Receptors, Ion Channels, and Signaling Mechanisms Underlying Microglial Dynamics

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Microglia, the innate immune cells of the CNS, play a pivotal role in brain injury and disease. Microglia are extremely motile; their highly ramified processes constantly survey the brain parenchyma, and they respond promptly to brain damage with targeted process movement toward the injury site. Microglia play a key role in brain development and function by pruning synapses during development, phagocytosing apoptotic newborn neurons, and regulating neuronal activity by direct microglia-neuron or indirect microglia-astrocyte-neuron interactions, which all depend on their process motility. This review highlights recent discoveries about microglial dynamics, focusing on the receptors, ion channels, and signaling pathways involved.

In the healthy CNS, microglia comprise up to 12% of all cells, with highest densities found in the hippocampus, substantia nigra, and basal ganglia (1). Microglia are brain-resident macrophages, but originate from primitive macrophages in the yolk sac during embryogenesis (2). During development, they invade the brain, where they proliferate into mature cells and form an independent population of brain immune cells, separated from peripheral macrophages by the blood-brain barrier (reviewed in Ref. 3). In contrast to the long-held view of microglia cells as being immune-competent cells that are normally “resting” and become activated only after brain inflammation or neurologically related diseases, even resting microglia constantly survey the brain parenchyma by sending out and retracting highly branched fine processes. This allows them to interact physiologically with cells in the brain parenchyma, as well as respond promptly to brain damage or injury (4, 5) and clear cellular debris or accumulating metabolic products by phagocytosis (6).

Functional Roles of Microglial Surveillance of the Brain

Microglia survey the brain to detect infection or cell damage or death (3). In addition, recent studies ascribe to microglial cells fundamental functions in the healthy developing and adult brain, affecting neuronal activity, synaptic connectivity, and programmed cell death. In vivo imaging has revealed that microglial processes do not just randomly screen the extracellular space but contact neuronal somata and synapses in an activity-dependent fashion (7, 8). Strikingly, the increased frequency of such contacts with highly active neurons is homeostatic, reducing both spontaneous and evoked neuronal activity (9). Microglia also indirectly alter excitatory neurotransmission by communicating with astrocytes via an ATP-dependent mechanism (10). Neuronal activity-dependent steering of microglia processes, furthermore, plays an important role in eliminating excessive synapses (synaptic pruning) in the developing brain, which is crucial for the proper maturation of synaptic circuits. Less active, “weaker” synapses are preferentially targeted and engulfed by microglial processes, and then phagocytosed by a mechanism involving the complement system (11). This synaptic pruning is critical for the maturation and strengthening of glutamatergic synapses (12, 13). Microglia also engulf and trigger apoptosis of newborn neurons, both during development and in adulthood. This is essential for regulating the number of neurons in the adult brain; progenitor cells produce neurons in excess, only a fraction of which mature and become integrated into neuronal circuits (14, 15). All of these newly discovered functions depend on the enormous process motility of non-activated, ramified microglia and their surveillance of the brain parenchyma.

In this review, we focus on the (still limited) research on microglia in vivo, or in situ using acute brain slices or retinal explants. This is because in vitro studies should be interpreted with caution, due to the highly reactive nature of microglia, which substantially alters their morphology and functional properties when exposed to culture conditions (16). We will compare the key features of microglial baseline surveillance and targeted motility (chemotaxis), and then describe the receptors and signaling pathways controlling both processes. Finally, we will discuss gaps in our understanding and highlight likely further research directions.

Different Modes of Microglial Motility

Microglial motility can be classified into two functionally and mechanistically distinct modes: (i) baseline surveillance (Figs. 1 and 2), with apparently random non-directed process movements, and (ii) microglia chemotaxis (Fig. 3), an “alert mode” in response to cell or tissue damage, with highly targeted movement of microglial processes toward the injury site. As we will review in detail below, there are similarities and differences between these two modes, as follows. First, both of these modes depend on cytoskeletal changes arising from highly motile bundles of actin filaments (17). High amounts of filamentous actin are present in microglial cells (18), and agents blocking actin polymerization such as cytochalasin B or latrunculin B inhibit both microglial baseline surveillance and chemotaxis in situ (19). Furthermore, the small Rho-GTPase Rac, a key regulator of actin dynamics (20), is highly enriched in the motile endings of microglial processes, and Rac down-regulation prevents directed process movement in response to neuronal activity (9). The remainder of this review focuses on signaling mechanisms...
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FIGURE 1. Factors contributing to baseline surveillance. Schematic representation illustrates the typical morphology of non-activated, ramified microglial cells as seen in vivo or acutely prepared brain slices in situ.

FIGURE 2. Possible control of baseline surveillance by astrocyte ATP. Upper panel, left: astrocytes (yellow) may tonically release ATP (blue shadow/circles) through connexin and/or pannexin hemichannels (black squares), which supports a ramified microglial morphology and baseline surveillance. The green- and brown-colored microglial cell illustrates its morphology at two different time points during baseline surveillance. Upper panel, right: blockade of astrocytic hemichannels (red crosses) or depletion of extracellular ATP by apyrase (purple circles) induces process retraction and demarangulation of microglial cells and thus leads to reduced baseline motility. Lower panel: close-up of microglial and astrocytic processes. Possible routes of ATP release from astrocytes include P2X receptors (e.g. P2X1), connexin (Cx), or pannexin (Px) hemichannels. The released ATP can then either directly activate microglial P2X or P2Y receptors or be degraded via ectonucleotidases (NT) into ADP and adenosine (Ado), which may act on microglial P2Y and P1 receptors. The precise molecular purinergic targets involved in maintaining baseline motility are still unknown.

upstream of the cytoskeleton. Second, the speed of process movement is similar for both motility modes. On the other hand, baseline surveillance operates isotropically (Figs. 1 and 2), with process extensions and retractions in all directions, whereas chemotaxis is anisotropic, involving retraction of processes on one side of the microglial cell and process extension on the other side (Fig. 3).

Microglia Chemotaxis

If freely scanning microglia detect brain damage (via activation of P2Y12 purinergic receptors or of fibrinogen-sensing CD11b/CD18 receptors, see below), they immediately extend processes toward the site of injury (Fig. 3), where they converge in less than 30 min to form a spherical shield preventing further spread of the affected area (4, 5, 19, 21). This robust chemotactic response is confined to microglial processes within ~50–80 μm from the injury, whereas the cell soma usually remains stationary over hours. The speed of process outgrowth is similar to that occurring normally during non-targeted baseline motility. Notably, as soon as processes start to grow toward a damaged area, their non-directional baseline motility ceases and processes on the other side of the soma from the damaged area retract, suggesting that the two motility modes are exclusively alternative mechanisms (19). Chemotraction of microglial processes by elevated concentrations of ATP (>1 mM) leaking from damaged cells was first shown in cultured microglia (22) and confirmed in situ and in vivo (4, 5, 19, 23). Other substances that induce microglial chemotaxis in vivo are NO (in the white matter (24)) and fibrinogen (25), which can leak from damaged blood vessels. In contrast to the few in vivo studies, numerous other chemoattractants have been shown to induce chemotaxis of cultured microglia in vitro, including various chemokines, tissue complement, and growth factors (26–28).

Mechanisms Involved in Microglial Chemotaxis

Unlike “native” microglial cells in situ, which respond to chemoattractants by sending out their processes, the less ramified and partly activated microglia in culture instead migrate by translocating their cell bodies (22, 28–30). Despite these differences, in vitro studies have established a crucial role for extracellular nucleotides like ATP as potent inducers of microglial chemotaxis and provided insights into the underlying mecha-
nisms. They are, however, unsuited to studying the interactions of microglia with the extracellular matrix and other brain cells, which are a key feature of targeted process motility.

**Purinergic Signaling Involved in Microglia Chemotaxis**

Microglia in situ use ionotropic P2X and metabotropic P2Y and P1 receptors to respond to extracellular nucleotides and nucleosides such as ATP (P2X6, P2X4, P2Y1), ADP (P2Y12, P2Y13), UTP (P2Y6, P2Y3), UDP (P2Y5), and adenosine (P1) (6, 23, 29, 31–35). ATP is the main nucleotide released upon tissue injury, and the extracellular concentration reached may be amplified by astrocytes releasing further ATP (4, 10). ATP signaling is diversified by enzymatic hydrolysis; the microglia-specific membrane-bound ectonucleotidase NTPDase1 (CD39), or the pyrophosphatase NPP1 (expressed on microglia but also on oligodendrocyte lineage cells (36)), converts ATP and ADP into AMP, which is then degraded into adenosine by ecto-5’-nucleotidases (CD73, on oligodendrocyte lineage cells (36)), whereas tissue-nonspecific alkaline phosphatase (TNAP, mainly on astrocytes (36)) converts ATP, ADP, and AMP into adenosine. This generates complex purinergic signaling with cellular effects including changes of membrane voltage, elevation of intracellular [Ca\(^{2+}\)], and activation of G protein-coupled second messenger cascades.

ATP/ADP-induced chemotaxis, dependent on G\(_i\)-coupled P2Y receptors, was first described in cultured microglia and later in vivo (4, 22), and knock-out of ADP-activated G\(_i\)-coupled P2Y\(_{12}\) greatly decreases chemotaxis (34). Expression of P2Y\(_{12}\) receptor protein on the surface of ramified microglia in vivo (34) is particularly enriched at the tips of the leading processes (34). ATP/ADP-induced chemotaxis, P2Y\(_{12}\) knock-out does not alter microglial baseline surveillance, suggesting that distinct mechanisms regulate the two types of motility.

Ion Channels and Non-purinergic Signaling Involved in Microglia Chemotaxis

While inducing microglial chemotaxis, activation of P2Y receptors also elicits a K\(^+\) current in microglia in situ. Blocking this with the non-selective K\(^+\) channel antagonist quinine abolished chemotaxis to ATP, implying that this current is important for ATP/ADP-mediated microglia motility (23). However, broad-spectrum blockers of Ca\(^{2+}\)-activated and voltage-gated K\(^+\) channels (4-aminopyridine, tetraethylammonium, and BaCl\(_2\)) had no effect on laser ablation-induced microglia chemotaxis in situ (19). Differences in the channel selectivity of the drugs used and in the stimuli triggering chemotaxis (ATP source versus ablation of cells releasing substances other than ATP) may underlie this difference.

Blocking volume-sensitive Cl\(^-\) channels prevented microglia chemotaxis in situ (19). Volume regulation may be essential for microglial cell processes navigating through narrow extracellular spaces. Surprisingly, however, microglial baseline motility was unaffected by Cl\(^-\) channel blockade, indicating differential regulation of the two types of motility (19). Volume-sensitive Cl\(^-\) channels (and channels with similar pharmacology, like pannexins (46)) also mediate ATP release from cells (47) and, if ATP-induced ATP release from astrocytes promotes microglial chemotaxis by amplifying local ATP signals (4, 10), Cl\(^-\) channel blockers may inhibit chemotaxis by reducing ATP release.

In addition to ATP, NO released by tissue damage is a microglial chemoattractant in spinal cord white matter in vivo, and blocking the NO/cGMP signaling pathway inhibited the chemotactic response to spinal cord injury (24). However, NO-evoked chemotaxis requires some ATP/ADP to be present.

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\(^2\) The abbreviation used is: PLC, phospholipase C.
because depletion of extracellular nucleotides with apyrase inhibited the response, whereas ATP still induced chemotaxis when NO signaling was abolished (24). Thus, ATP is the more potent chemotactant. Interestingly, a uniform distribution of ATP rendered the cells more ramified and motile, whereas a uniformly high NO level induced process retraction and adoption of an amoeboid-like phenotype.

In diseases with blood-brain barrier disruption such as multiple sclerosis, or small hemorrhages, the plasma protein fibrinogen, acting via its CD11b/CD18 receptor (integrin $\alpha_M\beta_2$), is another potent inducer of microglia chemotaxis (25), as shown in an in vivo model of multiple sclerosis and by injecting physiological concentrations of fibrinogen into the intact brain. Knocking out the binding motif of the CD11b/CD18 receptor led to a smaller cluster of microglia forming around blood vessels, and less microglial activation and axon damage (25).

Baseline Surveillance

Studies in vivo and in situ using animals (mouse, zebrafish) with genetically targeted microglia have demonstrated that microglial tissue surveillance in the healthy CNS is almost exclusively performed by their long, thin, and highly branched processes, which extend and retract at average velocities of $\sim2.5 \mu m/min$ (4, 5, 9, 23, 48), whereas thicker processes closer to the soma are less motile. Many processes show highly motile protrusions forming bulbous endings that are very changeable in size and have even greater motilities of $>4 \mu m/min$. In stark contrast to their fast moving processes, which span a territory of $\sim60 \mu m$ in cortex, their small somata ($\sim10 \mu m$ or less) are almost stationary, moving only a few $\mu m/hour$ (5). Their high process motility (as well as their high cell density in the brain) allows microglia to scan the entire brain parenchyma once every few hours (5). Although microglial scanning appears to be random, it follows a highly organized search routine, whereby an individual cell scans its own territory without any overlap or contact with neighboring microglia. Importantly, the motile nature of microglial cells in vivo and in situ has been characterized in studies using juvenile and young adult animals, but process ramification and motility decline in aged animals, which may lead to less efficient surveillance and perhaps attenuated protection from tissue damage (49).

Mechanisms Involved in Microglial Baseline Surveillance

Our current understanding of microglial baseline surveillance lacks deep mechanistic insight. Baseline surveillance not only depends on the density of microglia, the velocity of process movement, and the frequency at which extensions and retractions occur, but also on mechanisms regulating the architecture of microglial processes, such as the number of processes, process length, and degree of ramification (Fig. 1). The following mechanisms affect baseline surveillance.

Purinergic Control of Microglial Baseline Surveillance

Knock-out of the P2Y$_{12}$ receptors that mediate chemotaxis had no effect on baseline surveillance (34), but exposing brain tissue to apyrase, an ATP/ADP-degrading ecto-ATPase from potato, reversibly induced a rapid loss of microglial process ramification and greatly reduced baseline motility (Fig. 2) (4, 21, 50). This suggests that activation of a nucleotide receptor other than P2Y$_{12}$ may help to maintain baseline motility. Microglial process length was also reduced by broad-spectrum blockers of ionotropic and metabotropic purinergic receptors in mouse retinal explants (50). Thus, ambient levels of extracellular nucleotides seem necessary to drive a (still unknown) tonically active signaling mechanism in microglial cells that maintains their process length and motility. However, these experiments used very high concentrations of apyrase (100–300 units/ml) and should be interpreted with care as there may be nonspecific effects of apyrase at such high doses. Additionally, the enzyme was not focally but globally applied to the entire brain tissue, which may trigger unpredictable secondary effects.

Another indication that extracellular nucleotides regulate microglial process ramification came from mice lacking the ectonucleotidase CD39 (51), the hippocampal microglia of which had a less ramified morphology, with shorter processes covering a smaller cortical area than for wild-type mice, whereas their tissue density was unchanged. This might be explained by CD39 knock-out altering the extracellular levels of nucleotides and nucleosides, and thus altering tonic purinergic receptor activation, but the precise receptors and intracellular signaling mechanisms involved remain unknown.

If extracellular nucleotides regulate microglial baseline motility and process ramification, it is important to understand the mechanisms releasing nucleotides in brain tissue. In response to neuronal activity, astrocytes release ATP, which triggers astrocyte $Ca^{2+}$ waves. These release more ATP across the astrocytic network, promoting communication between astrocytes and to neurons (52). Astrocytes can amplify a local rise in extracellular [ATP] through this ATP-induced ATP release (53), facilitating microglial chemotaxis after focal brain damage (4) and enhancing excitatory neurotransmission following microglia activation by LPS (10). Astrocyte $Ca^{2+}$ waves can be sensed by some microglia via purinergic receptors that generate an outward membrane current (55). In line with astrocyte-microglia communication via ATP, blockade of gap junction hemichannels, which may mediate astrocytic ATP release (53, 54), facilitating microglial chemotaxis after focal brain damage (4) and enhancing excitatory neurotransmission following microglia activation by LPS (10), Astrocyte $Ca^{2+}$ waves can be sensed by some microglia via purinergic receptors that generate an outward membrane current (55). In line with astrocyte-microglia communication via ATP, blockade of gap junction hemichannels, which may mediate astrocytic ATP release (53, 54), facilitating microglial chemotaxis after focal brain damage (4) and enhancing excitatory neurotransmission following microglia activation by LPS (10), Astrocyte $Ca^{2+}$ waves can be sensed by some microglia via purinergic receptors that generate an outward membrane current (55). 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Regulation of Microglial Surveillance by Neuronal Activity

Microglial process length and motility can also be influenced by neurotransmitter signaling (5, 9, 37, 50, 58), but there are inconsistencies between the results obtained. Increases in neuronal activity evoked by glutamate (or analogues like NMDA, AMPA, or kainate), or by blocking GABAergic inhibition, led to microglial process extension and enhanced baseline motility, whereas block of AMPA receptors had the opposite effect (5, 37, 50, 75). Similarly, mice exposed to light deprivation leading to reduced neuronal activity in the visual cortex have less motile...
microglial processes, and light re-exposure increased their velocity back to control levels (7). However, in dark-adapted mice, microglia had an increased process area covering a larger extracellular space, which led to a higher frequency of synaptic contacts. Thus, the motility of microglial processes and their territory may be regulated inversely (7). In a more extreme model of visual deprivation (binocular eye enucleation), microglia in the visual cortex had shorter processes that made less frequent contacts with synapses (8), unlike the findings of Tremblay et al. (7). These differences may reflect the varied manipulations used, but in both studies, microglia actively responded to altered neuronal activity.

Similar findings were obtained in zebrafish larvae, where an increase of neuronal activity evoked process outgrowth toward neurons and, through enhanced microglial-neuron contacts, attenuated both spontaneous and evoked activity of previously highly active neurons (9). In contrast, reducing neuronal activity by blocking synaptic transmission with the Na+ channel blocker tetrodotoxin in situ did not affect process motility and baseline surveillance (5). Furthermore, contradicting the idea that process motility correlates with neuronal activity, high-frequency stimulation inducing long-term potentiation in hippocampal slices did not change microglial baseline surveillance (59).

The modulatory transmitter noradrenaline also affects microglial motility, reducing process length and baseline surveillance. Noradrenaline also abolished ATP-induced process outgrowth, suggesting a regulation of purinergic signaling by adrenergic signaling in microglia (58).

Instead of directly communicating to microglia, neurotransmitter signaling may affect microglial motility indirectly by modulating extracellular levels of nucleotides such as ATP, which evoke changes in morphology and process velocity (37, 50, 75). Indeed, in microglia in situ, only extracellular nucleotides such as ATP, ADP, and UDP evoke inward membrane currents through ionotropic P2X (presumably P2X4 and P2X7) receptors and outward K+ currents via G protein-coupled (P2Y) purinergic receptors (23, 31–33, 35, 50), whereas other neurotransmitters (glutamate, GABA, glycine, acetylcholine, dopamine, and noradrenaline) generate no current (23, 32, 33, 35, 59, 60, 75). This contradicts data from cultured microglia, showing expression of glutamate-sensing AMPA and kainate receptors, as well as glutamate transporters (61, 62). Supporting a role for ATP as the signal of neuronal activity for microglia, the reduction of microglial process length and motility seen with glutamate receptor blockade can be overcome and even reversed when ATP is present (50). Furthermore, NMDA-evoked outgrowth of microglial processes can be abolished by P2Y12 knock-out, inhibiting ATP hydrolysis, or omitting extracellular nucleotide concentrations. This may reflect co-release of ATP with principal neurotransmitters from neurons (63) or secondary neuronal ATP release following NMDA receptor activation (37, 50), or may involve communication to astrocytes, which release ATP in response to neuronal activity (52).

**Regulation of Microglia Baseline Surveillance by Fractalkine**

Activation of the microglial chemokine receptor CX3CR1 by its ligand CX3CL1 (fractalkine) is an important pathway for neuron-to-microglia communication, serving as a calming stimulus preventing microglia activation (64–66). Fractalkine is constitutively released by neurons and exists as a membrane-bound and -soluble ligand, thus promoting direct cell-cell signaling, but also long-range signaling to microglia. Fractalkine receptor deficiency leads to a transient reduction of microglial number during early postnatal development (12, 13), microglia-mediated neurotoxicity (65–67), and impaired phagocytosis of apoptotic newborn neurons, thus causing reduced neurogenesis and deficits in synaptic pruning associated with weaker synaptic transmission and impaired brain connectivity (68–70).

Fractalkine signaling promotes microglial motility because, in ex vivo retinal explants, CX3CR1 knock-out led to reduced microglial baseline surveillance. Although process velocity was reduced by ~30% for baseline surveillance and in response to tissue damage, microglial morphology and tissue density were unaltered (71). This is surprising as CX3CR1 receptor deficiency is known to transform microglia from a quiescent surveillance state into an activated state (65), with a larger cell soma and greatly reduced number of processes that are much shorter and less motile. Other chemokines, CD42 and CD200, also keep microglia in a non-activated, quiescent state, but their impact on microglial baseline motility is unknown.

**Gaps in Our Understanding and Future Directions**

Despite the advances reviewed above, the following issues need further work if we are to better understand microglial surveillance of the CNS. During baseline surveillance, it is still unclear what cues, if any, microglial processes follow when they invade and scan an area of the brain parenchyma, how they know which territory they have already surveyed (or whether it is occupied by another microglial cell), and how they meet the substantial energetic and membrane/cytoskeleton turnover requirements for their continuous surveillance. Mechanistically, it will be crucial to establish how microglia switch from baseline surveillance mode to targeted motility toward a threat. In chemotaxis, it is unclear how ATP, as a quickly hydrolyzed molecule with a very short half-time in tissue (~200 ms (72)), can act as a long-range signal to trigger prolonged process outgrowth in distant microglia. Indeed, the concept of extracellular purines such as ATP, ADP, UTP, or UDP acting as long-range chemoattractants for macrophages has been challenged (73). Because ATP functions in many cell types as an auto- or paracrine signaling molecule acting in the direct vicinity of its release site (74), a similar scenario may hold for microglia (during either baseline surveillance or chemotaxis). This would imply that an initially local ATP signal needs to be amplified across a much longer distance by employing a different signaling molecule or by enhancing the initial ATP response, perhaps by ATP-induced ATP release in astrocytes (4). Understanding how this works may give us insight into why chemotaxis is only
evoked when millimolar levels of ATP are released, yet is mediated by the ATP hydrolysis product ADP binding to a receptor (P2Y12) with a very high affinity. Finally, our review has focused on resting microglia. Further work is needed to reveal which other mechanisms exist to evoke a targeted response of microglial processes under pathological conditions when activated microglia have substantially down-regulated their P2Y12 receptor expression (29, 45). It will also be important to understand whether the properties of microglia generated from macrophages that invade the brain following injury differ from those of yolk sac-derived endogenous microglia.

Some of these gaps in our understanding have already been partly addressed in vitro. However, given the profound differences between cultured microglia and microglia in vivo, it is essential now to test current in vitro concepts under more physiological conditions and, more challengingly, to carry out future research investigating the properties of microglial cells in preparations in situ or in vivo from the beginning.

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