Neuronal network activity controls microglial process surveillance in awake mice via norepinephrine signaling

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Microglia dynamically survey the brain parenchyma. Microglial processes interact with neuronal elements; however, what role neuronal network activity plays in regulating microglial dynamics is not entirely clear. Most studies of microglial dynamics use either slice preparations or in vivo imaging in anesthetized mice. Here we demonstrate that microglia in awake mice have a relatively reduced process area and surveillance territory and that reduced neuronal activity under general anesthesia increases microglial process velocity, extension and territory surveillance. Similarly, reductions in local neuronal activity through sensory deprivation or optogenetic inhibition increase microglial process surveillance. Using pharmacological and chemogenetic approaches, we demonstrate that reduced norepinephrine signaling is necessary for these increases in microglial process surveillance. These findings indicate that under basal physiological conditions, noradrenergic tone in awake mice suppresses microglial process surveillance. Our results emphasize the importance of awake imaging for studying microglia–neuron interactions and demonstrate how neuronal activity influences microglial process dynamics.

Microglia are the resident immune cells of the CNS that interact with neuronal elements in the brain parenchyma1–3. Under normal conditions, microglia participate in synaptic pruning during brain development and in synaptic remodeling during learning4–6. Within neurological disorders such as pain, stroke, epilepsy, autoimmune diseases and neurodegeneration, microglia play key roles in pathology and can exert either detrimental or beneficial effects7. Multiple lines of evidence suggest that contact between microglial processes and neuronal dendrites can lead to changes in neuronal structure and function. Particularly during early developmental periods, microglial process contact can induce filipodia outgrowth from dendrites8,9. Conversely, in later development, microglial contact results in a higher probability of spine elimination7. This later interaction may be a key component of synaptic pruning and neuronal circuit formation10,11. In the mature brain, microglia can promote spine formation via BDNF signaling and thereby interact with the molecular mechanisms of learning and memory11. In addition, microglial processes can enhance dendritic calcium activity and may influence neuronal synchrony at the population level12. While microglial contact can influence neuronal structure and function across the developmental lifespan, it is unclear how microglial process dynamics are regulated in the basal state, particularly in the awake animal.

As surveilling immune cells, microglia dynamically respond to changes in the microenvironment with their remarkably motile processes13–15. To date, most studies of microglial process dynamics have occurred in slice preparations or under the constraints of general anesthesia in vivo. These studies showed that following laser-induced injury, local microglia quickly respond to damage by extending processes to isolate the injury site via a signaling mechanism comprising ATP and the P2Y12 receptor (encoded by Pty12 (also known as P2ry12))13,14,15,16. In addition, suppression of the neuronal network via tetrodotoxin (TTX) results in either reduced microglial–neuron contact16 or no significant changes in microglial process surveillance17. Conversely, enhancements of the neuronal network via interneuron inhibition can lead to increases in process sampling4. Under more extreme circumstances, suprathreshold neuronal activity or acute seizure induction can further induce microglial process extension and convergence toward hyperactive neurons18,19. Thus, dynamic microglial processes are able to sense brain injury and neuronal activity in basal and disease contexts. More specifically, microglial process dynamics appear to follow activity-dependent patterns in anesthetized animals.

Despite the importance of microglial process dynamics in neuronal interactions, we currently do not know how microglial processes behave under the most physiologically relevant conditions (that is, in the awake state). Using in vivo two-photon imaging in awake mice, we surprisingly find that microglial processes are less dynamic and survey a smaller territory before neuronal activity is suppressed by general anesthesia. In awake animals, other methods of neuronal suppression, including sensory deprivation (whisker trimming) and optogenetic inhibition, mimicked the relative increase in microglial process surveillance. We found that adrenergic, but not purinergic, signaling controls the microglial surveillance phenotype observed in the awake state. Accordingly, in the awake state, pharmacological inhibition of β2-adrenergic receptors or chemogenetic reductions of norepinephrine (NE) release from the locus coeruleus (LC) resulted in microglial process extension and increased surveillance territory. Thus, under awake conditions, microglial processes have constrained dynamics set by tonic NE tone. Under anesthesia, loss of NE tone becomes permissive for increasing microglia surveillance. Our findings in the awake animal demonstrate that microglial
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Next, we used Cx3cr1 knockout mice to examine microglial process interaction with neuronal dendrites in vivo. We found that microglial processes displayed longer contact duration and a larger contact area with dendrites under anesthesia compared to the awake condition (Supplementary Fig. 4b,c). These latter results suggest that increased microglial process surveillance could be translated into enhanced microglia–neuron interaction under anesthesia.

Whisker trimming increases microglial process surveillance in awake mice. Suppressed neuronal activity precedes the increase in microglial process surveillance under general anesthesia, which suggests that neuronal activity might negatively regulate microglial process dynamics. However, general anesthesia also alters metabolism, blood pressure and cardiac output\(^2\). For these reasons, we additionally employed unilateral whisker trimming as an approach to reduce neuronal activity and to study microglial process dynamics\(^3\). Whisker trimming is a sensory-deprivation method that suppresses neuronal activity in the contralateral barrel cortex, as demonstrated by calcium imaging in GCaMP6s-expressing cortical neurons (Supplementary Fig. 5a–c; Supplementary Video 3). In response to whisker trimming, microglia in the contralateral barrel cortex began to increase their process area and survey a greater territory of the barrel cortex, similarly to responses we observed for general anesthesia (Fig. 2a,b; Supplementary Video 4). Moreover, the latency for microglia to increase their surveillance after whisker trimming had a similar delayed-onset time course compared to anesthesia (Supplementary Fig. 5d,e). Sensory deprivation only increased process surveillance in microglia of the contralateral barrel cortex, with no effect observed for microglia of the ipsilateral barrel cortex (Fig. 2c,d). Therefore, these results demonstrate that reductions in region-specific sensory input selectively and negatively regulate microglial process surveillance.

Our whisker trimming experiments further substantiate the observation that reduced neuronal activity enhances microglial process surveillance. To determine whether sensory deprivation and general anesthesia increase microglial process surveillance through unique or shared mechanisms, we determined whether their combination had an additive effect on microglial surveillance. To this end, we performed unilateral whisker trimming followed by isoflurane inhalation after an additional 30 min. We found that whisker trimming increased microglial process surveillance, but the addition of isoflurane anesthesia did not further increase process surveillance (Fig. 2e,f). Thus, whisker trimming and isoflurane anesthesia do not have additive effects on microglial process surveillance, which suggests that they may share a similar mechanism for triggering an increase in microglial process dynamics.

Both pharmacological and optogenetic inhibition of neuronal network activity increase microglial process surveillance in awake mice. Results from the general anesthesia and sensory-deprivation paradigms indicate that neuronal activity regulates microglial process dynamics. To directly test this idea, we manipulated cortical network activity through pharmacological and optogenetic approaches and then examined microglial process surveillance. We used an intracerebral catheter and applied a potent and selective agonist for GABA\(_A\) receptors, muscimol (870 \(\mu\)M, 10 \(\mu\)l), to suppress neuronal network activity in the somatosensory cortex (Fig. 3a). Indeed, we found that muscimol application in awake mice reduced neuronal calcium activity in the somatosensory cortex (Supplementary Fig. 6; Supplementary Video 5). Coincident with the reduction in neuronal calcium activity, microglia increased their process surveillance (Fig. 3a,c; Supplementary Video 6). By contrast, intracerebral application of artificial cerebrospinal fluid (ACSF) did not alter microglial process surveillance (Fig. 3b). Similarly, we found that microglial surveillance increased after intracerebral application of TTX (10 \(\mu\)M) to inhibit neuronal firing in the awake animal (Supplementary Fig. 7). Thus, pharmacological inhibition
of local neuronal activity in the cortex is able to recapitulate the changes in microglial process dynamics induced by general anesthesia or whisker trimming.

Next, we employed an optogenetic strategy to suppress neuronal activity. Channelrhodopsin 2 (ChR2) was expressed in VGAT-positive GABAergic neuron populations using VGAT-ChR2 transgenic mice. Optogenetic activation of GABAergic cell populations in this transgenic line has previously been shown to suppress neuronal network activity\(^7\). Proper ChR2 expression and function in VGAT-positive interneurons was confirmed in our experiments by whole-cell patch-clamp recording in acute brain slices (Fig. 4a,b; Supplementary Fig. 8a,b). We found that in awake mice, activation of VGAT-ChR2 by 470-nm blue light (10 Hz, 10 min) increased microglial process surveillance (Fig. 4c,d; Supplementary Video 7; Fig. 8c,d). Building on findings obtained from the general anesthesia experiments ( Supplementary Fig. 3), transient and reversible network inhibition via optogenetic methods triggered a prolonged increase in microglial surveillance that was sustained for at least 20 min (Supplementary Fig. 8c,d). Together, these results demonstrate that inhibition of local cortical network activity by pharmacological or optogenetic means can trigger an increase in microglial process surveillance in awake mice.

NE signaling regulates microglial process surveillance in awake mice. Next, we investigated the molecular mechanisms underlying the observed increase in microglial process surveillance following neuronal activity suppression. To date, the ATP/ADP-sensing P2Y12 receptor has been shown to regulate microglial process extension in a variety of contexts\(^14,20,25\). To test whether P2Y12 receptor signaling mediates the observed increase in microglial process surveillance, we imaged process surveillance in P2Y12 receptor-deficient mice (Cx3cr1\(^{-/-}\);P2y12\(^{-/-}\)). Surprisingly, we found that in P2y12\(^{-/-}\) mice,
isoflurane anesthesia still increased microglial process surveillance (Fig. 5a,b). Therefore, unlike injury or hyperactivity-induced microglial process extension\textsuperscript{[25,26]}, the observed increase in microglial surveillance following isoflurane is not regulated by P2Y\textsubscript{12} signaling. Fractalkine signaling and its receptor CX3CR1 are reported to promote microglial process dynamics and microglia–neuron interactions\textsuperscript{[27]}. Therefore, we used Cx3cr1\textsuperscript{−/−} (Cx3cr1\textsuperscript{GFP/GFP}) mice to test whether CX3CR1 contributes to the increased anesthesia-induced microglial surveillance. We found that isoflurane anesthesia in Cx3cr1\textsuperscript{−/−} mice similarly increased microglial process surveillance (Fig. 5c,d), which suggests that fractalkine signaling is not involved in increasing microglial surveillance following anesthesia.

Previous studies have found that primary neurotransmitters such as glutamate and GABA cannot directly induce microglial process chemotaxis\textsuperscript{[20,28]}. For these reasons, we focused instead on investigating whether the neurotransmitters acetylcholine, dopamine, NE and serotonin affected microglial process dynamics. These neurotransmitters were specifically studied because their activity is significantly decreased by general anesthesia\textsuperscript{[29]}. We intracerebrally applied each neurotransmitter during isoflurane inhalation (Fig. 5e). Interestingly, we found that NE (3 mM, 10 µl) application, but not acetylcholine (5 mM, 10 µl) or serotonin (5 mM, 10 µl), prevented the increase in microglial process surveillance induced by isoflurane anesthesia (Fig. 5f). These results suggest that reduced NE signaling in the anesthetized condition selectively increases microglial process surveillance.

We further studied the regulation of microglial process surveillance by NE during whisker trimming and optogenetic inhibition.
adrenergic receptor antagonists on microglial dynamics (Supplementary Fig. 9). To explore whether adrenergic receptors control microglial process activity in awake mice, we examined the β2-adrenergic receptor antagonists propranolol (300 μM, 10 μl; Fig. 7b), a high-affinity antagonist of β1- and β2-receptors, and the highly selective β2-receptor antagonist ICI 118,551 (30 μM, 10 μl; Fig. 7c) induced an increase in microglial process surveillance in awake mice. In addition, an intracerebral injection of TTX (50 μM) followed by application of ICI 118,551 (10 μM) 30 min later did not further increase microglial surveillance (Supplementary Fig. 7). Thus, inhibition of β2-adrenergic receptors can increase microglial process dynamics.

Cortical NE tone is largely generated by noradrenergic neurons in the LC32, which project into the somatosensory cortex (Fig. 7d; Supplementary Video 8). Microglial processes are in close proximity to noradrenergic axons, as shown by labeling with adeno-associated viruses (AAVs; a combination of AAVrTH.Cre and AAV.FLEX.tdTomato; Fig. 7d). To directly study the effect of NE signaling on

β2-adrenergic receptors control microglial process surveillance in awake mice. Microglia are known to express high levels of β2-adrenergic receptors33. Their activation induces microglial process retraction in brain slices34. Using RNAscope in situ hybridization, we indeed found that most IBA1-positive microglia (97.6%) colocalized with β2-adrenergic receptor (Adrb2) mRNA (Supplementary Fig. 9). To explore whether adrenergic receptors control microglial process activity in awake mice, we examined the effects of β2-adrenergic receptor antagonists on microglial dynamics (Fig. 7a–c). Interestingly, both propranolol (300 μM, 10 μl; Fig. 7b), a high-affinity antagonist of β1- and β2-receptors, and the highly selective β2-receptor antagonist ICI 118,551 (30 μM, 10 μl; Fig. 7c) induced an increase in microglial process surveillance in awake mice. In addition, an intracerebral injection of TTX (50 μM) followed by application of ICI 118,551 (10 μM) 30 min later did not further increase microglial surveillance (Supplementary Fig. 7). Thus, inhibition of β2-adrenergic receptors can increase microglial process dynamics.

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Fig. 3 | Intracerebral application of muscimol increases microglial process surveillance. a. Left: schematic of placement of a drug infusion catheter in the subarachnoid space adjacent to the area of cranial window imaging. Right: images of microglia before (0 min) and 30 min after intracerebral administration of muscimol (870 μM). b. Changes in microglial process area and surveillance territory before and after administration of ACSF (area: 32, 0.0001, Fig. 3a–c). Interestingly, both propranolol (300 μM, 10 μl; Fig. 7b), a high-affinity antagonist of β1- and β2-receptors, and the highly selective β2-receptor antagonist ICI 118,551 (30 μM, 10 μl; Fig. 7c) induced an increase in microglial process surveillance in awake mice. In addition, an intracerebral injection of TTX (50 μM) followed by application of ICI 118,551 (10 μM) 30 min later did not further increase microglial surveillance (Supplementary Fig. 7). Thus, inhibition of β2-adrenergic receptors can increase microglial process dynamics.

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microglia, we used a chemogenetic approach to selectively suppress noradrenergic neurons in the LC by activating Gi-DREADD, which decreases cAMP levels and generally reduces neuronal firing rates. A Gi-DREADD-loxP-flanked mouse (R26-LSL-Gi-DREADD) was bred with the Cx3cr1<sup>GFP/+</sup> line. Expression of the Gi-DREADD receptor was confined to the LC through a targeted AAV-Cre injection (AAV9.rTH.PI.Cre.SV40) (Fig. 7e). In awake mice, the systemic application of the DREADD ligand clozapine-N-oxide (CNO; 5 mg per kg, intraperitoneally) increased microglial process surveillance in the somatosensory cortex in Gi-DREADD-expressing mice but not in their control littermates (Fig. 7f–h). We confirmed that CNO reduced the firing rate of Gi-DREADD-expressing noradrenergic neurons in the LC using acute brain slices (Supplementary Fig. 10a–d). In addition, to test potential off-target effects of CNO, we applied CNO in anesthetized Gi-DREADD mice and examined microglial process dynamics. We found that CNO did not further increase microglial surveillance in the anesthetized state (Supplementary Fig. 10e). Therefore, our results suggest that CNO targets Gi-DREADD to reduce endogenous NE release and subsequently increase microglial process surveillance.

To better access and mechanically study the LC–cortex circuitry, we performed several experiments in acute cortical slices. In slices, noradrenergic projections were well preserved (Supplementary Fig. 11a), but their connections to noradrenergic somata were severed during slice preparation (see Methods). Unlike the in vivo context (Supplementary Video 2), neuronal network activity was weak in cortical slices (Supplementary Fig. 11b,c; Supplementary Video 9). Bath application of a moderate dose of glutamate (50 μM) increased neuronal activity in cortical slices (Supplementary Fig. 11b,c; Supplementary Video 9), which resulted in reduced microglial surveillance (Fig. 8a). We further found that the β<sub>2</sub>-adrenergic receptor antagonist ICI 118,551 (10 μM) blocked the glutamate-induced decreases in microglial surveillance (Fig. 8b). Next, to test the direct effect of NE on microglial dynamics in cortical brain slices, we applied exogenous NE or chemogenetically induced endogenous NE release. Indeed, exogenous application of NE (30 μM) inhibited microglial process surveillance in cortical slices; this effect was abolished by ICI 118,551 (10 μM) (Fig. 8c). The application of ICI 118,551 (10 μM) alone did not affect microglial process surveillance, presumably due to the low activity of NE-releasing axons in brain slices (Fig. 8c). In slices prepared from mice with viral–induced expression of Gq-DREADD in LC adrenergic neurons and their projections, CNO was confirmed to increase neuronal firing (Fig. 8d,e). In addition, activation of noradrenergic terminals by CNO reduced microglial process surveillance (Fig. 8f). Therefore, our results suggest that local neuronal activity affects NE signaling, which is sufficient to regulate microglial process surveillance, even in the absence of LC somata.

Together, both β<sub>2</sub>-adrenergic receptor antagonism and chemogenetic inhibition of NE release can increase microglial process surveillance in awake mice. Inhibiting NE signaling therefore mirrors the effects of general anesthesia, sensory deprivation and optogenetic inhibition on microglial process surveillance. Overall, our results demonstrate that reduced NE signaling establishes a permissive microenvironment for enhanced microglial process surveillance. Meanwhile, tonic NE levels in the awake animal repress process surveillance and maintain a highly constrained microglial surveying state (Supplementary Fig. 12).
Fig. 6 | NE administration before whisker trimming or optogenetic inhibition prevents the increase in microglial process surveillance. a, Experimental paradigm: NE (or ACSF control solution) was administered intracerebrally immediately before contralateral whisker trimming. b, Representative images of microglial morphology before (0 min) and 30 min after whisker trimming, with either ACSF or NE applied. c, NE, but not ACSF, prevents changes in microglial process area (ACSF: 1.473 ± 0.072; NE: 1.048 ± 0.030) and surveillance territory (ACSF: 1.341 ± 0.058; NE: 1.038 ± 0.037) induced by contralateral whisker trimming (area: P < 0.0001, t(16) = 5.432; territory: P = 0.0005, t(16) = 4.395). d, Experimental paradigm: NE (or ACSF control solution) was administered intracerebrally immediately before optogenetic stimulation of VGAT-ChR2 transgenic mice. e, Representative images of microglial morphology before (0 min) and 30 min after optogenetic inhibition, with either ACSF of NE applied immediately before light stimulation. f, NE, but not ACSF, also prevents changes in microglial process area (ACSF: 1.496 ± 0.075; NE: 1.060 ± 0.045) and surveillance territory (ACSF: 1.523 ± 0.070; NE: 1.106 ± 0.033) induced by optogenetic inhibition (area: P = 0.0001, t(16) = 5; territory: P < 0.0001, t(16) = 5.334). N = 9 cells per 3 mice for each group; ***P ≤ 0.001, unpaired t-test (two-tailed). For b and e, experiments were repeated three times independently with similar results obtained.

Discussion

Microglia are the immune surveilling cells of the brain that shape neuronal circuitry in both health and disease. Multiple studies have demonstrated that the interactions between microglial processes and neuronal elements play a critical role in maintaining brain homeostasis. However, microglial process dynamics in vivo have been studied predominantly under anesthesia. Moreover, it is unclear whether neuronal network activity under awake conditions drive distinct microglial dynamics. Here, using in vivo imaging in awake mice, we showed that general anesthesia, sensory deprivation and optogenetic inhibition of local neuronal activity dramatically increases microglial process surveillance. We also pinpointed the underlying mechanism involved in the increased process surveillance, which arises from a reduction in NE signaling to microglial β2-adrenergic receptors. These results demonstrate that neuronal network activity inhibits microglial process dynamics through tonic NE signaling in awake mice.

Neuronal activity dictates microglial process dynamics. Prior studies of microglial imaging under anesthesia and of brain slices found that microglial process dynamics seem to follow neuronal activity patterns. Increased neuronal activity after bicuculline application, a GABA receptor antagonist, enhances microglial process sampling. In addition, neuronal hyperactivity triggered by NMDA receptor activation induces microglial process extension toward neuronal cell bodies and dendrites. Consistently, acute seizures increase microglial process dynamics and process convergence toward neuronal elements. During neuronal hyperactivity (for example, under seizure conditions), microglia can sense ATP release via the P2Y12 receptor and extend and converge their process toward an ATP source.

We expected that microglia in the awake animal would display more pronounced motility. Surprisingly, we found opposite results, showing that microglia exhibit less process surveillance in the awake condition than under general anesthesia. Consistently, introducing sensory deprivation or increased GABAergic network inhibition in awake animals resulted in similar increases in microglial process dynamics. Supporting this novel observation, a recent study reported that monocular deprivation induced greater microglial process ramification in the contralateral visual cortex. Together, our results reveal that neuronal activity negatively regulates microglial surveillance in awake mice. Therefore, in awake mice, microglial process dynamics are kept in a relatively constrained state.

Our results, in combination with previous studies that used general anesthesia, suggest that microglial process dynamics are not linearly dependent on neuronal activity (that is, increasing neuronal activity increases microglial process dynamics). Rather, microglial dynamics follows a U-shaped curve of regulation; that is, either increasing or decreasing neuronal activity relative to the awake state can increase microglial process dynamics. While microglial extension and convergence during hyperactivity or acute injury has been demonstrated through an ADP/ATP and P2Y12-receptor-dependent mechanism, we show here that a reduction in NE tone and β2-adrenergic activation (due to suppressed neuronal activity) results in microglial extension and territory surveillance. Although our results ruled out the contribution of P2Y12 receptor signaling in the increased territory surveillance, other studies have demonstrated that microglial P12Y12 and β2-adrenergic receptors control...
microglial process motility in vitro through interacting mechanisms\textsuperscript{31}. Overall, our study demonstrates that microglial dynamics are highly attuned to changes in the local microenvironment, but follow a U-shape style of regulation rather than linear activity-dependent regulation.

**Tonic NE and microglial process dynamics.** Here, we demonstrated that tonic NE signaling selectively regulates microglial process surveillance in vivo, which is based on several lines of evidence. First, of the four potential neuromodulators tested (acetylcholine, dopamine, NE and serotonin), only NE prevented anesthesia-mediated microglial process retraction, while the Gi-coupled P2Y\textsubscript{12} receptor, which also alters cAMP levels, induces microglial process retraction\textsuperscript{38}. Therefore, it seems that β\textsubscript{2}-adrenergic receptors are critical for microglial motility, migration, phagocytosis, proliferation and inflammatory responses\textsuperscript{36}. Our study corroborates the importance of noradrenergic regulation of microglia by providing evidence that tonic NE inhibits microglial process surveillance via β\textsubscript{2}-adrenergic receptors in awake mice.

While microglia represent an enriched cell population for functional β\textsubscript{2}-adrenergic receptor expression\textsuperscript{39}, the downstream signaling cascades that mediate β\textsubscript{2}-receptor-dependent process motility and surveillance are still undefined in microglia. It is known that the β\textsubscript{2}-adrenergic receptor is highly enriched in microglia compared with other cell types\textsuperscript{33}. Previous studies have also shown that microglial β\textsubscript{2}-adrenergic receptors are critical for microglial motility, migration, phagocytosis, proliferation and inflammatory responses\textsuperscript{36}. Our study corroborates the importance of noradrenergic regulation of microglia by providing evidence that tonic NE inhibits microglial process surveillance via β\textsubscript{2}-adrenergic receptors in awake mice.

![Fig. 7](image-url) **Blocking adrenergic receptors or lowering endogenous NE signaling increases microglial process surveillance.** a. Experimental paradigm: the β\textsubscript{1}/β\textsubscript{2}-adrenergic receptor antagonist propranolol (Prop) or the β\textsubscript{1}-receptor antagonist ICI 118,551 (ICI) was administered intracerebrally in awake mice. b, c. Both propranolol (b) (area: $P = 0.0002$, $t(8) = 6.331$; territory: $P = 0.0003$, $t(8) = 6.204$) and ICI 118,551 (c) (area: $P < 0.0001$, $t(8) = 9.024$; territory: $P = 0.0001$, $t(8) = 7.109$) infusion in awake mice trigger an increase in microglial process area and territory surveillance. d. Left: to determine the extent of noradrenergic innervation in the somatosensory cortex by LC neurons, td-Tomato expression was induced selectively in LC neurons using a specific Cre recombination strategy in Cx3cr1\textsuperscript{GFP/+} mice. Right: the fluorescent micrograph shows heavy noradrenergic innervation of the somatosensory cortex by LC projections. e. Experimental paradigm: the DREADD receptor agonist CNO was administered (intraperitoneally) to Gi-DREADD mice and control sibling littermates. f. Representative image of microglial morphology before (0 min) and 30 min after CNO administration in a Gi-DREADD mouse. g, h. CNO administration in control mice (g) does not alter microglial process area or territory surveillance (area: $P = 0.1193$, $t(8) = 1.744$; territory: $P = 0.1326$, $t(8) = 1.674$), while CNO administration in Gi-DREADD mice (h) increases microglial process area and territory surveillance (area: $P = 0.0001$, $t(8) = 6.815$; territory: $P = 0.0005$, $t(8) = 5.67$). N = 9 cells per 3 mice for each group; ***$P < 0.001$, paired t-test (two-tailed). For d and f, experiments were repeated three times independently with similar results obtained.
Fig. 8 | NE decreases microglial process surveillance in acute brain slices. a, b. Microglial morphology changes in acute brain slices before (0 min) and 30 min after bath application of 50 μM glutamate (a) (area: P < 0.0001; t(8) = 9.982; territory: P < 0.0001; t(8) = 8.027) or 50 μM glutamate with 10 μM CNO (Gq-DREADD) (b) (area: P = 0.625, t(8) = 0.509; territory: P = 0.264, t(8) = 1.202). c. Microglial morphology changes in acute brain slices before (0 min) and 30 min after bath application of 30 μM NE (area: P = 0.0003; t(8) = 6.127; territory: P < 0.0001; t(8) = 8.932); 30 μM NE with 10 μM CNO (Gq-DREADD) (area: P = 0.859, t(8) = 0.183; territory: P = 0.137, t(8) = 1.654) or 10 μM CNO 118,551 (area: P = 0.420, t(8) = 0.850; territory: P = 0.672, t(8) = 0.440). d, After a delay associated with CNO bath diffusion, Gq-DREADD neurons in the LC display a marked increase in firing frequency. Experiments were repeated three times independently with similar results obtained. e. Effects of CNO on firing rates are only observed in Gq-DREADD neurons in the LC, but not in control neurons (control: 0; Gq-DREADD: 593 ± 219; P = 0.002, t(10) = 11.8). N = 6 cells per 3 mice for each group; **P < 0.01, paired t-test (two-tailed). f. Microglial morphology changes in acute brain slices from mice expressing Gq-DREADD in the LC before (0 min) and 30 min after bath application of CNO (10 μM) (area: P = 0.0005; t(8) = 5.573; territory: P = 0.0004; t(8) = 5.797). N = 9 cells per 3 mice for each group; **P < 0.001, paired t-test (two-tailed), unless otherwise indicated. For a–d and f, experiments were repeated three times independently with similar results obtained.

cAMP controls microglial filopodia dynamics30. However, future studies of whether and how cAMP levels drive microglial process surveillance in vivo are still needed.

Our findings strike an interesting contrast to recent work investigating the impact of halogenated anesthetics, such as isoflurane, on microglial process dynamics. Tonic activation of the microglial two-pore domain channel THIK-1 was recently shown to control microglial process ramification and surveillance21. THIK-1 inhibition via isoflurane reduces microglial process surveillance in hippocampal slices. In contrast, our study indicates that isoflurane increases microglial process dynamics in the cortex of awake mice. This discrepancy could be due to microglial heterogeneity within different brain regions (hippocampus versus cortex), but could also be explained by the expected attenuation of NE tone that occurs when LC projections are severed during brain slice preparation. In brain slices, microglial surveillance is likely to be elevated in the absence of a strong NE tone. Potentially, when microglia are relieved of the dynamic constraints set by NE tone in slices, they are disposed to greater modulation in the microenvironment. In vivo, it is possible that the effects of isoflurane on NE tone serve as a stronger impetus for altering microglial dynamics than the effects of isoflurane on THIK-1 signaling. However, studies directly assessing β2-adrenergic and THIK-1 receptor signaling on microglial processes increase dendritic spine Ca2+ new spine formation during brain development and learning7,11. Therefore, microglial process surveillance may play a beneficial role in fine-tuning neuronal activity by reducing neuronal activity during seizures20,21 or by increasing neuronal synchronization during periods of reduced network activity12. Calibrating neuronal activity occurs in response to divergent neuromodulatory signals that exist in various brain states. While distinct receptors (that is, P2Y12 and β2) for these divergent neuromodulators have been identified in microglia, many more receptors are likely to exist and may aid in uniquely tuning microglial responses to the environment1. Toward this end, emerging tools for studying intracellular signaling mecha-
isms, such as cAMP levels, in vivo may greatly advance our ability to understand the complex downstream signaling mechanisms that mediate microglial responses to the microenvironment.

LC noradrenergic neurons innervate almost every region of the brain and could therefore have a widespread, potentially uniform, impact on microglial function. Under physiological conditions, NE regulates the sleep–wake cycle in addition to its role in memory and cognition. The loss of LC neurons is an early hallmark of neurodegeneration, as evident in Alzheimer’s disease and Parkinson’s disease. Meanwhile, chronic stress increases LC neuronal firing rates and brain NE concentrations. Therefore, the relative loss or gain in NE tone under these different pathophysiological contexts could serve as a powerful upstream modulator of microglial function across numerous brain regions. In turn, corresponding changes in microglial surveillance could produce profound consequences for the neuronal network over time in chronic conditions, based on the described roles for microglia in tuning neuronal activity. In addition, NE has been shown to reduce pro-inflammatory gene expression in microglia in various neurological disorders, including stroke, pain and neurodegeneration. In particular, NE can help resolve pathology in mouse models of Alzheimer’s disease by promoting microglial phagocytosis of amyloid-β and reducing amyloid-β-triggered inflammation. However, microglia are also reported to be inappropriately activated in Alzheimer’s disease and mediate early synapse loss. Apart from disease contexts, NE tone is reduced on a daily basis during sleep. It would be interesting to know whether reduced NE tone during sleep may ‘disinhibit’ microglial motility to allow greater synaptic remodeling to occur during sleep cycles, which might have implications for the consolation phase of learning and memory. However, we acknowledge that anesthesia and sleep are not mechanistically identical, and microglial dynamics during sleep will need to be explored in greater detail in future studies. Additionally, even with training, head-fixed animals can still remain stressed, thus potentially exaggerating the levels of NE in the awake state. Therefore, for normal animals that are awake but not head-fixed, microglial surveillance could be more pronounced than what we report here. Nevertheless, future studies are warranted to test the specific role of microglial adrenergic receptor signaling in sleep, memory and neurodegeneration.

In summary, we provide novel insights into microglial process dynamics under awake conditions and their regulation by neuronal activity and NE tone. Our results demonstrate that microglial process dynamics are fundamentally different in awake animals, emphasizing the importance of studying microglia in awake mice to establish the clearest picture for their basal physiology.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0511-3.

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Author contributions

Y.U.L., H.D. and L.-J.W. designed the studies. Y.U.L., A.D.U. and L.-J.W. wrote and revised the manuscript. Y.U.L. performed the electrophysiology experiments. Y.U.L., Y.Y., Y.L., U.B.E., T.C., J. Zheng, A.D.U., J. Zhu and D.B.B. performed animal surgery, image collection and data analyses. Y.U.L. and J. Zheng performed the in situ hybridization and immunofluorescence staining experiments.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Animals. Male and female mice, 2–3 months of age, were used in accordance with institutional guidelines. All experiments were approved by the Mayo Clinic Animal Care and Use Committee. Heterozygous Cx3cr1+/mice were used for all experiments. P2y12+/mice were originally obtained by M. Dailey at the University of Iowa. The following mice were purchased from the Jackson Laboratory: heterozygous R26-LSL-Gi-DREADD (Gi-DREADD; 026219); transgenic VGAT-ChR2 (014548); and R26-LSL-tTomato reporter mice (td-Tomato; 007909).

Heterozygous Gi-DREADD mice were bred to homozygous Cx3cr1−/− mice to obtain Gi-DREADD-expressing and -nonexpressing control offspring for experiments. Transgenic VGAT-ChR2 mice were also bred to homozygous Cx3cr1−/− mice to obtain offspring with (TG) and without (non-TG) transgene expression. Finally, P2y12−/− mice were bred to P2y12−/−:Cx3cr1−/− mice to obtain P2y12−/−:Cx3cr1−/− mice for imaging studies.

Viruses. To inject neuronal network activity in vivo, 0.3 μl of AAV9 CamKII.CaM2pm (G12289, Penn Vector Core) was injected into the somatosensory cortex (2-mm posterior and 1.5-mm lateral to bregma) or the barrel cortex (1.45-mm posterior and 3-mm lateral to bregma). To specifically target noradrenergic neurons, 0.3 μl of AAV9.εrt (Cre; G122845, Penn Vector Core) was injected into the LC (5.4-mm posterior, 0.9-mm lateral and 3.7-mm ventral to bregma) of Gi-DREADD or td-Tomato mice bred with Cx3cr1-GFP. To express Gq DREADD in noradrenergic neurons, we first made three viruses into the LC of Cx3cr1−/− mice: AAV9.εrt.PC;AAV9.DIO.hM3D(Gq) (44361, Addgene) and AAV9.FLEX.tdTomato (28306, Addgene).

Chronic window implantation. Mice were anesthetized with isoflurane (3% induction, 1.2% maintenance) and maintained on a heating pad during surgery. A dental drill (Osada Model EKL-M40) was used to drill a circular 3.3-mm diameter craniotomy. For the limb/trunk region of the somatosensory cortex, the craniotomy center was approximately 2-mm posterior and 1.5-mm lateral to bregma. For the barrel cortex, the craniotomy center was approximately −1.45-mm posterior and +3-mm lateral to bregma. A 3-mm glass coverslip previously sterilized in 70% ethanol was placed inside the craniotomy, then light-curing dental cement (Tetric EvoFlow) was applied around the glass coverslip and cured with a Kerr Demi Ultra LED Curing Light (Dental Health Products). The skull, excluding the region with the window, was then covered with IBond Total Etch glue (Heraeus) and cured with light-emitting diode light. Finally, a custom-skull, excluding the region with the window after implantation. A total volume of 10 μl of solution was applied through the catheter and into the brain. Neurotransmitters were exogenously applied to the following concentrations: 1 μM acetylcholine, 1 μM dopamine hydrochloride, 3 μM NE and 5 μM serotonin hydrochloride. The dose of muscimol (870 μM) and TTX (10 μM) represents the minimal dose needed to induce significant suppression of neuronal activity. CNO was intraperitoneally injected at 5 mg/kg to activate Gi-DREADD in awake mice.

Imaging data analyses. Images were analyzed using Image51. All microglia preselected via unbiased inclusion criteria were then analyzed. Inclusion criteria included a clearly identifiable soma near the center of the z-stack and multiple processes incorporated within the x–y imaging plane. Because rapid movements by the animal could lead to image distortion in the x–y plane, five images were selected for each animal (Supplementary Fig. 1c). At each level, the user selected the image with the least distortion for inclusion in a ‘sorted’ z-stack (Supplementary Fig. 1d). Images in this z-stack were then aligned along the z axis using the StackReg plugin to create a final z-stack used in analyses.

To obtain the total process area value, a maximum-intensity projection (MIP) algorithm was then applied to the final z-stack. After the z-stack was created from the final z-stack, a region of interest was manually drawn through the threshold to the MIP images, the Analyze particles tool was used to derive a process area value from this flattened image. To obtain a process survival territory value, a bounding box connecting the distal-most edges of the microglial processes was then mapped onto the MIP image. The area encompassed within this bounding box was defined as the microglial survival territory. Area and territory values in subsequent stacks were normalized to the values of the first stack to determine changes over time. Process velocity was measured using the Manual tracking plugin. Sholl analysis was performed in ImageJ with the Sholl analysis plugin. Z-stacks acquired between the 10 and 20 min mark were used to evaluate microglial dynamics in the awake condition. Z-stacks acquired between the 20 and 30 min mark after anesthesia (isoflurane, ketamine/xylazine and urethane) were used to evaluate microglial dynamics in the anesthetized conditions. Similarly, z-stacks were then analyzed to assess the stability of neuronal activity throughout a 30-min recording period and before and after whisker trimming (Supplementary Fig. 5a–c) or muscimol anesthesia (Supplementary Fig. 6).

To analyze interactions between microglial processes and neuronal dendrites, we used a spatial distribution analysis. Data analysis was performed blinded to the conditions of the experiments.

Electrophysiology. For verification of ChR2 function in VGAT-positive neurons or of Gi-DREADD function in noradrenergic neurons, we used single-cell activity in AC1- and AC2-containing ethyl carbamate (CEC) or cocaine-hydrochloride (VGAT-ChR2 mice) or the LC (Gi/Gq-DREADD-expressing mice). Transverse or sagittal brain slices were cut in chilled (−2°C to −4°C) cutting solution containing the following (in mM): 185 sucrose, 2.5 KCl, 1.2 NaH2PO4, 25 NaHCO3, 25 glucose, 10 MgCl2, and 0.5 CaCl2. Slices were then transferred to a chamber with ACSF containing the following (in mM): 150 NaCl, 2.5 KCl, 1.3 NaH2PO4, 26 NaHCO3, 10 glucose, 2 MgCl2, 2 CaCl2 (pH 7.3–7.4; osmolality: 300–310 mM). The chamber was saturated with 95% O2/5% CO2 to provide stable pH and continuous oxygenation. For recovery, slices were kept at 29–30°C for a 30-min period, then cooled and kept at room temperature for at least 1 h before recording. During recording, slices were continuously perfused with room temperature ACSF using a peristaltic bath perfusion system. Whole-cell recordings were performed using glass pipettes (3–5 MΩ) filled with the following intracellular solution containing (in mM): 121 KCl, 19 potassium-glucuronate, 5 NaCl, 4 MgCl2, 10 HEPES buffer, 0.1 EGTA, 4 Mg-ATP, Na2-GTP (pH 7.3–7.4; osmolality: 280–290 mOsm). Data were collected using a MultiClamp 700B amplifier (Molecular Devices). Signals were filtered at 2 kHz and digitized at 10 kHz with a Digidesign 1350 Data Acquisition System and recorded on a computer with pClamp software (Molecular Devices).
analyzed using the software pCLAMP 10 (Molecular Devices) or Mini Analysis 6.0.3 (Synaptosoft).

For field potential recording in the somatosensory cortex in awake mice, a specialized recording headplate was adhered to the mouse 2 weeks before recording. Following 3 days of training on the treadmill platform, a craniotomy was made above the somatosensory cortex under general anesthesia, as described above, except that a smaller glass coverslip (2 mm in diameter) was affixed over part of the craniotomy to provide an unobstructed region for micropipette insertion and recording (Supplementary Fig. 2e). After recovery from the surgery, mice were head-fixed and allowed to move on the treadmill. A glass pipettes filled with ACSF containing Alexa-594 was inserted through the dura and into the cortex under two-photon guidance. Data were acquired continuously in the awake and anesthetized conditions using the parameters described above for single-cell recording.

Two-photon imaging of acute brain slices. Transverse brain slices were used for microglial imaging. Acute brain slices were prepared as described above in the “Electrophysiology” section. The imaging protocol was the same as described above for “In vivo two-photon imaging”, except with modified imaging stack parameters (100–140 µm in depth, 2-µm step size). Data analyses from slice imaging are the same as for the in vivo experiments. Drugs were applied in a bath. To prepare isoflurane-enriched ACSF, carbogen gas was passed through an Anesthetic Vaporizer (Midmark Matrix, VIP 3000) at a constant flow rate of 500 ml min⁻¹ and then bubbled through the ACSF reservoir.

**RNAscope in situ hybridization and immunohistofluorescence staining.** To visualize β₂-adrenergic receptor (Adrb2) mRNA in situ, a RNAscope 2.5 HD Detection kit (Advanced Cell Diagnostics, 322360) was applied in accordance with the manufacturer’s protocol using fixed, frozen sections of 10-µm thickness. Briefly, sections were first dried for 30 min at 60 °C and cleared of OCT embedding matrix via a 5-min PBS wash. During pretreatment, the sections were incubated in hydrogen peroxide for 10 min at room temperature. The sections were rinsed in fresh tap water before observation. To further determine Adrb2 expression in microglia, the assay was continued with a modified immunofluorescence staining protocol following the manufacturer’s instructions. Fluorescent images were acquired using a confocal microscope (Zeiss LSM-810). Cells that were positive for IBA1 (Wako, 019-19741, 1:500 dilution) and colocalized with two or more mRNA puncta were counted as positive.

**Statistical analyses.** Statistical analyses utilized a paired t-test design with a two-tailed test of significance for changes within a cell across conditions. Comparisons between genotypes utilized an unpaired t-test design with a two-tailed test of significance. D’Agostino–Pearson tests were used to assess normality before applying a parametric or nonparametric t-test variant (paired: Wilcoxon matched-pairs rank test; unpaired: Mann–Whitney test). One-way analysis of variance (ANOVA) was used to compare three groups. All statistical testing was performed using GraphPad Prism (GraphPad Software). Data distribution was assumed to be normal, but this was not formally tested. Data are shown as individual values or expressed as the mean ± s.e.m. when grouped. A P value <0.05 was considered statistically significant. All P values that are not less than 0.0001 are provided as exact values, otherwise they are indicated as P<0.0001. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications.

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Software and code

Policy information about availability of computer code

| Data collection | ScanImage (Matlab based) for two-photon imaging acquisition; Axon 700B and pClamp10 for electrophysiological data collection. |
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| Data analysis   | ImageJ v1.52i (Fiji) for two-photon images analysis; Software Clampfit 10, Mini Analysis 6.0.3 for electrophysiological data analysis; GraphPad Prism 7 for figures and statistics. |

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| Data exclusions | No data were excluded from the analyses.                                                                                                                                                         |
| Replication    | All the mice with successful cranial glass windows surgery were used for imaging and replicated all the results described in the manuscript. The results were replicated by at least two investigators in our lab. |
| Randomization  | All cells analyzed were randomly selected from in vivo and in vitro samples.                                                                                                                        |
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| Wild animals       | No wild animals were used in this study.                                                                                                                                                         |
| Field-collected samples | No field-collected samples were used in this study.                                                                                     |
| Ethics oversight   | We performed all procedures involving live mice in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the protocols approved by the . All experiments were approved by the Mayo Clinic Animal Care and Use Committee. |

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