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ATP mediates rapid microglial response to local brain injury *in vivo*

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Parenchymal microglia are the principal immune cells of the brain. Time-lapse two-photon imaging of GFP-labeled microglia demonstrates that the fine termini of microglial processes are highly dynamic in the intact mouse cortex. Upon traumatic brain injury, microglial processes rapidly and autonomously converge on the site of injury without cell body movement, establishing a potential barrier between the healthy and injured tissue. This rapid chemotactic response can be mimicked by local injection of ATP and can be inhibited by the ATP-hydrolyzing enzyme apyrase or by blockers of G protein–coupled purinergic receptors and connexin channels, which are highly expressed in astrocytes. The baseline motility of microglial processes is also reduced significantly in the presence of apyrase and connexin channel inhibitors. Thus, extracellular ATP regulates microglial branch dynamics in the intact brain, and its release from the damaged tissue and surrounding astrocytes mediates a rapid microglial response towards injury.

Microglia in the cerebral cortex have a highly branched morphology, with each cell soma decorated by long processes with fine termini. They share many molecular structures with phagocytic macrophages and are thus expected to perform tissue surveillance in the nervous system[1,2]. Activated microglia are found in the brain under almost all pathological conditions and are involved in tissue repair, amplification of inflammatory effects and neuronal degeneration[3–5]. Microglia have been shown to phagocytose dead cells and clear the cellular debris in brain slices[6–9] and respond to various substances, including purine and pyrimidine analogs, complement factors, cytokines and chemokines in cell culture[10–13]. However, studies in brain slices are limited because the slicing procedure can inherently activate microglia and induce their transformation into a morphologically distinct and highly reactive state, therefore obscuring potentially important dynamic processes[9,14,15]. To investigate the dynamic properties of microglia and the mechanisms underlying their activation upon injury in the intact living brain, we took advantage of transgenic mice in which all microglia are fluorescently labeled after replacing the *Cx3cr1* gene with the gene encoding enhanced green fluorescent protein (EGFP) by homologous recombination in embryonic stem cells[16]. Using transcranial two-photon microscopy[17], we were able to image the behavior of GFP-expressing parenchymal microglia through the thinned skull of anesthetized heterozygous *Cx3cr1*$^{GFP/+}$ mice (see Methods). Our results indicate that microglial processes are highly dynamic in the intact brain and respond rapidly towards the site of traumatic injury. Furthermore, extracellular ATP released from astrocytes is essential in mediating both the rapid baseline dynamics and the injury-induced response of microglial processes.

RESULTS

Microglial processes are highly dynamic in the intact brain

We first examined the normal behavior of microglia in the living mouse brain through a thinned but intact area of the skull. Whereas microglial cell bodies and main branches were morphologically stable during the entire period of observation (hours), higher-order branches of the ramified processes underwent rapid extension and retraction over intervals of seconds to minutes, reaching up to several micrometers in length or retracting until they completely disappeared (**Fig. 1**; **Supplementary Video 1**; >30 cells in ten animals). Over a period of minutes to hours, many small processes appeared and disappeared without net change in the total number of branches (125 processes from five cells in three animals over 15 min; $P > 0.8$). This baseline dynamism of microglial processes is in sharp contrast to the stability of the surrounding neuronal processes[17] and is in line with their proposed function to sense and respond to tissue abnormalities.

Microglia respond rapidly to focal brain injury

To directly examine the response of microglia to brain injury, we took advantage of the focal properties of the two-photon laser and performed a small laser ablation, $\sim$15 μm in diameter, inside the cortex through the thinned skull (**Figs. 2,3;** see Methods). This type of injury

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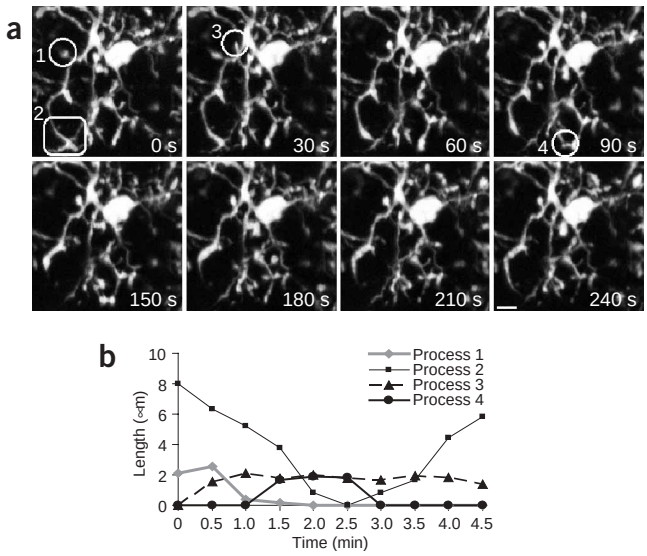

Figure 1 Transcranial two-photon imaging shows rapid dynamics of fine microglial processes. (**a**) Time-lapse imaging of the same microglial branches demonstrated rapid extension and retraction of fine microglial processes over seconds. Circles and rounded box indicate four representative processes that change in length and shape over time (**Supplementary Video 1**). (**b**) Length changes of the four processes marked in **a** as a function of time. Scale bar, 5 μm.

has been studied and involves non-linear processes that can be confined to the laser focus, much like the two-photon fluorescence excitation[18]. Time-lapse imaging showed that all the cells near the site of injury (cell bodies within ~50 μm from ablation) responded within the first minute post-ablation: the tips of their processes close to the site of injury appeared bulbous and slightly enlarged. Within the next few minutes, these cells extended their processes toward the damaged site at an average rate of 1.25 ± 0.06 μm/min (**Fig. 2b,c** and **Supplementary Video 2**; $n > 20$; five quantified in **Fig. 3g–i**). Approximately 30 min after the laser-induced injury, the processes of the nearby cells reached the damaged site and appeared to fuse together, forming a spherical containment around it (**Fig. 2d**). During this period, the same cells also retracted those processes that previously lay in directions opposite to the site of injury (**Fig. 2d,e**). Most of the cellular content of each of the immediate neighbors was directed towards the damaged site within the first 1–3 h (**Fig. 2e,f**) whereas the cell bodies remained at the same location for at least 10 h (**Supplementary Video 2**). Cells located further away (cell bodies between 75 and 125 μm from the ablation, 82 cells in six animals) also responded in a directional way, sending their processes towards the ablation without ever reaching the already-contained injury site (data not shown). In addition, when the laser ablation was performed on two or more sites very close to each other, we observed that the same cells that initially committed a number of their processes to the first ablation could still detect and send their remaining processes to the new ablation 10–20 min later (**Supplementary Video 3**). These results indicate that the site of laser-induced injury releases highly localized signals able to rapidly attract individual microglial processes.

To determine whether microglia can also respond to mechanical injury in a similar fashion, we induced local injury to a small area of the cortex (~50 μm in diameter) with a glass electrode through a small

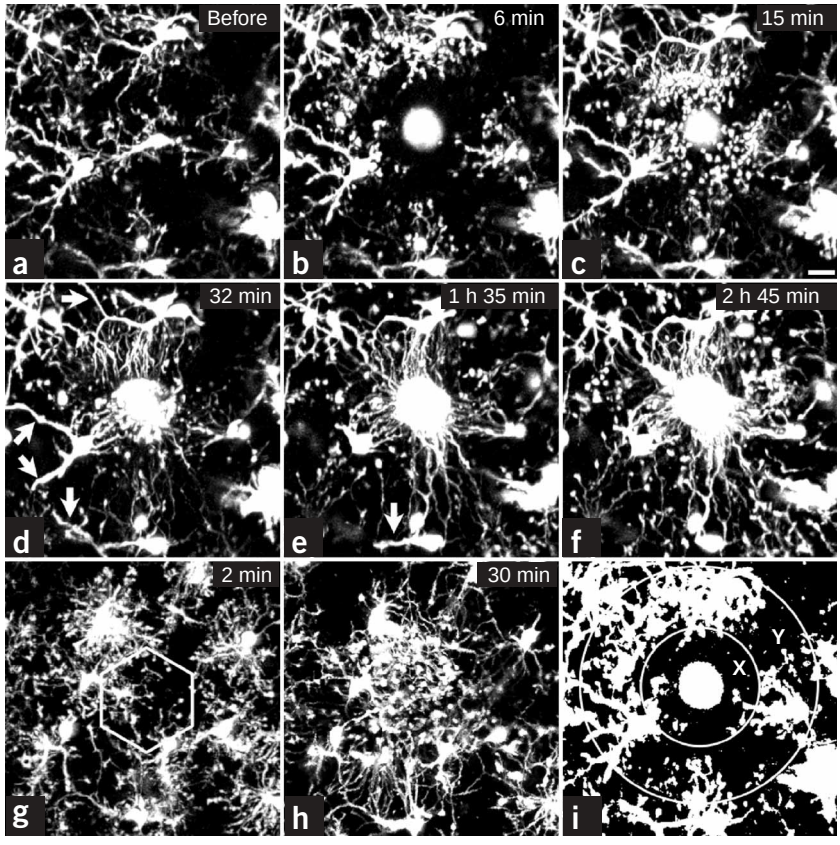

Figure 2 Microglial processes move rapidly towards the site of injury induced either by the two-photon laser, or mechanically with a glass electrode. (**a**–**f**) After we created a localized ablation inside the cortex (~15 μm in diameter) with a two-photon laser (**b**; see Methods), nearby microglial processes responded immediately with bulbous termini (**b**) and extended toward the ablation until they formed a spherical containment around it (**c**–**f**). At the same time, the same cells retracted those processes that lay in directions opposite to the site of injury (arrows in **d** and **e**; **Supplementary Video 2**). (**g**,**h**) Mechanical injury, induced with a glass electrode in a small area of the cortex marked with a hexagon (~50 μm in diameter), led to a similar response of microglial processes as with the laser-induced injury (**Supplementary Video 4**). (**i**) To quantify the microglial response toward the laser-induced injury, we measured the number of microglial processes entering from the outer area Y (70 μm in radius) into the inner area X (35 μm in radius) as a function of time. The number of white pixels in area X or Y were measured at each time point ($R_x(t)$ or $R_y(t)$), and the microglial response was defined as $R(t) = (R_x(t) - R_x(0))/R_y(0)$ (see Methods). The results of such quantification are shown in **Figure 3g**. Scale bar, 10 μm.

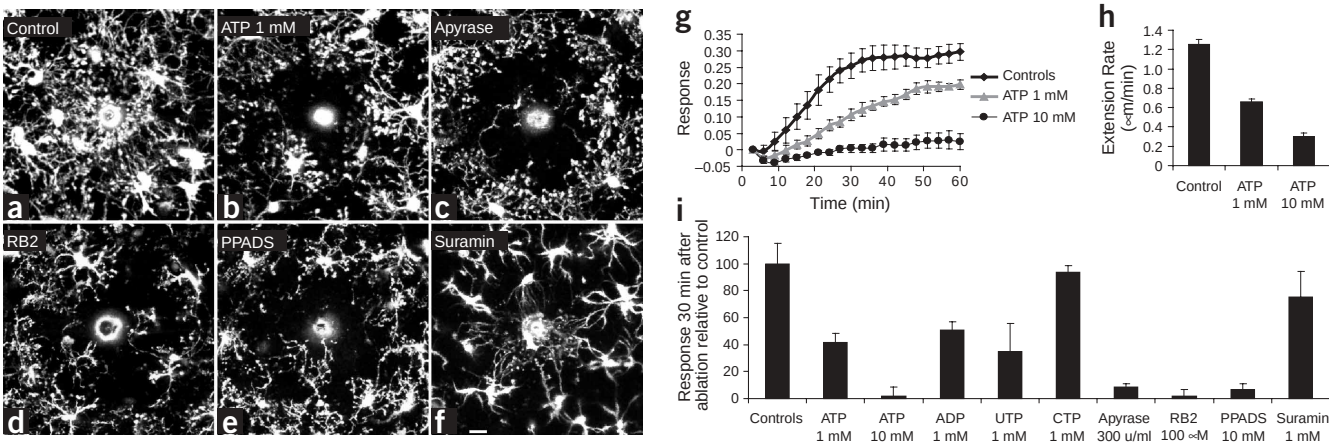

Figure 3 Extracellular ATP and activation of purinergic G protein–coupled receptors are necessary for rapid chemotactic microglial response. (**a**–**f**) Microglial response (30 min after laser ablation) in the presence of various compounds applied through a small craniotomy (see Methods): ACSF (**a**), 1 mM ATP (**b**), 300 U/ml apyrase (**c**), 100 μM reactive blue 2 (**d**), 10 mM PPADS (**e**) and 1 mM suramin (**f**). While many microglial processes reached the site of injury in **a** and **f**, few or none moved towards the ablation in **b**–**e**. (**g**–**i**) Quantification of microglial response to laser ablation (described in **Fig. 2i** and Methods) after a 45-min bath application of various compounds. (**g**) Detailed kinetics of microglial response over 1 h in the control (ACSF), 1 mM and 10 mM ATP solutions. The response in control reaches a plateau within ~35 min (when the converging processes have reached and contained the damage), whereas in the presence of 1–10 mM ATP, the response is smaller and slower. (**h**) Extension rate of microglial processes toward the laser ablation in control (ACSF), 1 mM ATP and 10 mM ATP solutions. (**i**) Microglial response measured at the 30-min time point after laser ablation in the presence of various compounds ($n = 3$–5 for each case). Scale bar, 10 μm.

craniotomy (see Methods). We observed that microglial processes assumed the bulbous morphology at their termini immediately upon injury and rapidly moved into the damaged tissue (**Fig. 2g**,**h**). The localized nature and the kinetics of the microglial response to mechanical damage were very similar to those observed in the laser ablation injury model (**Supplementary Video 4**). The rapid and highly directional responses of microglia to the exact location of both laser- and mechanically induced injuries suggest that the same signaling cascade is involved in attracting the microglial processes in both models. Owing to the relative ease in controlling the extent of injury with a focused laser beam, we performed all the subsequent experiments using laser-induced ablation.

ATP mediates rapid microglial response towards injury

Previous studies of microglia in culture have shown that ATP signaling through G protein–coupled P2Y receptors induces rapid microglial membrane ruffling and whole-cell migration[11]. To test whether ATP released as a result of tissue injury is important in mediating microglial response, we applied 100 μM, 1 mM or 10 mM ATP in artificial cerebrospinal fluid (ACSF) directly through a small craniotomy (see Methods), followed by laser ablation. We reasoned that if ATP released from the injured tissue were responsible for initiating the observed microglial response, such a response should be prevented by eliminating the ATP concentration gradient or by saturating ATP receptors after bathing the tissue with a high concentration of ATP, comparable to the intracellular ATP concentration[19]. Indeed, we found that applying 1–10 mM (but not 100 μM) ATP significantly reduced both the extent and speed of the microglial response towards the injury site (**Fig. 3b**,**g**–**i**; the extension rate of processes was 0.66 ± 0.03 μm/min and 0.29 ± 0.04 μm/min in the presence of 1 mM and 10 mM ATP, respectively ($n = 3$–5 for each concentration), and the extension rate of the control was 1.25 ± 0.06 μm/min; $P < 0.001$). Similar to ATP application, 1 mM ADP or UTP also significantly reduced the microglial response, but 1 mM CTP had no effect (**Fig. 3i**, $n = 3$–5 for each case). These results suggest that factors such as ATP, ADP and UTP released from the injured tissue are involved in attracting microglial processes towards the injury site.

To further test the role of ATP and ADP in the microglial response, we performed the laser ablation in the presence of apyrase, an ATPase that hydrolyzes both extracellular ATP and ADP[19,20]. Similar to application of ATP and ADP, application of 300 U/ml apyrase also substantially reduced the microglial response to laser ablation (**Fig. 3c**,**i**; extension rate of processes was 0.5 ± 0.03 μm/min, $n = 5$; $P < 0.001$), suggesting that the presence of ATP and/or ADP in the extracellular space is necessary for the rapid microglial response towards the site of injury. In addition, the rapid baseline motility of microglial processes slowed in the presence of 300 U/ml apyrase: over a 15-min interval, the average absolute length change of the fine dynamic processes was 1.91 ± 0.25 μm after applying apyrase (36 processes, four cells from four animals), significantly smaller than the control with ACSF solution (2.73 ± 0.29 μm, 35 processes, five cells from three animals; $P < 0.003$), suggesting that extracellular ATP and/or ADP is also involved in regulating the baseline motility of microglial processes.

P2Y receptor activation is necessary for microglial response

ATP, ADP and UTP are potent agonists for P2X ligand-gated ion channels and P2Y G protein–coupled receptors[21]. To investigate the involvement of activation of these receptors in mediating the microglial response, we applied various P2 receptor inhibitors directly to the cortex through a small craniotomy and recorded their effect on microglial dynamics after laser ablation. Two P2Y inhibitors, reactive blue 2 (100 μM) and PPADS (10 mM), markedly reduced the number and motility of microglial processes toward the ablations, while up to 1 mM suramin (mainly a P2X inhibitor) was not effective in preventing the laser-induced response (**Fig. 3d**–**f**, i; $n = 3$–4 for each inhibitor). Together, these results suggest that activation of P2Y G protein–coupled receptors by ATP, ADP or UTP, either on microglia or cells in the surrounding tissue (see discussion below), is necessary for the rapid microglial response to injury.

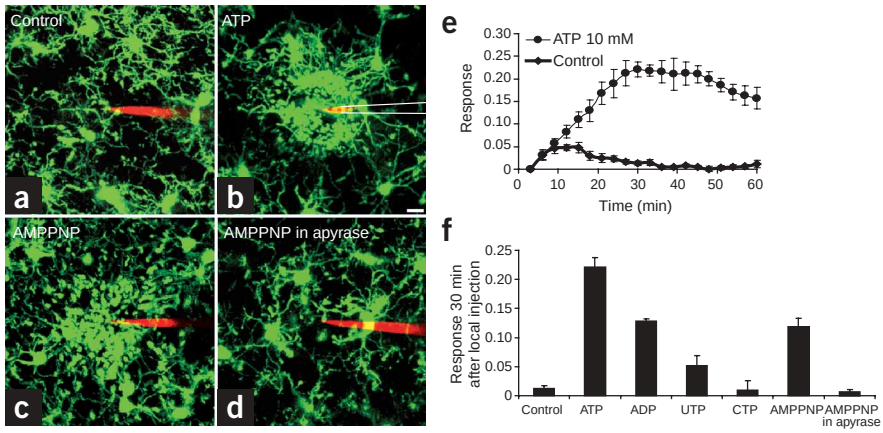

Figure 4 ATP-induced ATP release is essential for rapid microglial response. (**a**–**d**) Microglial response to local injection of ATP or AMPPNP (non-hydrolyzable ATP analogue) 30 min after insertion of the glass electrode through a craniotomy. Whereas a control microelectrode containing ACSF and rhodamine-dextran (**a**) caused little or no microglial response, similar electrodes containing 10 mM ATP (**b**) or 10 mM AMPPNP (**c**) induced rapid extension of microglial processes towards the tip of the electrode (**Supplementary Video 5**). However, a microelectrode containing 10 mM AMPPNP in the presence of 300 U/ml apyrase (**d**; applied 1 h before the electrode was inserted) did not elicit a response towards the tip. (**e**) Detailed kinetics of microglial response towards control electrode (**a**) and towards electrodes containing 10 mM ATP (**b**). (**f**) Microglial response measured as described in **Figure 2i**, 30 min after the insertion of electrodes containing only ACSF (control) or 10 mM of various compounds in ACSF ($n = 3$–5 for each case). For the last bar graph, electrodes containing 10 mM AMPPNP were inserted in the brain after a solution of 300 U/ml apyrase was applied for 1 h. Scale bar, 10 μm.

Local release of ATP induces rapid microglial response

To test whether ATP is sufficient to induce a microglial response similar to the one we observed after laser ablation, we inserted a sharp microelectrode containing ATP (tip diameter <1 μm) into the cortex and monitored the surrounding microglial behavior (**Fig. 4**). We found that simply inserting an ACSF-containing electrode into the cortex (without moving it laterally to cause tissue damage) induced no significant response from the surrounding microglia (**Fig. 4a**,**e**,**f**; $n = 5$). However, a similar electrode containing 10 mM ATP in an ACSF solution was able to mimic in time, range and kinetics the rapid response of microglial processes observed following laser ablation (**Fig. 4b**; 15 injections in ten mice, three quantified in **Fig. 4e**,**f**). Within the first minute after the electrode was inserted, many nearby microglial processes showed enlarged termini and began extending toward the tip of the electrode (extension rate of processes toward the tip was on average 1.10 ± 0.05 μm/min; 30 processes in three quantified experiments). Within ~45 min, a large number of processes converged and tightly surrounded the tip of the electrode (**Supplementary Video 5**). If the electrode was withdrawn from the cortex 10–15 min after insertion while the microglial response was underway, the convergence of the processes towards the previous location of the tip stopped immediately (data not shown), suggesting that the constant release of ATP from the tip of the electrode was necessary for the chemotactic response of microglial processes. Notably, when electrodes containing a high concentration of ATP (100 mM) were used, the surrounding microglial processes responded rapidly at the beginning but slowed significantly and formed a sphere midway around the tip, possibly because ATP receptors within the sphere were saturated by the high concentration of ATP released from the tip of the electrode ($n = 4$, data not shown).

Similar to the response to ATP, release of 10 mM ADP through an electrode also induced a rapid response from microglial processes (**Fig. 4f**; $n = 4$); 10 mM UTP induced a smaller response (**Fig. 4f**; $n = 5$), and we observed little or no response with 10 mM CTP (**Fig. 4f**; $n = 4$). Since ATP in the extracellular space is hydrolyzed very rapidly by ectonucleases[19,20], we further tested whether ATP alone could attract microglia by injecting non-hydrolyzable ATP analogues (AMPPNP or ATPγS) into the cortex. We found that local application of 10 mM AMPPNP caused a chemotactic response similar in range and size to that with ATP application (**Fig. 4c**,**f**; average extension rate towards the tip was 1.07 ± 0.05 μm/min; 30 processes, $n = 4$), and 10 mM ATPγS induced a smaller response ($n = 3$, data not shown).

ATP-induced ATP release is essential for microglial response

The above experiments indicate that ATP released from either the damaged tissue or an electrode can trigger the rapid response of microglial processes towards the source of ATP. Many lines of evidence have shown that extracellular ATP released from astrocytes, and subsequent activation of purinergic receptors, mediate intercellular communication among astrocytes as well as communication between astrocytes and microglia, as indicated by calcium wave propagation[22–27]. In addition, ATP can induce ATP release from astrocytes, and such a regenerative ATP release is important for the spreading of Ca^{2+} waves[23,25,28]. To test whether ATP-induced ATP release is necessary for the rapid microglial response, we applied 300 U/ml apyrase on the cortex through a small craniotomy for 1 h and then tested the effect of non-hydrolyzable ATP (either AMPPNP or ATPγS at 10 mM) released from a microelectrode. Although a non-hydrolyzable ATP-containing electrode alone can rapidly attract microglial processes (**Fig. 4c**,**f**), no response was observed in the presence of apyrase (**Fig. 4d**,**f**; $n = 5$). Furthermore, neither inserting a microelectrode containing high concentration of AMPPNP (up to 100 mM) nor continuously injecting 10 mM AMPPNP was able to reverse the inhibitory effect of apyrase on the microglial response towards the electrode tip ($n = 4$ for each, data not shown). As only the endogenous ATP, not the non-hydrolyzable ATP, was rapidly degraded by the applied apyrase in the extracellular space, this suggests that non-hydrolyzable ATP–induced release of endogenous ATP is required for attracting microglial processes. In addition, ATP released from the electrode appears to function as a trigger for further release of ATP and possibly other factors from the surrounding tissue (astrocytes, neurons, etc.) that are important for microglial response, because constant release of ATP from the electrode is not able to attract microglial processes directly in the presence of apyrase.

Connexin hemichannels are important for microglial dynamics

Because ATP induces ATP release from astrocytes, and such an induction serves as an important mechanism for inter-astrocyte communication[23,25,28], the above experiments suggest that astrocytes and their release of ATP are important for the microglial response upon injury. It has been shown that ATP release from cultured astrocytes and

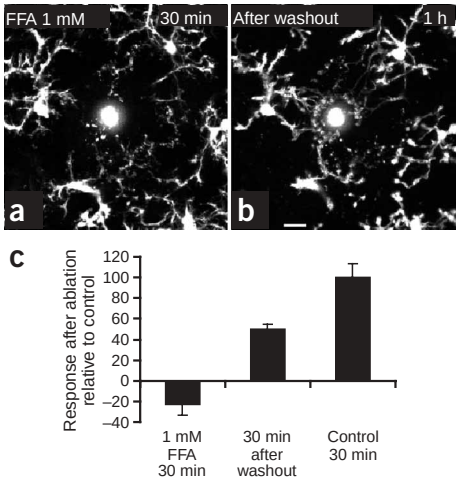

Figure 5 The microglial response towards the laser ablation is inhibited after blocking connexin hemichannels, the conduits of ATP. (**a**) Very few or no microglial processes extended towards the site of injury within the first 30 min after laser ablation in the presence of 1 mM flufenamic acid (FFA) applied through a small craniotomy. (**b**) Microglial processes extended toward the same site of injury 30–60 min after FFA was washed out. (**c**) Quantification of microglial response in the presence of 1 mM FFA and 30 min after washout. Scale bar, 10 μm.

glioma cell lines involves connexin hemichannels[24,25,29–32]. Adult astrocytes express very high levels of connexin proteins[33], and the connexin channel blocker flufenamic acid (FFA)[34] inhibits ATP release from astrocytes[25,30]. To further explore the involvement of ATP and astrocytes in microglial response, we applied connexin channel inhibitors such as FFA or carbenoxolone (CBX) before laser ablation. We first found that the baseline motility of microglial processes was abolished within 30 min after applying 250 μM CBX or within 10 min after applying 1 mM FFA. In the presence of FFA, the average absolute length change of the small processes is 0.19 ± 0.04 μm within 15–30 min (33 processes from ten cells, three animals), significantly smaller than that of the control (2.73 ± 0.29 μm; $P < 0.0001$), suggesting that connexin channels are important for the dynamics of microglial processes in their native state, possibly by releasing ATP and/or other factors. In addition, 30 min after laser ablation, we did not observe any microglial processes moving towards the injury site in the presence of either 250 μM CBX ($n = 3$, not shown) or 1 mM FFA (**Fig. 5**; $n = 7$). The directional responses of microglia towards an electrode containing 10 mM ATP were also completely abolished in the presence of CBX or FFA ($n = 4$ for each inhibitor; data not shown). Notably, after washing out FFA with ACSF, the microglial processes extended towards the site of ablation that was performed in the presence of 1 mM FFA ~30 min earlier (**Fig. 5**; $n = 7$). This result indicates that the inhibitory effect of FFA is reversible and that the signals attracting microglial processes persist for at least 30 min after laser ablation is performed. Together, these experiments suggest that astrocytes are involved in releasing ATP through connexin channels and are important for mediating the rapid microglial response to local brain trauma.

DISCUSSION

By taking advantage of transcranial two-photon imaging and GFP knock-in mice, we have been able to observe, for the first time, rapid dynamics of microglial processes in the intact living brain. In addition, within the first minutes of laser-induced or mechanical injury, microglial processes respond and move directly towards the site of injury. The directional convergence of microglial processes towards the trauma implies the presence of a gradient of one or more highly diffusible and abundant molecules that mediate this phenomenon. For at least 30 min after the laser ablation, the signals attracting the microglial processes still remain (**Fig. 5a**,**b**), suggesting a role of the surrounding tissue in preserving and regenerating the signals that fuel the observed response.

ATP is an important signaling molecule mediating interactions among various cell types in the brain[10,35,36]. Massive release of purines occurs after metabolic stress and trauma[36,37], and high levels of ATP persist in the peritraumatic zone for many hours after the insult[38]. ATP, acting through purinergic receptors, can induce membrane ruffling and ramification of cultured microglia[11] and can stimulate them to release various biologically active substances[10]. Our results demonstrate that extracellular ATP and activation of P2Y receptors are necessary for the rapid microglial response towards the injury site, suggesting that extracellular ATP could itself be the chemoattractant responsible for the directional extension of the processes by activating P2Y receptors on microglia. Alternatively, ATP may activate microglia and/or act as a trigger (through purinergic mechanisms) to induce the release of unidentified chemoattractant(s) from the surrounding tissue. Our results with non-hydrolyzable ATP injection and apyrase application indicate that (i) release of endogenous ATP triggered by non-hydrolyzable ATP (or ATP) is necessary for the microglial response, and (ii) ATP may not be the endogenous chemoattractant, because non-hydrolyzable ATP from a point source (an electrode) in the presence of apyrase is not sufficient to attract the microglial processes.

Although neurons, oligodendrocytes and endothelial cells are likely to release large amounts of ATP upon injury and thereby contribute to microglial reaction, two lines of evidence suggest that astrocytes are important in mediating the rapid and widespread microglial response. First, our results indicate that this response involves ATP-triggered ATP release from the surrounding tissue; such a regenerative release of ATP has been shown to be an important mechanism for long-range signaling among astrocytes[23,25,28]. Second, we have found that connexin channel inhibitors block microglial response reversibly. Connexin channels are highly expressed in astrocytes but not microglia in the resting state[33] and are involved in ATP release from astrocytes[25,30]. Together, our results suggest that the initial ATP release from the damaged tissue triggers further release of ATP and other factors from the surrounding astrocytes, a process requiring the opening of connexin channels and the activation of purinergic receptors. Although factors other than ATP may be important for directional chemotaxis of microglial processes, ATP-triggered ATP release from the surrounding astrocytes is key in mediating such a response.

Furthermore, the baseline motility of microglial processes in the intact brain is also modulated by the same ATP signaling mechanisms mediating injury-induced microglial responses, because baseline dynamism slows significantly in the presence of apyrase and connexin channel inhibitors. The high baseline motility of microglial processes may reflect the fluctuation of the ATP concentration in the surrounding tissue. It is worth noting that owing to technical limitations, we imaged microglial behavior only in anesthetized mice. As anesthetics can induce microglial activation in the brain[39], the degree to which the baseline microglial dynamics is altered by anesthetics and/or the state of anesthesia remains to be determined. Upon injury and release of intracellular ATP, the immediate chemotactic response of microglial processes seems to be specific for microglia, as we did not observe any obvious motile responses of nearby neuronal processes within hours of a laser ablation in the cortex of mice with YFP-labeled

neurons (D.D., J.V.K., M.L.D., W.-B.G., unpublished observations). It is also important to point out that not all brain pathologies would trigger a rapid microglial response as in the case of traumatic injury. For example, the ATP concentration near sites of focal ischemia and other types of vascular injury are likely to be low owing to the lack of oxygen in the surrounding cells, and the amounts of ATP released from the injured tissue does not appear to be sufficient to generate a rapid microglial response (J.G., D.D., W.-B.G., unpublished observations). The functional significance of the immediate microglial response towards sites of traumatic injury remains to be elucidated. Such a response may allow for rapid isolation of damaged areas and could be particularly important when trauma-associated local bleeding occurs inside the brain and immediate containment of the damage is a priority for restoring a stable environment for the nearby neurons.

METHODS

Transgenic mice. Heterozygous *Cx3cr1*$^{GFP/+}$ mice were used for all experiments[16]. *Cx3cr1* (encoding fractalkine receptor) is expressed in microglia, some monocytes and dendritic cells. No obvious defects of parenchymal microglia were found in homozygous mutant *Cx3cr1*$^{GFP/GFP}$ mice[16], and the rapid microglial response to laser-induced injury was also observed in these mice. These mice were housed and bred in Skirball Institute's animal facilities, and all experiments were done in accordance with protocols approved by the institutional animal committee.

***In vivo* imaging of microglia with two-photon microscopy.** GFP-labeled microglia were imaged by two-photon microscopy, either through a thinned intact skull as described previously[17] or through a small craniotomy. Briefly, adult transgenic mice were anesthetized intraperitoneally with ketamine (200 mg/kg body weight) and xylazine (30 mg/kg body weight) in 0.9% NaCl solution. For transcranial imaging, a region (~1 mm in diameter) over barrel or motor cortex was first thinned with a high-speed drill under a dissecting microscope and then scraped with a microsurgical blade to a final thickness of <20 μm. For imaging through a window in the skull, a thinned region (~1–2 mm in diameter) was opened either with a needle or forceps. A drop (~200 μl) of artificial mouse cerebrospinal fluid (ACSF) was applied on the exposed region for the duration of the experiment. The skull surrounding either the thinned region or the open skull window was attached to a custom-made steel plate to reduce respiratory-induced movement. The animal was placed under either a Bio-Rad multi-photon microscope (Radiance 2001) or a custom-made two-photon microscope described previously[17].

The Ti-sapphire laser was tuned to the excitation wavelength for GFP (890 nm). A stack of image planes with a step size of 0.75–2 μm was acquired using a water-immersion objective (Olympus 60×, 0.9 N.A.; Nikon 60× 1.0 N.A. or 40×, 0.8 N.A.) at zoom of 1.0–3.0. The maximum imaging depth was ~200 μm from the pial surface. Images were acquired using low laser power (<30 mW at the sample) and a low-pass emission filter (<700 nm).

Two-photon laser ablation and mechanical injury inside the cortex. A highly localized injury was achieved by focusing a two-photon laser beam (~1 μm in size) in the superficial layer of the cortex either through a thinned, intact skull or with a small piece of the skull removed. The wavelength of the two-photon laser was set at 780 nm and the laser power was ~60–80 mW at the sample. The beam was parked at the desired position for approximately 1–3 s to create a small injury site as indicated by a bright autofluorescent sphere (~15 μm in diameter) around the focal point of the beam (**Figs. 2–4**). The injury was confined to the area ~15–20 μm in diameter around the laser focal point, because microglia within this area lost their GFP immediately after laser ablation, whereas those ~30 μm from the injury site still responded to the ablation. The laser-induced focal ablation is a useful injury model, as the site and degree of injury are easy to control, and the response of microglia toward the injury is highly reproducible.

Mechanical injury inside the cortex was generated with a glass microelectrode (~1 μm tip diameter). The electrode was loaded with an ACSF solution containing 3% rhodamine-dextran (10 kDa, Molecular Probes) to make it visible under a fluorescent microscope. The electrode was inserted into the exposed cortex through an open skull window using a micromanipulator. The local mechanical injury was generated by moving the fine control of the micromanipulator laterally within a region ~50 μm in diameter (**Fig. 2g,h**).

Drug application through an open window in the skull. Various reagents were dissolved in ACSF, and a small drop of the solution (~200 μl) containing the compound of interest was applied directly onto the cortex through the open window. Imaging started 10–45 min after drug application. Because of rapid diffusion of small molecules within the cortex[40], we estimated that the effective drug concentration in the cortex was ~10 times lower than that in the solution directly applied to the cortex. For the local drug application, the reagent was added to the ACSF-rhodamine solution and loaded in a sharp glass electrode (1.0 mm outer diameter; 10–12 MΩ resistance as measured in ACSF). A picospritzer (General Valve Incorporation) was used to release a small volume of the solution at 20–40 psi for 20–50 ms.

Image processing and quantification. All image processing was done using US National Institutes of Health Image J software. All *z*-stacks of images were projected along the *z*-axis to recreate a two-dimensional (2D) representation of 3D structures. Time-lapse movies were generated by *z*-projections of stacks of images taken sequentially over time. In making both the movies and figures, we made sure that the 3D image stacks at all time points pass above and below the processes studied.

The laser ablation appeared as a small autofluorescent sphere ~15 μm in diameter (**Fig. 2b,i**). Following the ablation, the processes of neighboring microglia invaded the area around the site of injury and eventually reached the sphere. To quantify the extent and speed of microglial responses to laser-induced injury, we measured the number of microglial processes entering from the outer area Y (70 μm in radius) into the inner area X (35 μm in radius) surrounding the ablation site as a function of time (**Fig. 2i**). To account for signal intensity differences among different experiments, we thresholded every image so that all processes had the maximum value (255), and all background was set to 0 (**Fig. 2i** shows the thresholded version of **Fig. 2b**). We then counted the number of white pixels in area X over time ($R_x(t)$) and compared it with the first picture taken immediately after the ablation ($R_x(0)$). The number of white pixels corresponds to the region covered by processes within the area X, and its increase over time provides a measure of the microglial response. To account for the variability in the number of microglia located in the outer area Y in different experiments, we calculated the microglial response relative to the number of processes in the outer area Y immediately after the ablation ($R_y(0)$). The microglial response at any time point ($R(t)$) is therefore given by $R(t) = (R_x(t) - R_x(0))/R_y(0)$.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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