation by bromodeoxyuridine (BrdU) injection. BrdU rarely co-localized with cortical microglia, though robust co-localization was detected in the dentate gyrus (DG) as expected (Askew et al., 2017; Tay et al., 2017) (Figures 1D and 1E). Similar minimal co-localization in the cortex was observed for microglia with Ki67 (Figure S3). We investigated contributions
by receptors implicated in microglial proliferation and translocation/chemotaxis: Trem2 (Zhang et al., 2014; Zheng et al., 2017) and CX3CR1 (Cardona et al., 2006; Liang et al., 2009), respectively. Trem2/−/− microglia showed a 59% reduction in cortical (Figure 1G) and an 87% reduction in DG (data not shown) proliferation. Trem2/−/−, but not CX3CR1/−/−, mice had reduced microglial numbers in 2-month-old mice (Figure 1G). However, neither genotype differed from wild-type in microglial rearrangement (Figure 1H) or “gained” and “lost” cells (Figure 1I). This indicates that the mechanism underlying microglial rearrangement couples “gained” and “lost” cells independent of proliferation. Carefully monitoring microglia daily, we noted both static and, presumably, translocating cells (Figure 1J).

Acute Experimental Modulation of the Microglial Landscape

Next, we sought to determine whether the observed daily rearrangement could be experimentally altered. We first tested...
the possibility that systemic activation of microglia could alter their rearrangement via intraperitoneal (i.p.) lipopolysaccharide (LPS) (2 mg/kg) treatment. However, cortical microglia did not alter their daily rearrangement within 24 hr of LPS treatment (Figures S2D and S2E). Then, we turned our attention to systemic brain hyperactivity to modulate the microglial landscape. Our previous work showed that i.p. KA-induced seizures altered microglial morphologies (Eyo et al., 2014, 2017). Therefore, we used this treatment paradigm to experimentally alter brain activity and found that severe (stage-5), but not mild (stage-3), seizures transiently increased microglial rearrangement especially within the first 24 hr (Figures 2A–2C). By the third day of seizures, basal levels were restored and maintained thereafter for at least a month (Figures S2D and S2E). Under these conditions, microglial translocations could also be detected (Figure 2F). Together, these results indicate that global increases in neuronal activity resulting from seizures increased microglial somatic rearrangements.

To complement this global approach, we tested the effect of more localized brain manipulations on the rearrangement of the microglial landscape. First, we performed a localized laser-induced tissue injury and monitored the microglial landscape at 6-hr intervals and report obvious somatic translocations within the first few days, with cells translocating the most within the first 12 hr of the injury (Figures 3A and 3B; Video S1). Then we monitored the microglial landscape in the barrel cortex following whisker trimming and also observed increased changes in the microglial landscape of whisker-trimmed mice (Figures 3C–3E). Thus, changes in brain activities globally and locally alter the microglial landscape.

**P2Y12 Receptors Regulate the Microglial Landscape**

Finally, we sought to determine a molecular regulator of the changing microglial landscape. Though we showed that Trem2 receptors regulate microglial proliferation (Figure 1G) and CX3CR1 receptors are known to regulate microglial process activity (Liang et al., 2009), we ruled out roles for these two receptors in the microglial landscape rearrangement (Figure 1H). Considering established roles for microglial P2Y12Rs in microglial process movements in situ (Haynes et al., 2006; Wu et al., 2007) and soma movements in vitro (Honda et al., 2001), we tested its involvement in regulating the daily microglial landscape.

P2Y12Rs are highly and uniquely expressed by brain microglia in the healthy brain (Hickman et al., 2013; Zhang et al., 2014). We found an upregulation of these receptors on...
microglia following KA-induced seizures and whisker trimming in the mouse cortex (data not shown). Remarkably, P2Y12−/− mice exhibited significantly reduced microglial rearrangement under basal conditions (Figures 4A and 4B), with a similar percent of “gained” and “lost” cells (Figure 4C) and no significant difference in microglial proliferation (Figure 4D). Consistently, following seizures and whisker trimming, there was a significant reduction in rearranged microglia in P2Y12−/− mice (Figures 4E and 4F; Figure S4). Translocation in wild-type microglia occurred in a fashion where days of movement were often interrupted by a day (or days) of stasis (Figure 4G; Video S2), but microglial translocation was rarely observed in P2Y12−/− mice (Figure 4G; Video S3).

We classified cell behavior into (a) “death” (cells missing from the FOV compared to the previous day and that were not present in any other region of the FOV; Figure 4H; Video S4, left); (b) “proliferation” (cells emerging into the FOV compared to the previous day and that did not come from any other region of the FOV; Figure 4I; Video S4, right); and (c) “translocation,” as defined previously. Most cell changes belonged to the translocation category (5.8 ± 0.2%; n = 21 FOVs from 7 mice), while the rest were split between the “death” (2.1 ± 0.3%) and “proliferation” (1.3 ± 0.3%) categories. P2Y12−/− mice exhibited similar death (1.6 ± 0.2%) and proliferation (1.2 ± 0.3%) categories but showed significantly reduced translocating cells (1.0 ± 0.2%; n = 23 FOVs from 7 mice; Figure 4J). Therefore, we conclude that P2Y12 receptors regulate the microglial landscape predominantly through translocation mechanisms.

**DISCUSSION**

Microglia carry out elaborate surveillance of the brain by their never-resting process extensions and retractions (Davalos et al., 2005; Nimmerjahn et al., 2005). These processes interact with neuronal elements (Kato et al., 2016; Li et al., 2012), while microglial somata are assumed to be sessile. Evidence suggest a high capacity for soma translocation in murine microglia ex vivo (Eyo et al., 2016) and zebrafish microglia in vivo (Svahn et al., 2013) during early development. In addition, microglia in the adult Alzheimer’s brain exhibit locomotory capacities (Füger et al., 2017; Fuhrmann et al., 2010). Our findings suggest that a small population of microglia retain this ability in the healthy adult brain. Thus, microglial surveillance of the brain also (at least, for some microglia) includes cellular translocation.

Recently, it was suggested that the microglial landscape is altered predominantly by a coupling of proliferation and apoptosis (Askew et al., 2017), whereas other approaches suggested that cortical microglial proliferation is minimal (Tay et al., 2017). Our results indicate that proliferation and death are not the predominant mechanisms regulating the changing microglial landscape for three reasons. First, the degree of cortical proliferation is much too low, between 0.45% from our fixed tissue studies and 1.3% from our chronic in vivo imaging studies, when compared to the degree of daily re-arranged cells (10%–15%). Second, the percentage of daily translocating cells (6.8%), when doubled as “gained” and “lost” cells, would account for the 10%–15% daily rearrangement, with little contribution from proliferation or apoptosis. Finally, genetic ablation of
Trem2, while reducing microglial proliferation (Zheng et al., 2017), did not significantly alter the basal daily re-arrangement. Our proliferation numbers from daily imaging (1.3%) is likely an overestimation, because some of the cells we identified as suddenly appearing in the FOV may have simply migrated into the FOV. Conversely, our proliferation quantification with BrdU (0.45%) is likely an underestimation, as we gave just one pulse of BrdU, which circulates for shorter than 24 hr and would miss some of the proliferating cells within that time period. Alternative to proliferation, we provide robust evidence of translocating microglia that accounts for the bulk of the daily changes in the microglial landscape.

Finally, we provide genetic evidence that, while proliferation and cell death are unaffected by a P2Y12R deficiency, translocation capacities are significantly reduced. P2Y12Rs are a molecular signature for brain microglia, as revealed by transcriptome studies (Hickman et al., 2013; Zhang et al., 2014). They function as the predominant receptor in microglial process chemotaxis toward injury (Haynes et al., 2006) and can heal the compromised blood-brain barrier by this chemotactic mechanism (Lou et al., 2016). Moreover, they mediate various microglial-to-neuronal physical interactions (Eyo et al., 2014, 2015, 2017) and are critical for synaptic plasticity in the visual system (Sipe et al., 2016). Since P2Y12Rs are required for microglial...
translocation, as shown in the present study, we would predict that they are important for adequate housekeeping surveillance functions of microglia. In summary, our findings demonstrate that, even in the homeostatic state, microglia should be better thought of as not merely stationary surveying cells but, more appropriately, as patrolling cells with migratory capacities.

**EXPERIMENTAL PROCEDURES**

**Animals**

Both male and female adult mice, 2 to 5 months of age, were used in accordance with institutional guidelines, as approved by the Institutional Animal and Care Committee (IACUC) animal care and use committee at the Mayo Clinic and Rutgers University. Heterozygous (CX3CR1GFP/F) reporter mice expressing GFP under control of the fractalkine receptor (CX3CR1) promoter (Jung et al., 2000) and transgenic mice (Thy1YFP reporter) expressing YFP (Feng et al., 2000) in a subset of pyramidal neurons under the control of the Thy1 promoter were purchased from the Jackson Laboratory. For some experiments, two-photons imaging of either kainic acid at 22–24 mg/kg or pilocarpine at 260–280 mg/kg. For P2Y12-deficient mice, kainic acid was administered at 18–20 mg/kg to allow robust seizures without death, as these mice are more susceptible to kainic-acid-induced seizures (Eyo et al., 2014). Seizure behavior was monitored under a modified Racine scale as follows: (1) freezing behavior; (2) rigid posture with raised tail; (3) continuous head bobbing and forepaws shaking; (4) rearing, falling, and jumping; (5) continuous occurrence of level 4; and (6) loss of posture and generalized convulsion activity (Eyo et al., 2014). Mice that progressed to at least stage 3 were used for subsequent chronic daily imaging of mild (stages 3/4) to severe (stages 5/6) seizures.

**Chronic Window Implantation**

Mice were anesthetized with isoflurane (5% for induction; 1.5% for maintenance and surgery) combined with oxygen fitted into a custom-made stereotaxic frame. Mice were maintained on a heating pad during surgery and then received a local subcutaneous injection of 15–20 mL 0.25% bupivicane and 0.1% lidocaine in 5% dextrose solution and mounted for imaging. In summary, our findings demonstrated

**LPS Treatment**

Mice with implanted windows were monitored for at least 3 days under basal conditions and subsequently received i.p. injections of LPS at 2 mg/kg.

**Experimental Manipulations**

**Seizure Induction**

Mice with implanted windows were monitored for at least 5 days under basal conditions and received i.p. injections of either kainic acid at 22–24 mg/kg or pilocarpine at 260–280 mg/kg. For P2Y12-deficient mice, kainic acid was administered at 18–20 mg/kg to allow robust seizures without death, as these mice are more susceptible to kainic-acid-induced seizures (Eyo et al., 2014). Seizure behavior was monitored under a modified Racine scale as follows: (1) freezing behavior; (2) rigid posture with raised tail; (3) continuous head bobbing and forepaws shaking; (4) rearing, falling, and jumping; (5) continuous occurrence of level 4; and (6) loss of posture and generalized convulsion activity (Eyo et al., 2014). Mice that progressed to at least stage 3 were used for subsequent chronic daily imaging of mild (stages 3/4) to severe (stages 5/6) seizures.

**Whisker Trimming**

Mice with implanted windows were monitored for at least 5 days under basal conditions, and under anesthesia, all whiskers on the contralateral side to the implanted window were trimmed daily for 5 consecutive days with a clipper (Wahl BravMini).

**Brdu Labeling and Analysis**

Brdu was used to label proliferating and recently post-mitotic cells in the brain. The BrdU solution was diluted in 1 M PBS just before use at a concentration of 10 mg/mL and intraperitoneally administered at 100 mg/kg in naive mice or 24 hr after mouse manipulation (seizures or whisker trimming). Mice were sacrificed 3 hr after the BrdU injection by perfusion, first with PBS and then with 4% paraformaldehyde (PFA). The brains were then incubated in 4% PFA overnight and transferred to 30% sucrose solution for at least 2 days. Brains were then cryosectioned to 15–20 μm thickness and attached to glass slides. For BrdU immunohistochemistry, the slides with brain sections were hydrated in Tris-buffered saline (TBS) for 10 s and transferred to a 50% formamide in 2× saline sodium citrate (SSC) solution at 65°C for 2 hr. Slides with brain sections were then placed in a 2× SSC solution at room temperature for 15 min and were transferred to a 2 N HCl solution at 37°C for 20 min and then a 0.1 M borate buffer at room temperature for 10 min. Slides with brain sections were then washed three times in TBS (pH 7.6) in room temperature for 10 min each. Next, brain sections were blocked with 3% normal goat serum (NGS) in 1× TBS + 0.3% Triton-X at room temperature for 1 hr and then overnight at 4°C in the primary antibody Iba1 (Wako Pure Chemicals Industries, 1:500) or anti-BrdU (Sigma, 1: 500), washed 3 times in 1× TBS for 5 min each, and then incubated in the secondary antibody donkey anti-rabbit (1:500) or donkey anti-mouse (1:500) at room temperature for 2 hr. Slides with brain sections were then rinsed three times in 1× TBS for 5 min each and mounted for imaging on an EVOS fluorescence microscope. Images of Iba1+ and BrdU-labeled tissues were collected at 10× magnification. Microglial proliferation was determined by assessing the colocalization of the BrdU signal with the Iba1 signal for microglia.

**Landscape Rearrangement Analysis**

For cell rearrangement analyses, microglia in FOVs from consecutive days within a volume of 60 × 330 × 330 μm between ~50 and 120 μm (180–240 μm) from the brain surface were compared. Individual cells were identified and marked with numbers from the previous day and transposed to the next day. Cell bodies were regarded as stable if they maintained their position within a 2-cell-body distance from the previous day (~10–15 μm). Cell bodies that were either absent or present at a distance of 2 cell bodies or more were marked as either “gained” if they were not there on the previous day or “lost” if they were there on the previous day but absent on the next day. The percent change of “rearranged” cells was determined as the number of both from each mouse, z stack images were collected at 1- to 2-μm intervals in several FOVs. For repeated imaging, blood vessels were used as gross landmarks, and dendrites were used as fine landmarks. To perform a general laser injury, we focused the laser 66× and parked it at 250 mW at 900 nm for 1–3 s.
“gained” and “lost” cells divided by the total number of cells in each FOV \( \times 100 \). Similar analysis, but specific for either “gained” or “lost” cells, was performed to determine the percent rearrangement of those cell groups (Figures 1 and 4C).

**Statistical Analysis**

Data were collected from at least 3 and up to 8 FOVs per mice and pooled together. Three to seven mice were used for each set of experiments. In our re-arrangement studies comparing changes in the microglial landscape before and after treatment, power analysis for paired t tests \((\alpha = 0.05, \beta = 0.2)\) was performed to establish the sufficiency of the sample size using GraphPad software. The power values achieved were as follows: (1) for an effect size of 30% change (from \(-13\%\) in control) in microglial rearrangement in the limb/trunk cortex, the power value was 0.90 for kainic acid (KA) treatment \((n = 6\) mice; combined SD: 9.83); (2) for an effect size of 20% change (from \(-13\%\) in control) in microglial rearrangement in the limb/trunk cortex, the power value was 0.80 for pilocarpine treatment \((n = 3\) mice, combined SD: 8.58); Student’s t test was used for statistical analysis, Bonferroni correction was used for multiple comparisons, and significance was determined with a p value of 0.05 or less.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and four videos and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.04.001.

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**AUTHOR CONTRIBUTIONS**

U.B.E., P.X., and L.-J.W. designed the study and wrote the manuscript, with help from all authors. U.B.E. performed most of the experiments; M. Mo, M.-H.Y., and J.L. performed some experiments; U.B.E., J.L., M. Murugan, M.-H.Y., and R.Y. performed data analysis; D.J.M. provided training for the chronic window implantation studies and valuable discussions for the project.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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