

Intracellular Ca^{2+} dynamics are required for microglial process motility during surveillance and in response to injury

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Abstract

Microglia, the resident immune cells of the central nervous system, play pivotal roles in brain surveillance and injury responses through highly dynamic process motility. Here, we investigated the role of Ca^{2+} signaling in mediating microglial process motility under both physiological and pathological conditions in vivo. Using dual-laser two-photon microscopy in a transgenic mouse model expressing red fluorescent protein (mCherry) and the Ca^{2+} indicator (GCaMP6m) specifically in microglia, we observed autonomous Ca^{2+} microdomains that operate asynchronously within individual processes, exhibiting significantly more frequent and localized Ca^{2+} transients compared to non-motile structures. Upon focal laser-induced cortical injury, we observed rapid Ca^{2+} waves in microglia, with processes extending toward the lesion site displaying correlated Ca^{2+} activity. Attenuation of Ca^{2+} transients by BAPTA-AM, a Ca^{2+} chelator, impaired both homeostatic and laser-induced motility. Importantly, microglia-specific genetic overexpression of plasma membrane Ca^{2+} ATPase 2 in vivo markedly reduced both homeostatic surveillance and injury-induced motility. Together, our findings provide evidence that microglial intracellular Ca^{2+} signaling is required for process motility in vivo, supporting a model in which local Ca^{2+} dynamics enable surveillance and injury-directed remodeling.

Keywords Microglia · Ca^{2+} signaling · Surveillance · Motility · In vivo · Ca^{2+} wave · Laser injury · PMCA2

Introduction

Microglia, the resident immune cells of the central nervous system (CNS), play crucial roles in maintaining brain homeostasis, orchestrating responses to pathological challenges, and modulating neuronal activity [1, 2]. Through the dynamic extension and retraction of their highly ramified processes, microglia continuously survey the brain, enabling rapid detection of and response to disturbances [3, 4]. In response to injury, microglial

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processes converge toward the lesion site at speeds of $\sim 2 \mu\text{m}/\text{min}$, a response largely driven by extracellular ATP and dependent on the purinergic P2Y₁₂ receptor [4, 5]. This rapid convergence is considered neuroprotective, as it limits lesion expansion [6].

In addition to large motile processes, microglia utilize thin, actin-dependent filopodia for rapid nanoscale sensing. These distinct modes of motility are differentially regulated: P2Y₁₂ activation promotes major process extension and filopodial collapse [5], whereas elevated cyclic AMP exerts the opposite effects [7]. However, the intracellular mechanisms linking receptor activation to cytoskeletal remodeling and process motility remain incompletely understood.

In other migratory cells, such as fibroblasts and macrophages, localized calcium (Ca^{2+}) transients at the leading edge orchestrate actomyosin contractility and focal adhesion turnover, driving directional movement [8–10]. Recent work in microglia has implicated type-II myosins in morphological regulation [11], suggesting that conserved Ca^{2+} dependent mechanisms may also contribute to microglial motility. However, *in vivo* studies have reported low basal Ca^{2+} activity in microglia despite active surveillance [12, 13], raising questions about the functional relevance of Ca^{2+} signaling for microglial dynamics.

Recent evidence has begun to refine this view. *In vivo* imaging in awake mice revealed that Ca^{2+} signaling is preferentially associated with extending, rather than stable or retracting, microglial processes [14]. While this study established an important correlation between process-level Ca^{2+} and extension, the fine-scale spatiotemporal organization of this signaling remains unknown. Moreover, although Ca^{2+} has been implicated in migration in other models [15] and is associated with the injury response [16], these findings remained largely correlative and did not establish whether Ca^{2+} signaling is required for microglial process motility. Thus, a microglia-intrinsic requirement for Ca^{2+} signaling during surveillance and injury-directed remodeling *in vivo* has remained unresolved.

Here, we addressed these questions using the dual-reporter C2M2G mouse line (Csf1r-2A-mCherry-2A-GCaMP6m), which enables simultaneous high-resolution two-photon imaging of microglial morphology and Ca^{2+} dynamics *in vivo*. This approach enabled us to identify spatially confined, asynchronous Ca^{2+} microdomains within microglial processes and to examine their temporal coupling with motility. By combining this model with pharmacological Ca^{2+} chelation and microglia-specific overexpression of the Ca^{2+} extrusion pump plasma membrane Ca^{2+} ATPase (PMCA) 2, we directly tested whether intracellular Ca^{2+} signaling is required for microglial process motility during physiological surveillance and laser-induced acute injury.

Our findings show that microglial process motility is closely associated with Ca^{2+} transients under both baseline and laser-induced acute injury conditions. The perturbation of Ca^{2+} dynamics markedly impaired process motility, establishing a central role for Ca^{2+} in microglial surveillance and the injury response. Together, these results establish a requirement for intracellular Ca^{2+} signaling in microglial process motility *in vivo*.

Results

Microglial processes exhibit compartmentalized, unsynchronized Ca^{2+} activity in microdomains

To investigate microglial Ca^{2+} dynamics *in vivo*, we utilized our previously described C2M2G transgenic mouse line [17], which enables simultaneous visualization of microglial morphology (via mCherry) and Ca^{2+} activity (via GCaMP6m) by two-photon microscopy (Fig. 1A). Imaging was performed under anesthesia through a cranial window in cortical layer 1, approximately $100 \mu\text{m}$ beneath the pial surface, thereby restricting our analysis to parenchymal microglia rather than superficial meningeal macrophages. Microglia displayed characteristic ramified morphology and were readily identifiable by mCherry fluorescence (Fig. 1B). Motion-corrected GCaMP6m fluorescence ($\Delta F/F_{\text{corr}}$) within mCherry-positive microglial compartments revealed spontaneous Ca^{2+} transients under basal conditions (Fig. 1C).

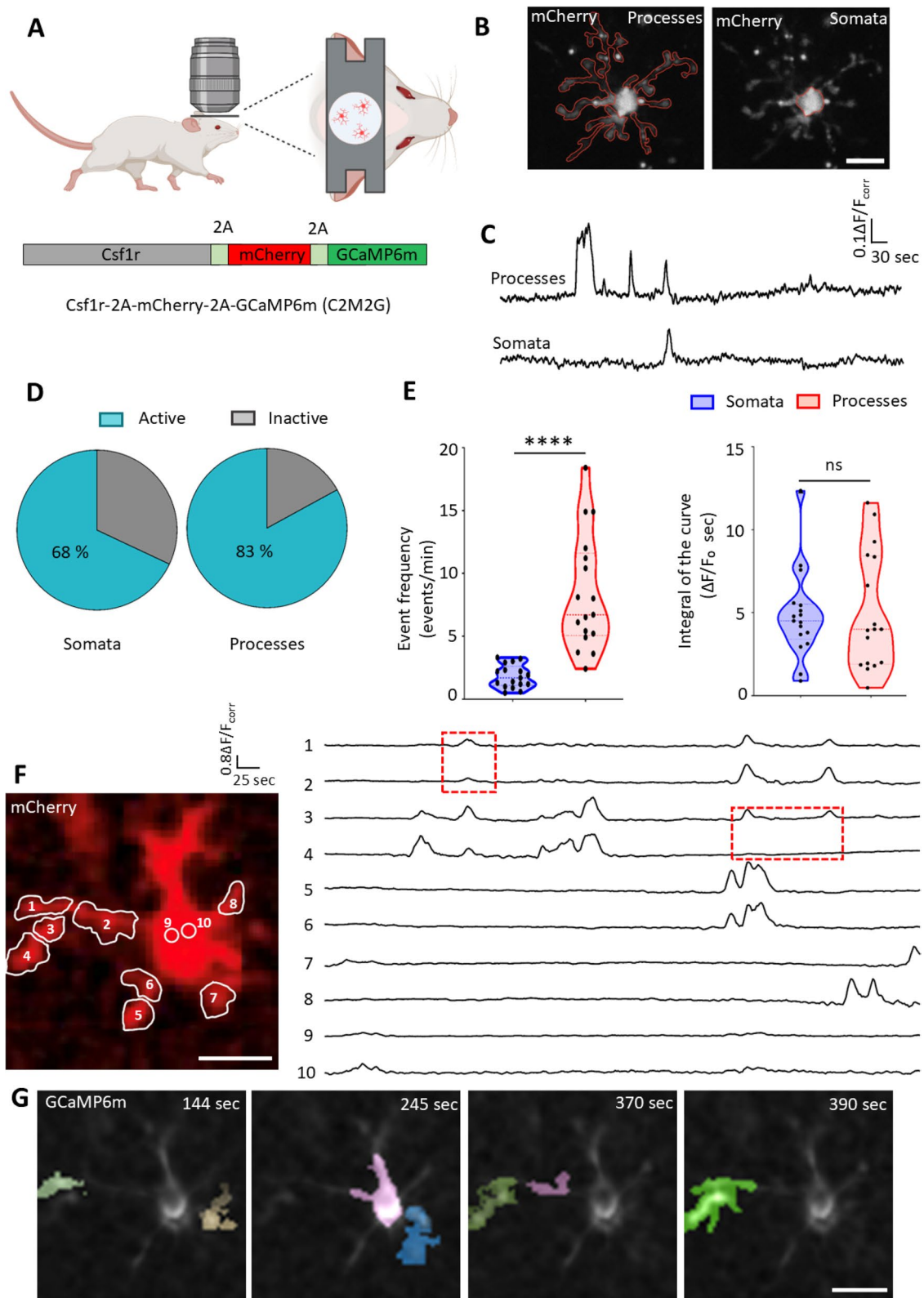


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Fig. 1 Microglial processes exhibit compartmentalized, unsynchronized Ca^{2+} activity in microdomains in vivo. **A** Scheme of in vivo two-photon imaging in C2M2G transgenic mice expressing mCherry to visualize microglial morphology and GCaMP6m to monitor Ca^{2+} dynamics through a cranial window over the somatosensory cortex. The Csflr-2A-mCherry-2A-GCaMP6m reporter strategy is shown below. Created with BioRender.com. **B** Representative two-photon images of a layer 1 microglial cell imaged approximately 100 μm below the pial surface under anesthesia. ROIs were defined for the somata (right) and individual processes (left). Scale bar, 20 μm . **C** Representative motion-corrected GCaMP6m $\Delta F/F_{\text{corr}}$ traces extracted from the ROIs in (B), illustrating spontaneous Ca^{2+} transients in the somata and processes under basal conditions. **D** Pie charts showing the mean percentage of active (cyan; ≥ 1 detectable Ca^{2+} transient) and inactive (gray; no detectable Ca^{2+} transient) somata (left) and processes (right) during a 10-min recording. On average, $68 \pm 4.60\%$ of the somata and $83 \pm 3.04\%$ of the processes were active (mean $\pm$ SEM). Data are from $n = 17$ imaging sessions/FOVs from 6 mice. **E** Quantitative comparison of Ca^{2+} event frequency (left) and integral of the curve (IC) (right) between somata (blue) and processes (red). Process compartments exhibited a significantly higher event frequency than somata, whereas IC did not differ significantly. Each point represents one imaging session/FOV. Statistical analysis was performed using a paired two-tailed t-test; **** $P < 0.0001$; ns, not significant; $n = 17$ imaging sessions/FOVs from 6 mice, with each imaging session lasting 10 min. The triangle in the IC plot indicates one value identified as an outlier using ROUT ($Q = 1\%$) and excluded from the statistical analysis; the corresponding matched observation was therefore excluded from the paired IC comparison ($n = 16$ matched pairs). **F** Representative high-resolution imaging of Ca^{2+} activity in microglial compartments. Left: mCherry image with numbered ROIs (1–8, processes; 9–10, somata). Right: Corresponding motion-corrected GCaMP6m $\Delta F/F_{\text{corr}}$ traces from the numbered ROIs. The traces illustrate spatially confined, unsynchronized Ca^{2+} transients in processes. In addition, representative $\Delta F/F_{\text{corr}}$ traces also illustrate asynchronous Ca^{2+} transients within a single process. The rectangular red dashed boxes highlight representative periods of localized, asynchronous activity occurring within the same branch of the single process. Scale bar, 10 μm . **G** Representative time-lapse images of GCaMP6m showing spontaneous, event-based Ca^{2+} transients (pseudo-color) in various compartments of individual microglia, as shown in Fig. 1F. The images demonstrate compartmentalized activity, characterized by unsynchronized, localized Ca^{2+} signals occurring in processes. Timestamps are shown in the upper right of each panel. Scale bar, 10 μm

Quantitative analysis of individual events—assessing frequency, peak amplitude, duration, rise and decay time, and the integral of the curve (IC)—was performed separately for the somata and processes. Over a 10-min recording period, $68 \pm 4.60\%$ of all somata and $83 \pm 3.04\%$ of the processes exhibited at least one detectable Ca^{2+} transient (Fig. 1D).

No significant differences were detected in peak amplitude, duration, rise time, decay time, or IC between Ca^{2+} events in somatic and process compartments (Fig. 1E, Fig. S1A–D, Table S1). In contrast, event frequency was significantly higher in processes than in somata (8.38 ± 1.11 vs. 1.81 ± 0.22 events/min, respectively; $P < 0.0001$, Fig. 1E). This discrepancy suggested that microglial processes harbor Ca^{2+} microdomains that operate independently, thereby increasing the overall event rate. We detected no significant sex-related difference in somatic or process Ca^{2+} event frequency in the present cohort (Fig. S1E, F). Because each mouse contributed 2–5 spatially distinct FOVs and each recording was treated as one data point, we also tested whether sequential FOV acquisition introduced a systematic time-dependent change in baseline Ca^{2+} activity. To this end, we compared Ca^{2+} event frequency between the first and last baseline recording within individual animals. No significant difference was detected in either somatic or process Ca^{2+} event frequency, supporting the stability of the baseline imaging protocol across sequential FOV acquisitions (Fig. S1G, H).

To examine the spatial organization of Ca^{2+} signals across processes, we first determined whether spontaneous Ca^{2+} activity is coordinated within a given individual microglial cell. We therefore defined each major process of a microglial cell as a region of interest (ROI) and extracted its Ca^{2+} ($\Delta F/F_{\text{corr}}$) trace (Fig. S2). Under basal conditions, Ca^{2+} activity showed no obvious synchronization across different processes within a cell (Fig. S2), consistent with previous observations that process Ca^{2+} activity is locally organized [14].

We next asked whether process-restricted Ca^{2+} activity reflects uniform activation over the entire process, or whether smaller subcompartments can be identified within a given branch of the process. We therefore segmented contiguous regions along individual processes using manually delineated ROIs and compared their $\Delta F/F_{\text{corr}}$ traces at higher resolution (Fig. 1F). This approach revealed spatially confined events that were not necessarily

synchronized across adjacent segments of the same process. For example, Ca^{2+} transients detected in ROI 1 were absent in the neighboring ROI 2, and events detected in ROI 3 did not uniformly propagate into ROI 4, indicating functional subcompartmentalization within a single process. Thus, even within a single continuous process, Ca^{2+} activity can be locally restricted rather than spreading throughout the entire process. To validate this interpretation with an event-based approach, we employed AQuA2 [18], which resolves spatially restricted Ca^{2+} transients confined to discrete subregions within individual processes. These microdomains generated independent Ca^{2+} events without propagating to neighboring segments, establishing functionally autonomous compartments within a single process (Fig. 1G). The present dataset was not designed to quantify the proportion of localized versus propagating events or to infer a preferred propagation direction. Nevertheless, our interpretation is broadly consistent with recent in vivo work showing that microglial Ca^{2+} activity often remains localized at its site of origin and, when it spreads, can show directional bias rather than isotropic propagation [19]. The spatial restriction of these signals that we found resembles the microdomains described in Bergmann glial processes [20, 21].

We also tested whether Ca^{2+} activity differs between anatomically proximal and distal process compartments. Process ROIs were classified as primary processes directly continuous with the somata or as distal higher-order fine processes emerging from primary branches. We then extracted and compared Ca^{2+} event properties between these two compartments. Primary processes were located closer to the somata than non-primary processes (i.e., fine processes) ($8.82 \pm 0.67 \mu\text{m}$ vs $18.68 \pm 1.69 \mu\text{m}$; Fig. S3A, B). A space–time scatter plot representation showed Ca^{2+} events in both compartments throughout the recording period without obvious temporal clustering (Fig. S3B). Although this analysis demonstrates localized Ca^{2+} activity in process compartments at varying distances from the soma, it was designed to compare anatomical compartments rather than to determine whether the distance of a Ca^{2+} event from the soma predicts process movement. Quantitative analysis revealed no significant differences between primary and fine process compartments in event frequency, peak amplitude, duration, rise time, decay time, or event IC (Fig. S3C–H). Thus, despite their different anatomical positions, the measured kinetics and amplitudes of Ca^{2+} events were similar in proximal and fine process compartments.

Ca^{2+} dynamics are associated with microglial process motility

We next asked whether intracellular Ca^{2+} signaling is functionally linked to the structural plasticity of microglial processes. All imaging was conducted under anesthesia, a condition known to exhibit enhanced motility [22, 23], allowing for robust detection of dynamic remodeling events. Microglial somata were largely stable over the analyzed intervals; thus, we focused our movement analysis on processes.

Microglial processes continuously survey the brain parenchyma through dynamic extension and retraction of fine filopodia [3]. Consistent with this [3], we observed active filopodial motility under homeostatic conditions, as revealed by mCherry fluorescence, in our C2M2G transgenic mouse model, indicating ongoing surveillance of the local environment (Fig. 2A).

To quantify motility in an unbiased manner, we excluded somatic regions and applied an image-subtraction strategy. Specifically, for each 2-min interval, a maximum-intensity projection of the mCherry time series was subtracted from the initial frame of the same interval, yielding a binary map of structures that changed position, namely motile structures, while the remaining mCherry-positive signal was classified as non-motile structures (Fig. 2B). Using this approach, $18.64 \pm 0.75\%$ of the process structures were classified as motile within a 2 min baseline surveillance period. We then compared the motion-corrected Ca^{2+} signals between motile and non-motile structures within the same 2-min intervals. The motile structures were strongly enriched for Ca^{2+} transients: $12.06 \pm 0.41\%$ of motile structures displayed Ca^{2+} activity compared with $7.39 \pm 0.05\%$ of non-motile structures ($P < 0.0001$; Fig. 2C). These data demonstrate an association between local Ca^{2+} activity and microglial process motility under homeostatic conditions. Finally, we asked whether Ca^{2+} activity preferentially associates with specific modes of process dynamics. Processes were classified as extending, retracting, or stable (Fig. 2D). Extending processes exhibited a significantly higher Ca^{2+} event frequency than retracting or stable processes ($P < 0.01$), whereas retracting and stable processes were not significantly different (Fig. 2D). These data indicate

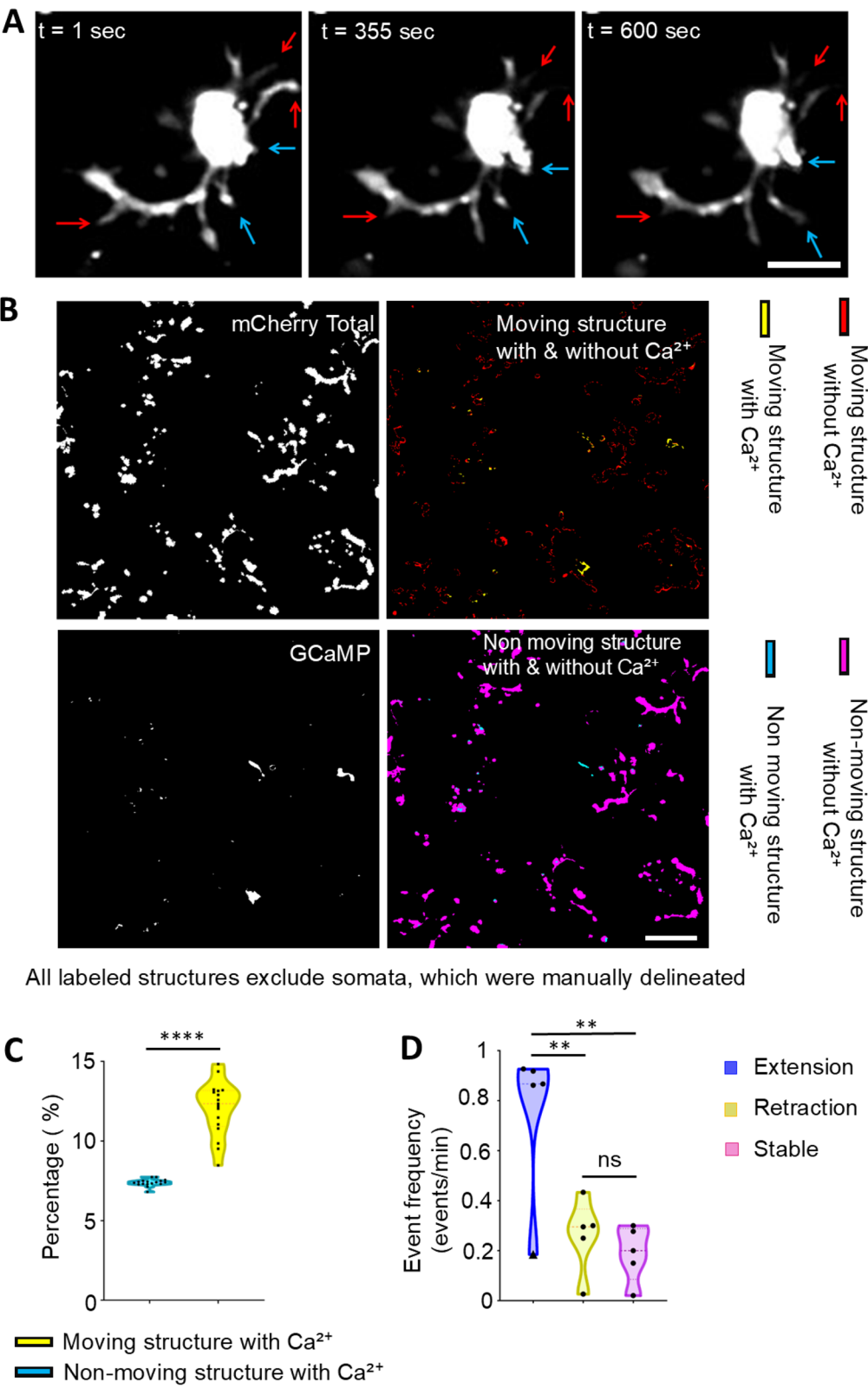


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Fig. 2 Microglial process motility is associated with intracellular Ca^{2+} transients in vivo. **A** Representative time-lapse two-photon images of a microglial cell labeled with mCherry at 1 s, 355 s, and 600 s under homeostatic conditions. Red arrows indicate examples of process extension, whereas cyan arrows indicate examples of process retraction during the imaging period. Scale bar, 10 μm . **B** Identification of moving and non-moving microglial process structures over a 2 min interval. Top left: binary map of total mCherry-positive structures after exclusion of somata, which were manually delineated. Top right: moving structures classified according to the presence (yellow) or absence (red) of Ca^{2+} activity. Bottom left: binary map of GCaMP6m-positive structures. Bottom right: non-moving structures classified according to the presence (cyan) or absence (magenta) of Ca^{2+} activity. All labeled structures exclude somata. Scale bar, 20 μm . **C** Quantification of the percentage of moving and non-moving microglial structures exhibiting motion-corrected GCaMP6m Ca^{2+} activity during 2 min intervals. Moving structures were significantly more likely to exhibit Ca^{2+} transients than non-moving structures. Individual points represent imaging session/FOV-level values, with moving and non-moving measurements paired within the same imaging session/FOV. Data were analyzed using a paired two-tailed t-test; **** $P < 0.0001$; $n = 17$ imaging sessions/FOVs from 6 mice. **D** Quantification of Ca^{2+} event frequency associated with process extension, retraction, and stable processes. Individual points represent matched FOV/session-level values; $n = 5$ FOVs from 5 mice. One extension value was identified as an outlier using ROUT ($Q = 1\%$) and is shown as a black triangle; this value was excluded from statistical analysis, whereas retained values are shown as circles. Data were analyzed using a mixed-effects model followed by Sidak's multiple-comparisons test. Extension-associated Ca^{2+} activity was significantly higher than retraction-associated and stable-process activity, whereas retraction-associated and stable-process activity did not differ significantly. ** $P < 0.01$; ns, not significant

that, under our experimental conditions, Ca^{2+} activity is more closely associated with process extension than with retracting or stable processes, consistent with previous work reporting enriched Ca^{2+} signaling in extending microglial branches [14].

Coordinated microglial process outgrowth after laser lesion is accompanied by local Ca^{2+} transients

To explore whether microglial process outgrowth in response to injury is likewise accompanied by Ca^{2+} signaling, we next examined microglial responses to focal laser-induced lesions (Supplementary Videos S1–S4). As previously reported [3, 4], laser-induced injury triggers rapid and highly coordinated extension of microglial processes toward the lesion site (Fig. 3A). The tips of extending processes were clearly detectable through the mCherry fluorescence signal, whereas the shaft connecting the tip with the cell body was commonly below the detection threshold. Thus, we predominantly detected the movement of the process tips.

We quantified the motility response to a small focal laser lesion (lesion area: $33.18 \mu\text{m}^2$) within a larger field of view (field area: $64,742 \mu\text{m}^2$) and compared the fraction of motile process structures before and after photoablation within the same continuous recordings. Somatic regions were manually excluded to avoid bias from the largely immobile cell bodies. Despite the lesion occupying only a small portion of the imaged area, the proportion of motile structures across the entire field increased significantly after injury ($18.64 \pm 0.75\%$ vs $24.81 \pm 1.17\%$, $P < 0.0001$; Fig. S4A). Sex-stratified analysis did not reveal any significant sex-dependent differences in the percentage of motile microglial structures in either the pre-laser or post-laser condition in the present cohort (Fig. S5).

To examine the relationship between process outgrowth and Ca^{2+} signaling after injury, we selected three ROIs ($63 \mu\text{m}^2$ each) along the trajectory of advancing process tips toward the laser lesion and analyzed the mCherry channel together with the motion-corrected GCaMP6m $\Delta F/F$ ($\Delta F/F_{\text{corr}}$) signal (Fig. 3B). Time-lapse imaging of the mCherry channel captured the progressive advance of process tips into successive ROIs (Fig. 3B, left). Spatial maps of $\Delta F/F_{\text{corr}}$ revealed localized Ca^{2+} activity associated with advancing process tips (Fig. 3B, middle), and ROI traces showed that entry of an mCherry-labeled tip into a given ROI coincided with a local increase in $\Delta F/F_{\text{corr}}$ (Fig. 3B, right). These observations indicate that injury-directed microglial process outgrowth is accompanied by localized Ca^{2+} transients at the leading edge of extending processes.

To further quantify this association, we tracked extending processes over time and classified ROIs based on the appearance of newly emerging motile structures (Fig. S4B). Injury-evoked Ca^{2+} activity was then analyzed over defined time periods in these ROIs: pre-lesion (-10 to 0 min), early (0 to 10 min), middle (10 to 20 min),

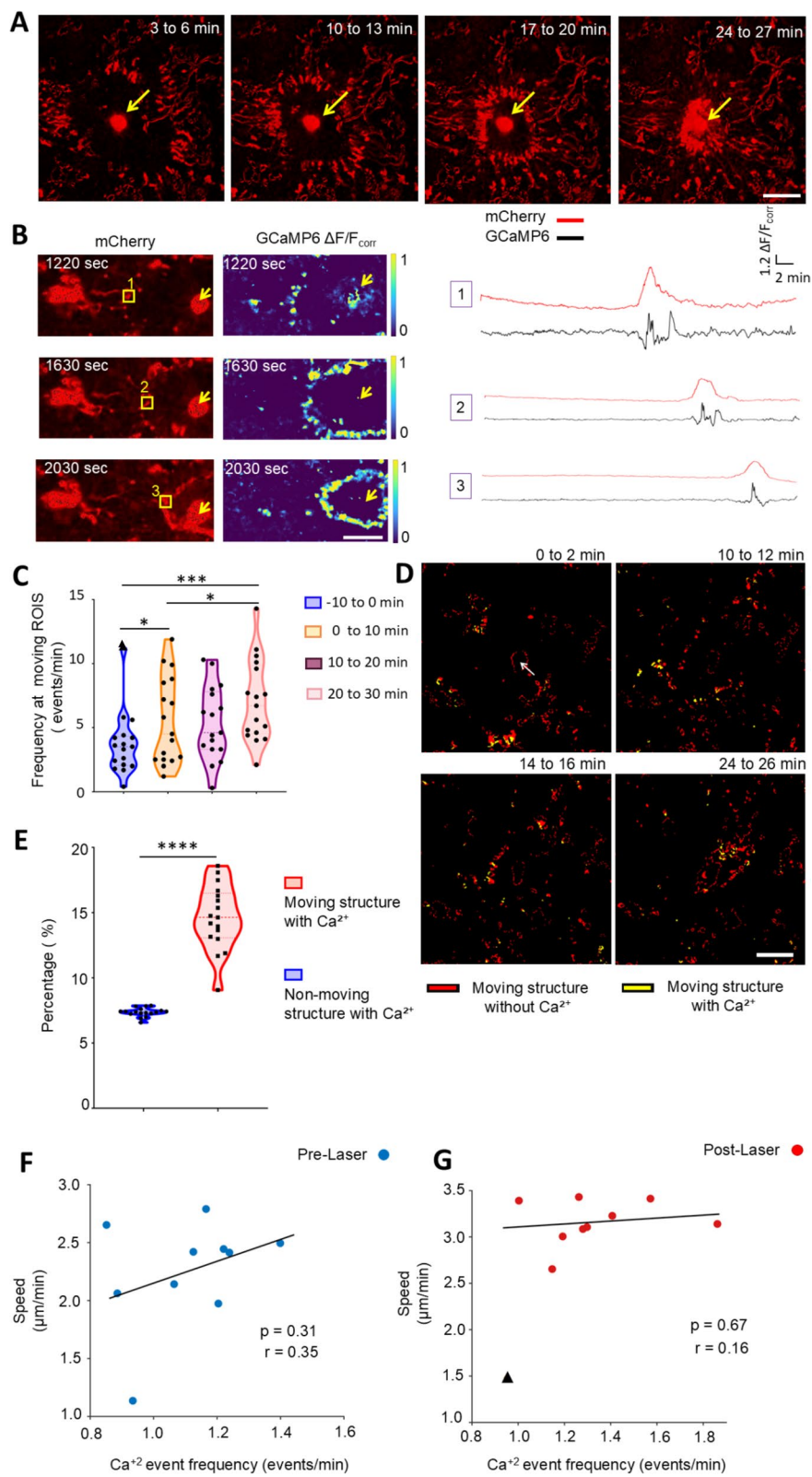


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Fig. 3 Coordinated microglial process extension and local Ca^{2+} transients in response to focal cortical injury. **A** Time-lapse maximum-intensity projections of mCherry-labeled microglia over the indicated post-lesion intervals (3–6 min, 10–13 min, 17–20 min, and 24–27 min), showing progressive extension of microglial processes toward the laser lesion site (yellow arrows). Scale bar, 25 μm . **B** Representative dual-channel analysis of an advancing microglial process tip following laser injury. Left: mCherry images at the indicated time points, with ROIs 1–3 positioned sequentially along the trajectory of the advancing process tip toward the lesion (yellow arrows). Middle: corresponding motion-corrected GCaMP6m $\Delta F/F_{\text{corr}}$ images showing localized Ca^{2+} signals associated with the advancing process tip. Right: fluorescence traces extracted from the correspondingly numbered ROIs, showing the mCherry signal (red) and the motion-corrected GCaMP6m $\Delta F/F_{\text{corr}}$ signal (black). Entry of the extending process tip into each ROI, reflected by an increase in mCherry fluorescence, was accompanied by a localized increase in the motion-corrected GCaMP6m $\Delta F/F_{\text{corr}}$ signal. Scale bar, 20 μm . **C** Quantification of Ca^{2+} event frequency at moving ROIs across the pre-lesion (–10 to 0 min, blue) and post-lesion intervals (0–10 min, orange; 10–20 min, purple; 20–30 min, pink). Individual points represent matched imaging session/FOV-level values; $n = 17$ time-lapse recordings/FOVs from 6 mice. One value in the pre-lesion interval was identified as an outlier using ROUT ($Q = 1\%$) and is shown as a black triangle; this value was excluded from statistical analysis, whereas retained values are shown as circles. Because outlier exclusion created one missing matched value, data were analyzed using a mixed-effects model followed by Sidak's multiple-comparisons test for planned comparisons. Ca^{2+} event frequency increased after injury, as indicated on the graph. Each recording consisted of a 10-min pre-lesion baseline and a 30-min post-lesion period. $*P < 0.05$, $***P < 0.001$. **D** Representative spatial maps of moving microglial structures during the indicated post-lesion intervals (0–2 min, 10–12 min, 14–16 min, and 24–26 min). Yellow indicates moving structures associated with Ca^{2+} activity, whereas red indicates moving structures without detectable Ca^{2+} activity. These maps highlight progressive recruitment of Ca^{2+} -active structures toward the lesion site (white arrow). Scale bar, 50 μm . **E** Quantification of the percentage of moving and non-moving structures exhibiting motion-corrected GCaMP6m Ca^{2+} activity during the post-lesion period. Individual points represent matched time-lapse recording/FOV-level values, with moving and non-moving measurements paired within the same recording/FOV. Moving structures were significantly more likely to exhibit Ca^{2+} activity than non-moving structures. Data were analyzed using a paired two-tailed t-test; $n = 17$ time-lapse recordings/FOVs from 6 mice. Each recording consisted of a 30-min post-lesion period. $****P < 0.0001$. **F, G** Correlation between Ca^{2+} event frequency at moving ROIs and process speed before and after laser injury. (F) Pre-laser condition showed no significant correlation between Ca^{2+} event frequency and process speed (Pearson $r = 0.35$, $P = 0.31$). (G) Post-laser condition also showed no significant correlation (Pearson $r = 0.16$, $P = 0.67$). Black lines indicate linear regression fits. The black triangle in (G) indicates one value identified as an outlier using ROUT ($Q = 1\%$) and excluded from the post-laser correlation analysis. Pearson correlation analysis was performed using a dataset of $n = 10$ FOVs from 7 mice; all 10 FOVs were included in the pre-laser analysis, whereas 9 FOVs were included in the post-laser analysis after outlier exclusion

and late (20 to 30 min). This analysis revealed a progressive increase in Ca^{2+} event frequency within motile structures, reaching significance during the late phase relative to both pre-lesion baseline and early responses (Fig. 3C). Using the image-subtraction strategy as in Fig. 2C, we separated motile from non-motile compartments and found that moving structures exhibited higher Ca^{2+} activity than non-moving structures during the post-lesion period ($14.61 \pm 0.60\%$ vs. $7.36 \pm 0.08\%$, $P < 0.0001$, Fig. 3D, E). These findings underscore an association between intracellular Ca^{2+} signaling and microglial process motility in response to focal brain injury.

Finally, we asked whether the magnitude of process movement relates to Ca^{2+} activity. Because basal surveillance and injury-directed extension may represent partially distinct motility states, we analyzed the pre-laser and post-laser periods separately. During the pre-laser baseline period, process tip speed showed no significant correlation with Ca^{2+} event frequency ($r = 0.35$, $P = 0.31$; Fig. 3F). Similarly, during the post-laser period, process tip speed showed no significant relationship with Ca^{2+} event frequency ($r = 0.16$, $P = 0.67$; Fig. 3G). As a supplementary analysis, we also show the relationship between process tip speed and Ca^{2+} event frequency combining both pre-laser and post-laser (Fig. S6, $r = 0.55$, $P = 0.01$); however, this pooled analysis is interpreted only as a descriptive across-observation summary, not as evidence for a single shared motility rule across baseline and injury conditions.

Spatially graded and temporally phased Ca^{2+} dynamics in relatively stable microglial compartments following laser lesion

Having characterized the Ca^{2+} activity of motile microglial processes, we next focused on relatively stable compartments, namely somata and major processes not involved in directed outgrowth, to determine how these

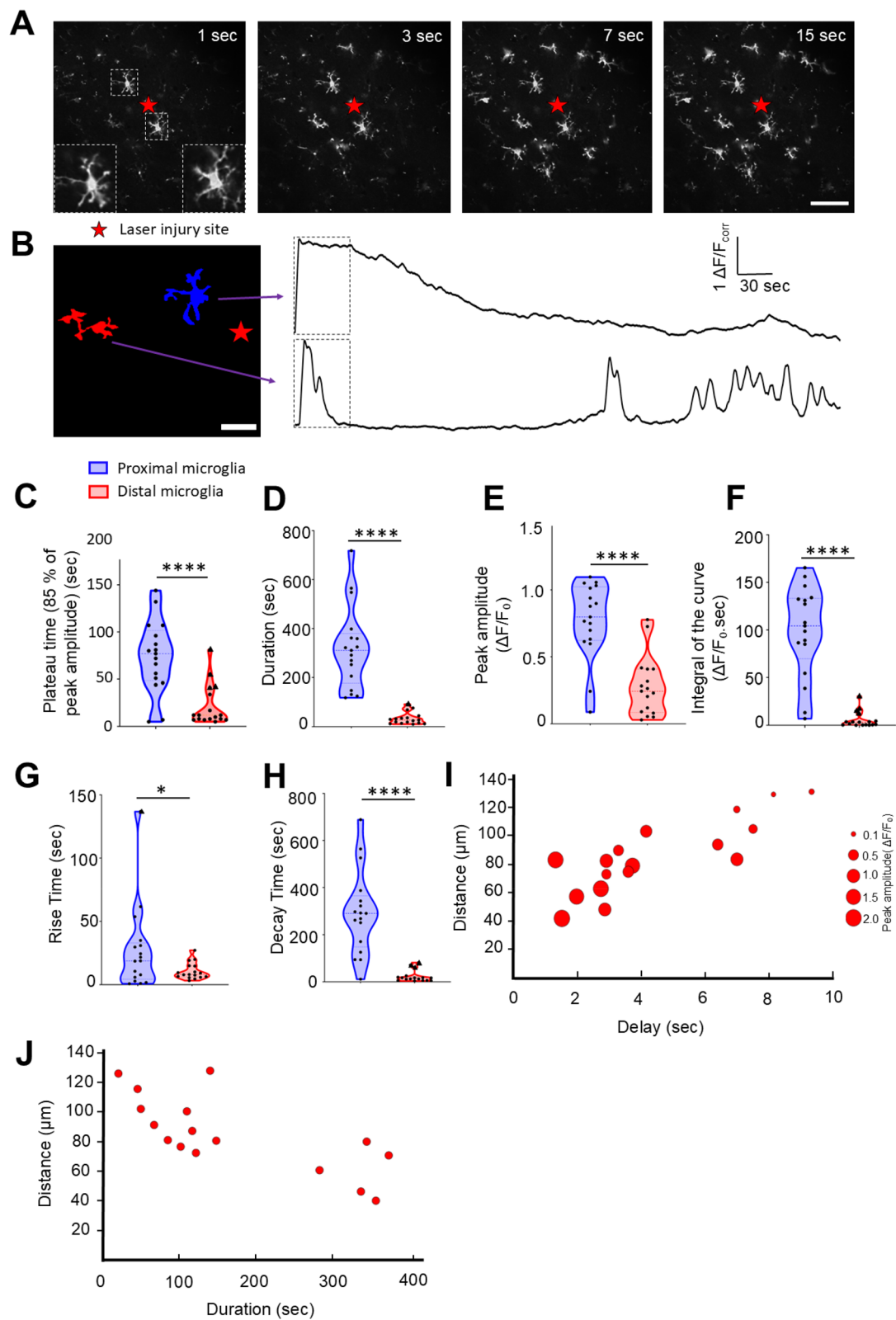


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Fig. 4 Distance-dependent microglial Ca^{2+} signaling dynamics following focal cortical injury. **A** Time-series two-photon images showing propagation of an injury-evoked Ca^{2+} wave among microglia at 1 s, 3 s, 7 s, and 15 s after a focal laser lesion. The red star indicates the laser injury site. Higher-magnification images in the first image illustrate that the early injury-evoked Ca^{2+} response involves both somatic and process compartments. Scale bar, 50 μm . **B** Representative example of two microglia located at different distances from the lesion site and their corresponding GCaMP6m $\Delta\text{F}/\text{F}_{\text{corr}}$ traces. The proximal microglial cell (blue; $\sim 35 \mu\text{m}$ from the lesion) exhibited a larger and more sustained Ca^{2+} response than the distal microglial cell (red; $\sim 108 \mu\text{m}$ from the lesion). Boxes indicate the early post-lesion response window. Scale bar, 25 μm . **C–H** Quantitative comparison of injury-evoked Ca^{2+} transient parameters between proximal and distal microglia, including plateau time at 85% of peak amplitude (C), event duration (D), peak amplitude (E), integral of the curve (IC) (F), rise time (G), and decay time (H). Proximal microglia (mean distance: $34.7 \pm 16.8 \mu\text{m}$, mean $\pm$ SD) exhibited significantly larger and more sustained Ca^{2+} responses than distal microglia (mean distance: $137.2 \pm 21.9 \mu\text{m}$, mean $\pm$ SD). Individual points represent matched time-lapse recording/FOV-level values from 17 recordings/FOVs from 6 mice. Outliers, when identified, were detected using ROUT with $Q=1\%$ and are shown as black triangles; these values were excluded from statistical analysis, whereas other values are shown as circles. Statistical analysis was performed using paired two-tailed t-tests or Wilcoxon matched-pairs signed-rank tests according to the distribution of paired differences. Each recording consisted of a 30-min post-lesion period. $*P<0.05$, $****P<0.0001$. **I** Scatter plot from one representative imaging session showing the relationship between latency to Ca^{2+} response onset (delay) and distance from the lesion site. Circle size is proportional to peak amplitude ($\Delta\text{F}/\text{F}_0$), as indicated in the scale at right. **J** Scatter plot from the same representative imaging session showing the relationship between event duration and distance from the lesion site, illustrating that the duration of the initial injury-evoked Ca^{2+} response tends to decrease with increasing distance from the lesion. Panels I and J show individual microglial ROIs from one representative imaging session and are intended to illustrate the spatial gradient of the initial injury-evoked Ca^{2+} response; these panels were not used as the primary statistical unit for group-level inference

domains respond to acute cortical injury. Individual microglia, including somata and proximal processes, were defined as ROIs and monitored over time (Fig. S7). Immediately following laser-induced injury, a robust Ca^{2+} wave initiated in microglia located near the lesion site and propagated outward to more distal cells within 15 s (Fig. 4A). We defined this as the initial Ca^{2+} response to injury and analyzed its spatiotemporal properties. Notably, this initial Ca^{2+} response involved both the somata and processes simultaneously, indicating a coordinated initial response within individual microglia. To better illustrate this point, a higher-magnification view of single microglia in Fig. 4A shows that the early injury-evoked Ca^{2+} response involves both somatic and process compartments. Thus, laser-induced injury changes the organization of microglial Ca^{2+} activity from predominantly local, process-restricted events under basal conditions to an early, broader Ca^{2+} response involving the entire cell. We now describe this as a two-stage injury response: an early, widespread Ca^{2+} wave that spreads outward from the lesion core, involving both the somata and processes simultaneously, followed by more localized Ca^{2+} activity associated with directed process outgrowth toward the injury site. Microglia situated within approximately 35 μm of the lesion core ("proximal microglia"; $34.67 \pm 16.84 \mu\text{m}$, mean $\pm$ SD) responded rapidly, with Ca^{2+} elevations detected 2.2 ± 1.41 s post-injury. This response was sustained, maintaining an elevated plateau that decayed to 85% of the peak amplitude after 74 ± 9.20 s and gradually returned to baseline within approximately 5 min (320.45 ± 40.44 s) (Fig. 4B–D). In contrast, microglia located more than 100 μm away from the lesion site ("distal microglia"; $137.17 \pm 21.92 \mu\text{m}$, mean $\pm$ SD) presented significantly delayed and attenuated initial Ca^{2+} response. Ca^{2+} transients in these cells started 8.6 ± 1.9 s post-injury, with faster decay kinetics—returning to 85% of the peak amplitude within 21.53 ± 5.25 s and reaching baseline in 33.37 ± 5.87 s (Fig. 4B–D).

Compared with distal microglia, proximal microglia presented a significantly higher Ca^{2+} peak amplitude (Fig. 4E), a larger integral of the transient event (IC, Fig. 4F), a longer rise time (Fig. 4G), and a longer decay time of this initial Ca^{2+} response (Fig. 4H). Furthermore, plotting latency, amplitude, and duration against distance to the lesion site illustrated clear spatial gradients, with increasing distance associated with longer latency, reduced amplitude, and shortened duration (Fig. 4I, J). Together, the proximal–distal comparisons and representative spatial plots support the presence of temporally and spatially graded initial Ca^{2+} responses following acute cortical

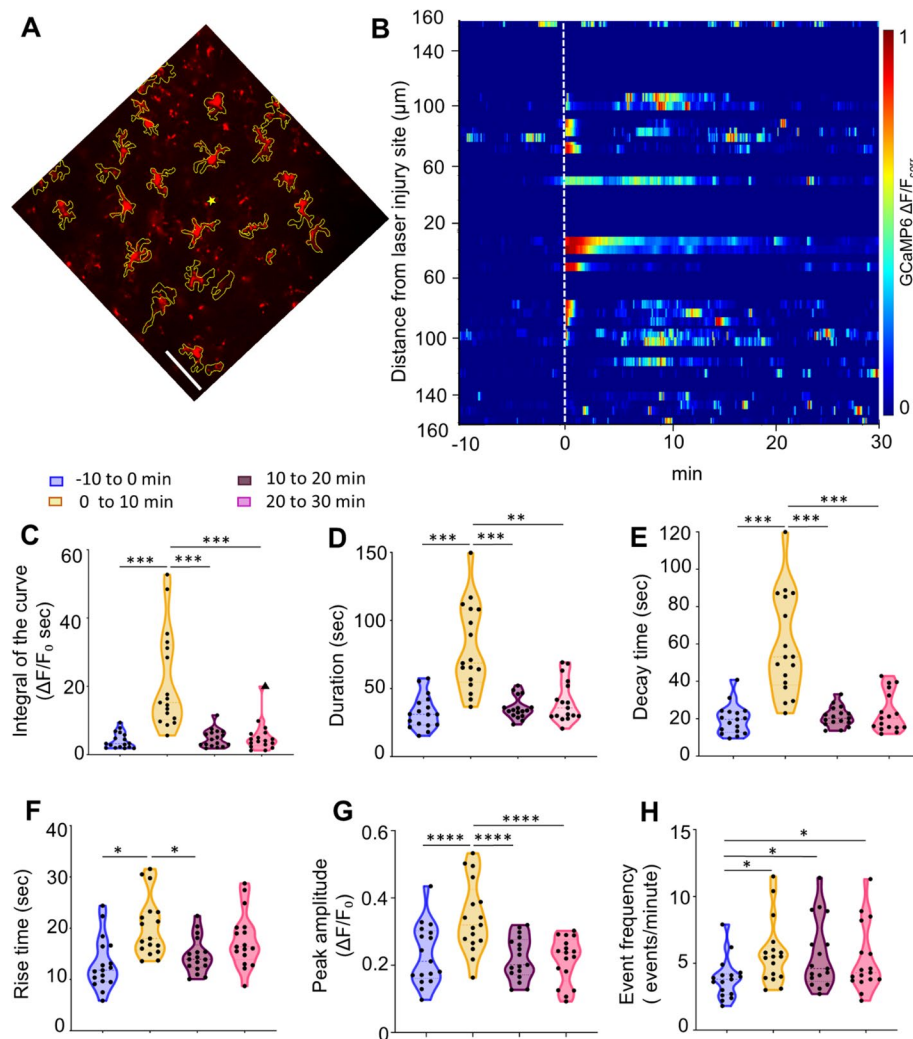


Fig. 5 Temporal evolution of microglial Ca^{2+} transients following focal cortical injury. **A** Representative mCherry fluorescence image of the imaging field surrounding the laser lesion. Individual microglial ROIs are outlined in yellow, and the lesion site is marked by a yellow star. Scale bar, 50 μm . **B** Spatiotemporal heatmap of microglial GCaMP6m $\Delta F/F_{\text{corr}}$ activity before and after laser injury. Each horizontal row represents an individual microglial ROI, arranged according to its distance from the laser injury site on the y-axis. The x-axis shows time, including the 10 min pre-lesion baseline and 30 min post-lesion period. The moment of laser injury is indicated by the white dashed line at 0 min. Color represents the amplitude of the Ca^{2+} signal ($\Delta F/F_{\text{corr}}$), with warmer colors indicating higher activity. **C–H** Quantitative comparison of Ca^{2+} transient parameters across pre-lesion and post-lesion phases, including integral of the curve (IC) (C), event duration (D), decay time (E), rise time (F), peak amplitude (G), and event frequency (H). The early post-lesion phase showed greater IC, longer duration, prolonged decay time, increased rise time, higher peak amplitude, and higher event frequency relative to the indicated comparison phases. Individual points represent matched time-lapse recording/FOV-level values. Outliers, when identified, were detected using ROUT with $Q=1\%$ and are shown as black triangles; these values were excluded before statistical analysis, whereas other values are shown as circles. Panels with complete matched observations were analyzed using repeated-measures one-way ANOVA with Geisser–Greenhouse correction, followed by Sidak’s multiple-comparisons test. Panels with missing matched values after outlier exclusion were analyzed using a mixed-effects model, followed by Sidak’s multiple-comparisons test. Data were obtained from $n=17$ time-lapse recordings/FOVs from 6 mice, with each recording consisting of a 10-min pre-lesion baseline followed by a 30-min post-lesion period. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.



(See figure on previous page.)

Fig. 6 Pharmacological and microglia-specific genetic attenuation of Ca^{2+} signaling reduces microglial process motility during surveillance and after focal cortical injury. **A** Quantification of the percentage of moving microglial structures before and after laser injury in Saline-Control, BAPTA-AM-treated, Control::C2M2G, and CalEx::C2M2G mice. Saline-Control refers to saline-treated C2M2G mice used for the BAPTA-AM pharmacological comparison. Laser injury significantly increased the percentage of moving structures in Saline-Control and Control::C2M2G recordings, whereas this increase was not significant after BAPTA-AM treatment or in CalEx::C2M2G mice. The percentage of moving structures was significantly reduced at both pre-laser and post-laser time points in BAPTA-AM-treated and CalEx::C2M2G recordings compared with their respective controls, as indicated on the graph. Saline-Control: $n = 17$ FOVs from 6 mice; BAPTA-AM: $n = 5$ FOVs from 5 mice; Control::C2M2G: $n = 17$ FOVs from 6 mice; CalEx::C2M2G: $n = 8$ FOVs from 5 mice. **B** Quantification of microglial process speed before and after laser injury. Process speed increased after injury in Saline-Control and Control::C2M2G recordings but not in BAPTA-AM-treated or CalEx::C2M2G recordings. Process speed was reduced in both Ca^{2+} -attenuated conditions compared with their respective controls, as indicated on the graph. Saline-Control: $n = 10$ FOVs from 7 mice; BAPTA-AM: $n = 5$ FOVs from 5 mice; Control::C2M2G: $n = 11$ FOVs from 5 mice; CalEx::C2M2G: pre-laser $n = 17$ FOVs and post-laser $n = 7$ FOVs from 5 mice. **C** Percentage of moving and non-moving structures associated with spontaneous Ca^{2+} transients under basal conditions in Control::C2M2G and CalEx::C2M2G mice. Moving structures were more frequently associated with Ca^{2+} activity than non-moving structures, while CalEx::C2M2G microglia showed a reduced proportion of Ca^{2+} -associated moving structures compared with controls. Control::C2M2G: $n = 17$ FOVs from 6 mice; CalEx::C2M2G: $n = 8$ FOVs from 5 mice. **D** Representative time-lapse mCherry images showing microglial responses before and after focal laser injury in Control::C2M2G and CalEx::C2M2G mice. Images are shown under basal conditions and at the indicated times after injury. The lesion site is marked by the white circle and arrow. Control::C2M2G microglia showed progressive process recruitment toward the lesion, whereas CalEx::C2M2G microglia showed reduced and delayed recruitment. Scale bar, 50 μm . **E** Quantification of the microglial response index over time after focal laser injury in Control::C2M2G and CalEx::C2M2G mice. Control::C2M2G microglia showed stronger and more sustained accumulation of processes at the lesion site, whereas CalEx::C2M2G microglia showed significantly attenuated recruitment. $n = 5$ FOVs from 5 mice per group; two-way repeated-measures ANOVA followed by Bonferroni's multiple-comparisons test. Data are shown as violin plots, with individual values representing FOV/imaging sessions in (A–C), and as mean $\pm$ SEM in (E). Outliers, when identified, were detected using ROUT with $Q = 1\%$ and are shown as black triangles; these values were excluded before statistical analysis, whereas retained values are shown as circles. Because outlier exclusion resulted in missing matched values, statistical analyses in (A–C) were performed using two-way mixed-effects models, followed by Sidak's multiple-comparisons test. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

injury. At the tissue level, this initial Ca^{2+} response propagates outward from the lesion core toward more distal microglia.

To assess the temporal evolution of Ca^{2+} activity beyond this initial wave, we next monitored the same microglial subdomains over a 30-min period following injury. As described above, the post-lesion interval was segmented into early (0–10 min), middle (10–20 min), and late (20–30 min) phases, with the 10-min pre-lesion period serving as the baseline. To visualize the spatiotemporal dynamics of this response, we first identified individual microglial ROIs (Fig. 5A). For each ROI, GCaMP6m $\Delta\text{F}/\text{F}_{\text{corr}}$ signal of the Ca^{2+} transient was calculated and plotted against its spatial distance from the lesion site and time of occurrence (Fig. 5B).

Beyond the initial Ca^{2+} response, Ca^{2+} events during the early post-lesion phase (0–10 min) exhibited significantly greater IC, longer event duration, and prolonged decay times compared to both pre-lesion and middle phases (Fig. 5C–E). The rise time was significantly higher in the early phase than in the pre-lesion phase (Fig. 5F). In addition, peak amplitude was significantly increased during the early post-lesion phase compared with the indicated phases, while event frequency was significantly elevated during the post-lesion period (Fig. 5G, H). These findings indicate that, in relatively stable microglial compartments, injury triggers an early phase of prolonged Ca^{2+} events accompanied by increased peak amplitude and event frequency. Notably, by the late phase (20–30 min), the kinetic parameters and peak amplitude had largely returned toward baseline, whereas Ca^{2+} event frequency remained elevated relative to baseline (Fig. 5C–H).

Together, this phase-specific profile highlights a transient, spatially confined Ca^{2+} response in relatively stable microglial compartments, distinct from the sustained Ca^{2+} activity observed in actively migrating processes.

Ca²⁺ signaling is required for microglial process motility in homeostasis and in response to focal cortical injury

Having established an association between microglial Ca²⁺ activity and process motility under baseline and injury conditions, we next tested whether intracellular Ca²⁺ signaling is required for this motility. To do so, we used two complementary approaches. First, we acutely reduced intracellular Ca²⁺ signaling by applying the membrane-permeable Ca²⁺ chelator BAPTA-AM through the cranial window (Fig. S8A-E; Supplementary Videos S5-S8). Second, we used a microglia-specific genetic strategy, CalEx::C2M2G mice, in which PMCA2 is overexpressed in microglia to enhance Ca²⁺ extrusion [24]. CalEx::C2M2G mice were compared with tamoxifen-treated littermate Control::C2M2G mice (Fig. S9A).

We first confirmed that both approaches reduced microglial Ca²⁺ signaling. BAPTA-AM markedly reduced spontaneous Ca²⁺ transients compared with saline-treated controls (Fig. S8A-E; Supplementary Videos S5-S8). BAPTA-AM also altered microglial morphology, as shown by reduced Sholl intersections at intermediate and distal distances from the somata (Fig. S8A, B). In the genetic model, CalEx::C2M2G microglia showed reduced spontaneous Ca²⁺ event duration, integral of the response curve, and decay time compared with Control::C2M2G microglia (Fig. S9B; Supplementary Videos S9, S10), consistent with our previous characterization of this model [24].

We next analyzed the same motility readouts side by side in four datasets: saline-treated controls, BAPTA-AM-treated mice, Control::C2M2G, and CalEx::C2M2G mice. Using the image-subtraction strategy described for Fig. 2C, laser injury significantly increased the percentage of moving microglial structures in saline-treated controls and in Control::C2M2G mice. In contrast, this injury-induced increase was not observed after BAPTA-AM treatment or in CalEx::C2M2G mice (Fig. 6A, Fig. S8F). Direct comparison within each time period showed that both Ca²⁺-attenuated conditions had significantly fewer moving structures than their corresponding controls during the pre-lesion baseline as well as after laser injury (Fig. 6A). Thus, acute Ca²⁺ chelation and microglia-specific enhancement of Ca²⁺ extrusion converged on the same phenotype: impaired microglial process motility during both baseline surveillance and the injury response.

We obtained a similar result when microglial process speed was analyzed as an independent motility readout. Process speed increased after focal laser injury in saline-treated controls and Control::C2M2G mice, but not after BAPTA-AM treatment or in CalEx::C2M2G mice (Fig. 6B). Direct comparison within each time period showed that process speed was significantly reduced in both BAPTA-AM-treated mice and CalEx::C2M2G mice compared with their respective controls during the pre-lesion baseline as well as after laser injury (Fig. 6B). These data show that Ca²⁺ attenuation reduces not only the fraction of moving structures but also the speed of process movement during both baseline surveillance and the injury response.

We then asked whether the residual motility observed after Ca²⁺ attenuation remained associated with detectable Ca²⁺ activity. After BAPTA-AM treatment, residual Ca²⁺ activity was still preferentially associated with the remaining motile structures compared with non-motile structures (Fig. S10). Similarly, in the genetic dataset, moving structures were more frequently associated with Ca²⁺ activity than non-moving structures in both Control::C2M2G and CalEx::C2M2G mice (Fig. 6C). However, CalEx::C2M2G microglia showed a reduced proportion of Ca²⁺-associated moving structures compared with Control::C2M2G microglia (Fig. 6C). These findings support the interpretation that local Ca²⁺ activity remains linked to process movement, while reducing Ca²⁺ dynamics limits the extent of motile behavior.

Finally, we examined coordinated microglial process recruitment toward the laser lesion over time in the genetic model. Control::C2M2G microglia progressively accumulated around the lesion site, whereas CalEx::C2M2G microglia showed weaker recruitment and failed to form a comparable containment response during the imaging period (Fig. 6D, E; Supplementary Videos S11, S12). The microglial response index was significantly reduced in CalEx::C2M2G mice compared with Control::C2M2G mice (Fig. 6E), supporting the conclusion that microglia-specific attenuation of Ca²⁺ dynamics impairs the coordinated injury-directed process response.

Together, the pharmacological and genetic Ca^{2+} attenuation data demonstrate that intracellular Ca^{2+} signaling is required for efficient microglial process motility during baseline surveillance and for process recruitment toward focal cortical injury. Acute Ca^{2+} chelation and microglia-specific Ca^{2+} attenuation produced convergent defects in moving-structure fraction, process speed, and injury-directed recruitment, supporting a functional requirement for intracellular Ca^{2+} dynamics in microglial process motility in vivo.

Discussion

Microglial process motility is a defining feature of brain surveillance and the rapid response to focal tissue damage [3, 4]. Yet, the intracellular signals that enable this motility in vivo have remained incompletely resolved, in part because microglial Ca^{2+} activity was long considered sparse under basal conditions [12, 16]. In this study, we combined in vivo dual-laser two-photon microscopy with the C2M2G dual-reporter line to simultaneously monitor microglial morphology (mCherry) and Ca^{2+} activity (GCaMP6m) at subcellular resolution. This study reveals that microglial Ca^{2+} signaling is predominantly organized within processes, frequently confined to autonomous microdomains, and closely linked to structural remodeling. Importantly, both pharmacological Ca^{2+} chelation and microglia-specific genetic enhancement of Ca^{2+} extrusion via PMCA2 [24] establish that intracellular Ca^{2+} dynamics are not merely associated with, but required for microglial process motility during homeostatic surveillance and after focal cortical injury.

Process microdomains and compartmentalized Ca^{2+} signaling

Earlier in vivo work reported infrequent spontaneous Ca^{2+} events in resting microglia, with the majority of cells remaining silent over comparable observation windows [12, 16]. In contrast, we detected Ca^{2+} transients in the majority of somata and processes within 10 min. This difference could reflect both technical and experimental factors. The C2M2G line provides microglia-specific genetic labeling and improved detectability of low-amplitude signals, and our analysis deliberately emphasizes process compartments where microglial Ca^{2+} activity is most prominent. At the same time, the acute cranial-window condition, superficial imaging depth, and anesthesia can influence microglial physiology and may elevate apparent event rates [14, 25, 26]. For this reason, we interpret absolute event frequencies cautiously. The central conclusions of this study rely on relative comparisons within the same preparation and on perturbations of Ca^{2+} dynamics, rather than on absolute baseline rates.

Our data show microglial Ca^{2+} activity is locally compartmentalized. Ca^{2+} events are often unsynchronized across different processes within the same cell, consistent with previous observations that Ca^{2+} signaling is tuned to local cues and neuronal activity states [14]. We further resolve a second level of compartmentalization within individual branches: Ca^{2+} events can be confined to subregions of a single continuous process, forming autonomous microdomains that do not necessarily propagate into adjacent segments of the same process. This microdomain architecture provides a parsimonious explanation for why processes exhibit higher aggregate event frequencies than somata and resembles microdomain signaling described in other glial processes [20, 21]. Together, these findings support a model in which microglial Ca^{2+} signaling is fundamentally decentralized and spatially restricted.

Ca^{2+} activity is coupled to motility

During baseline surveillance, motile process structures were strongly enriched for Ca^{2+} transients relative to non-motile structures, and extending processes showed higher Ca^{2+} event frequency than retracting or stable structures. This is consistent with prior work reporting that process extension is preferentially associated with Ca^{2+} activity [14]. Process extension and retraction may not be controlled by identical intracellular mechanisms. This fits with a broader model in which local Ca^{2+} microdomains support active protrusion by regulating actin

remodeling, leading-edge dynamics, and directional movement, as described in other migrating cells where localized Ca^{2+} signals guide cell migration [8]. By contrast, retraction may rely more on loss of protrusive actin polymerization, actin depolymerization, membrane tension, and actomyosin-based contraction. In addition, receptor-linked pathways may actively contribute to microglial process retraction. For example, adenosine A2A receptor signaling can promote retraction during inflammation [27], and high-intensity purinergic stimulation can promote process withdrawal through P2Y1/P2Y13-dependent mechanisms [28]. Under basal conditions, surveillance dynamics are also shaped by THIK-1-dependent membrane potential control [29]. Thus, the reduced Ca^{2+} event frequency in retracting structures does not imply that retraction is Ca^{2+} -independent; rather, it may be driven in part by Ca^{2+} -independent cytoskeletal mechanisms. Following injury, Ca^{2+} activity remained enriched in motile compartments. Although process speed showed positive associations with Ca^{2+} event frequency during both the pre-laser and post-laser periods, neither correlation reached statistical significance when the two conditions were analyzed separately. A pooled analysis combining pre-laser and post-laser observations revealed a significant positive correlation between process speed and Ca^{2+} event frequency ($r=0.55$, $P=0.01$); however, because baseline surveillance and injury-directed extension represent distinct motility states, we interpret this pooled relationship descriptively. Therefore, we interpret the speed- Ca^{2+} relationship cautiously and base our main conclusion on the motile versus non-motile comparisons and the loss-of-function experiments. These observations extend earlier studies in mice and zebrafish linking Ca^{2+} transients to microglial motility during injury or inflammatory conditions [14–16, 30].

The increase in motile structures after laser injury is consistent with the well-established ATP/ADP-P2Y12-dependent recruitment of microglial processes toward focal tissue damage [4, 5]. Additional injury-associated receptor systems may further shape this response. CX3CL1-CX3CR1/fractalkine signaling has been implicated in microglial process dynamics after focal injury and can engage IP3 receptor-dependent Ca^{2+} signaling in microglial cells [31]. Similarly, TREM2 supports microglial chemotaxis and process outgrowth toward focal neuronal damage, and TREM2-DAP12-SYK signaling can activate PLC γ 2, leading to PIP2 hydrolysis, IP3 generation, and intracellular Ca^{2+} release [32, 33]. Thus, injury-induced process activation is likely driven by converging receptor pathways that modulate intracellular Ca^{2+} signaling. Our data place Ca^{2+} downstream of these injury-associated pathways as a common execution signal for cytoskeletal remodeling and directed process motility, rather than assigning the enhanced motility to a single upstream receptor.

A recurring technical concern in motility-linked Ca^{2+} analysis is that movement of a labeled structure through a fixed ROI can generate fluorescence changes unrelated to Ca^{2+} . To mitigate this, we used additional dual-channel correction strategies throughout the dataset that leverage the activity-independent mCherry signal to estimate shared motion- and area-related fluctuations in the GCaMP channel. This mCherry-based correction reduces the likelihood that our results are explained solely by structural artifacts. The consistent enrichment of Ca^{2+} activity in motile compartments, together with the loss-of-function experiments, provides a stronger foundation for mechanistic interpretation.

Notably, not every moving structure exhibited a suprathreshold Ca^{2+} transient within the analyzed window. This does not argue against Ca^{2+} dependence. Our event detection emphasizes discrete transients and may miss low-amplitude, highly localized, or temporally offset signals. Moreover, distinct movement modes may not share identical Ca^{2+} signatures. These considerations support the view that Ca^{2+} acts as an enabling intracellular signal for motility, even if a resolvable transient is not detected during every individual movement episode. The comparable Ca^{2+} event frequency between primary and distal process ROIs should not be interpreted as evidence against a coupling between Ca^{2+} activity and motility. This analysis classified processes according to anatomical branch order, not movement state. Distal process ROIs contained the entire visible higher-order branch, including motile tips, stable shafts, and non-moving segments. Therefore, Ca^{2+} events confined to small moving microdomains can be diluted when averaged across the whole branch. Conversely, primary processes can show spontaneous Ca^{2+} events without obvious movement, indicating that Ca^{2+} signaling also supports functions beyond motility.

Ca²⁺ is required for microglial motility *in vivo*

The robust evidence for a functional link between motility and Ca²⁺ activity comes from perturbing intracellular Ca²⁺ dynamics. Acute application of BAPTA-AM reduced spontaneous Ca²⁺ activity in microglia and impaired both baseline motility and injury-directed process outgrowth. This phenotype was recapitulated by a microglia-specific genetic approach, PMCA2 overexpression (CalEx), which enhances Ca²⁺ extrusion and reduces Ca²⁺ dynamics while maintaining core microglial homeostatic features [24]. The convergence of acute pharmacology and a microglia-intrinsic genetic manipulation support a cell-autonomous requirement for intracellular Ca²⁺ signaling in microglial process motility.

After injury, extracellular ATP is a dominant attractant cue that drives process recruitment [4, 5]. ATP activates purinergic receptors linked to intracellular Ca²⁺ signals. The increase in the total percentage of motile structures after lesion is therefore consistent with recruitment of a broader injury-triggered motility program. Basal surveillance and post-lesion directed extension likely engage overlapping but non-identical upstream pathways: basal surveillance may reflect low-threshold local cues, whereas injury produces a stronger ATP-rich damage signal that recruits coordinated process extension toward the lesion. Our data indicate that Ca²⁺ is a necessary effector of this program, because process extension is strongly impaired when intracellular Ca²⁺ is buffered or extrusion is enhanced, despite the continued presence of the injury context. We acknowledge key limitations. BAPTA-AM lacks cell specificity and could alter the local injury milieu. Conversely, chronic Ca²⁺ attenuation in CalEx could in principle influence long-term signaling states. However, the rapid impairment of minute-scale injury-directed outgrowth, together with prior evidence that CalEx microglia retain homeostatic markers and density [25], supports a primary, microglia-intrinsic requirement for Ca²⁺ dynamics in the execution of motility.

Downstream effectors of Ca²⁺ microdomains

How might Ca²⁺ microdomains be translated into movement? Microglial shape change and motility require actin remodeling and actomyosin force generation, and Ca²⁺ is a canonical regulator of contractile and cytoskeletal systems [34]. Microglia express the machinery required for force generation and actin turnover, including non-muscle myosin II and F-actin regulators [35]. Our findings therefore support a model in which process-local Ca²⁺ microdomains gate local cytoskeletal remodeling, enabling selective protrusion and stabilization of specific branches. Ca²⁺-sensitive actin-binding proteins such as cofilin represent plausible downstream nodes that could couple microdomain signaling to rapid actin turnover and membrane protrusion. A useful analogy is the axonal growth cone, where spatially restricted Ca²⁺ signals steer cytoskeletal remodeling during directional motility [36]. Testing these ideas will require microglia-specific perturbations of candidate Ca²⁺-responsive cytoskeletal regulators *in vivo*, ideally combined with simultaneous imaging of Ca²⁺ microdomains and cytoskeletal dynamics.

Upstream pathways that generate Ca²⁺ microdomains

An equally important question concerns the upstream drivers of process microdomains. Under physiological conditions, tonic release of nucleotides and activity-dependent neurotransmitters could engage purinergic receptors, neurotransmitter receptors expressed in microglia, and generate localized Ca²⁺ activity. In contrast, injury produces a large ATP surge that strongly activates purinergic signaling and is expected to recruit additional Ca²⁺ sources and receptor classes [4, 5]. It is plausible that metabotropic P2Y signaling contributes to chemotactic guidance and sustained directional extension, whereas ionotropic P2X signaling contributes to rapid Ca²⁺ influx and the initial injury-evoked Ca²⁺ wave. Our study was not designed to assign receptor dominance across states, and we did not apply receptor antagonists. Instead, our data place intracellular Ca²⁺ downstream of these pathways as a common execution signal, because motility is impaired when intracellular Ca²⁺ is buffered or extrusion is enhanced. Future work combining receptor-specific perturbations with microdomain-resolved Ca²⁺ imaging will be required to define how surveillance and injury cues are translated into distinct spatial Ca²⁺ programs.

Limitations and future directions

Several limitations should be emphasized. First, imaging was performed under Zoletil/xylazine anesthesia and in an acute cranial-window preparation. Both anesthesia and acute imaging conditions can modulate microglial motility and Ca^{2+} activity [14, 25, 26]. Zoletil/xylazine was chosen because it provides stable conditions for high-resolution two-photon imaging and reproducible laser injury paradigms and has been used in microglial imaging studies [37, 38]. While all experimental groups were recorded under identical conditions, making it unlikely that anesthesia explains differences between genotypes or treatments, anesthesia may still influence absolute activity levels. Chronic window preparations and awake imaging will therefore be important to define absolute microglial Ca^{2+} activities under less perturbed conditions. In the pharmacological experiment, BAPTA-AM was prepared from a DMSO stock and diluted in saline before topical application. Because a separate DMSO-only vehicle control was not included, we cannot fully exclude vehicle-related effects in this experiment. However, the convergent reduction in microglial process motility observed in the microglia-specific CalEx::C2M2G model supports the interpretation that attenuation of intracellular Ca^{2+} dynamics impairs microglial motility.

Second, our time resolution limits inferences about rapid temporal ordering. Our sampling does not allow us to determine whether individual process-local Ca^{2+} events propagate toward or away from the lesion during the chemotactic phase. We also did not compute a single population-level lag between Ca^{2+} signals and movement, and the acquisition rate (1 Hz) may be insufficient to resolve high frequency events. More importantly, a fixed lag is not required to establish necessity, since our central inference is grounded in loss-of-function logic. Future analyses that track tips as moving ROIs and quantify Ca^{2+} in a tip-centered coordinate system, followed by event-triggered or cross-correlation analyses, will sharpen temporal relationships without changing the main conclusion.

Third, our experiments were performed in somatosensory cortex, where microglia are strongly influenced by network state [14, 22, 23]. We did not apply direct sensory stimulation, but it is plausible that sensory-evoked changes in neuronal activity modulate motility by shaping the frequency or amplitude of localized Ca^{2+} microdomains. Testing this will require awake paradigms with controlled sensory input and habituation.

Fourth, most quantitative analyses used the imaging session/FOV as the statistical unit. For Ca^{2+} analyses, event measurements were first quantified and normalized at the ROI level, and the mean of ROI-level values within each FOV/session was used for statistical analysis. Although multiple FOVs from the same mouse were spatially distinct and non-overlapping, this design does not fully substitute for a larger mouse-level cohort. We therefore report mouse number and FOV/session number separately for each analysis in Table S2 and interpret session-level analyses in light of this sampling structure. Although both male and female mice were included, the sex-stratified analyses were exploratory and not powered to detect subtle sex-dependent effects.

Conclusions

In sum, this study supports a requirement for intracellular Ca^{2+} dynamics in efficient microglial process motility in vivo. The identification of autonomous process microdomains provides a mechanistic framework that reconciles robust surveillance behavior with limited somatic signaling and offers an entry point to dissect how microglia translate physiological activity and tissue damage into neuroimmune behavior. The Ca^{2+} -attenuated microglial genetic tool provides a tractable platform to further dissect microglial execution programs in neuroinflammation and disease.

Materials and methods

Animals and housing conditions

All animal procedures were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. The mice were maintained under standard laboratory conditions (12-h light/dark cycle, 22–24 °C, 50–60% humidity) with ad libitum access to food and water.

For all in vivo experiments, we used the previously described C2M2G transgenic mouse line, including both male and female mice aged 8–12 weeks. In this line, the *Csf1r* promoter drives co-expression of the Ca^{2+} indicator GCaMP6m and the red fluorescent reporter mCherry, enabling simultaneous visualization of microglial morphology and intracellular Ca^{2+} dynamics in vivo. This reporter line has been validated previously [17, 24]. PMCA2^{fllox/+} mice were obtained from the laboratory of Dr. Baljit Khakh (David Geffen School of Medicine, UCLA) and crossed with Cx3cr1-Cre^{ERT2/+} (Jackson Laboratory, Stock No. 020940) to generate PMCA2^{fllox±}::Cx3cr1^{CreERT2±} (CalEx mice). CalEx mice were then bred with C2M2G mice to generate CalEx::C2M2G (PMCA2^{fllox±}::Cx3cr1^{CreERT2±}::*Csf1r*-2A-mCherry-2A-GCaMP6m^{+/+}) and littermate Control::C2M2G (Cx3cr1^{CreERT2±}::*Csf1r*-2A-mCherry-2A-GCaMP6m^{+/+}) mice. For induction of PMCA2 overexpression in microglia, 5-week-old CalEx::C2M2G mice and littermate controls were treated with tamoxifen (75 mg/kg, i.p.) daily for 5 consecutive days (Fig. S9A), as extensively described in our recent article [24].

Stereotaxic surgery

Mice were anesthetized by intraperitoneal injection of a mixture of Zoletil® 50 (50 mg/mL; Virbac, Carros, France) and xylazine (10 mg/mL; Sigma-Aldrich, St. Louis, USA) diluted in saline and administered at 0.1 mL per 10 g body weight. Mice were then secured in a stereotaxic frame (RWD Instruments, Shenzhen, China), and body temperature was maintained at 37 °C using a heating pad. Following hair removal and disinfection with 10% povidone-iodine (Shandong Lircon Medical Technology, Dezhou, China), the scalp was excised, and the periosteum was carefully removed. The skull surface was dried with sterile cotton swabs, and a 4-mm-diameter cranial window was generated over the right somatosensory cortex via a high-speed dental drill.

The exposed cortical surface was irrigated with sterile saline. A 4-mm glass coverslip was placed over the window, which was sealed with 3 M Vetbond tissue adhesive (3 M, Saint Paul, USA), and reinforced with dental cement (Leonine Biotechnology, Shenzhen, China). In experiments involving BAPTA-AM treatment, BAPTA-AM was applied to the cortical surface prior to coverslip placement. BAPTA-AM stock solution (5 mM) was prepared in DMSO and diluted 1:200 in saline to a final working concentration of 25 µM. Then, it was applied topically to the exposed cortical surface before coverslip placement, in an amount sufficient to completely cover the cranial window/exposed brain surface. A custom head plate was affixed simultaneously with dental cement to stabilize the preparation for imaging. After surgery, the animals were subjected to two-photon imaging. Before imaging, mice received an intraperitoneal supplemental anesthetic dose equal to half of the initial surgical dose administered during surgery.

In vivo two-photon imaging

Two-photon imaging was performed via an Olympus FVMPE-RS microscope (Tokyo, Japan) equipped with an InSight X3 laser (Spectra-Physics, Andover, USA). Images were acquired through a 25× water immersion objective (NA 1.05, XLPLN25XWMP2; Olympus). GCaMP6m and mCherry fluorescence were excited at 920 nm and 1045 nm, respectively. To ensure our imaging was restricted to parenchymal microglia, all in vivo two-photon imaging was performed at a depth of approximately 100 µm below the pial surface, targeting cortical layer 1. This depth physically excludes superficial dural and subarachnoid macrophages residing in the meninges. Although

the Csf1r-driven C2M2G line can label other myeloid populations, including border-associated macrophages, our imaging depth and field selection were chosen specifically to restrict analysis to parenchymal microglia. All imaging was performed under continuous anesthesia. Imaging sessions were initiated within approximately 30 min after completion of the stereotaxic surgery.

Time-lapse Ca^{2+} imaging for baseline and laser-lesion experiments was performed using a galvanometer scanner in one-way mode. Baseline and laser-lesion Ca^{2+} imaging was performed as single-plane time series to maximize temporal resolution. Because microglial Ca^{2+} signals in our preparation evolve on a relatively slow timescale, we prioritized acquisition at approximately 1 frame/sec to continuously capture both process morphology and associated Ca^{2+} transients within the same field of view. We acknowledge that this approach does not capture out-of-plane dynamics, but it provided the temporal sampling needed for the present analyses. Images were acquired at 512×512 pixels with a spatial resolution of $0.497 \mu\text{m}/\text{pixel}$ and a scan speed of $2.0 \mu\text{s}/\text{pixel}$. We employed two distinct imaging protocols across spatially distinct, non-overlapping FOVs. To ensure robust sampling of physiological surveillance, a subset of imaging sessions was dedicated exclusively to baseline recordings (10-min continuous image recording) with no subsequent lesion. In separate FOVs, injury-response sessions were conducted; these consisted of a 10-min pre-lesion baseline, followed by a focal laser injury (1 s) and a subsequent 30-min post-lesion recording. Consequently, in some analyses, the total sample size (N) for baseline observations is larger than for post-lesion observations, as the baseline dataset aggregates data from both the independent surveillance-only recordings and the pre-lesion phases of the injury-response recordings. The total imaging duration under anesthesia did not exceed 2.5 h per mouse. Each mouse contributed 2–5 spatially distinct, non-overlapping FOVs, depending on image stability and quality. Recordings were excluded if the mCherry channel showed obvious lateral drift, z-plane displacement, unstable morphology, or physiological instability. The resulting mouse numbers and FOV/session sample sizes for each analysis are summarized in Table S2. In addition, to distinguish morphological differences between BAPTA-AM-treated and control mice, z-stack images were acquired over a depth of $13 \mu\text{m}$ with a $2 \mu\text{m}$ step size. Maximum-intensity projections were generated from these images and subsequently used for Sholl analysis in Fiji (ImageJ, National Institutes of Health, Bethesda, MD, USA) with the Sholl Analysis plugin to quantify the number of process intersections at increasing distances from the somata of each microglial cell.

To assess baseline microglial motility in the context of the PMCA2 mouse line, we imaged microglial processes in control (Control::C2M2G) and PMCA2 (CalEx::C2M2G) mice (Fig. S9A). Z-stacks spanning $16 \mu\text{m}$ in depth ($2 \mu\text{m}$ step size) were acquired every 3 min for 1 h in the mCherry channel, which was used to visualize microglial processes. Three-dimensional z-stacks were processed in Fiji, and maximum-intensity projections were generated for two-dimensional analysis. To quantify injury-directed process motility, the integrated density and spatial distribution of the mCherry fluorescence signal were measured within a circular ROI ($60 \mu\text{m}$ diameter) on the lesion. A response index reflecting microglial migration toward the injury site was then calculated based on the integrated density of the mCherry signal within this ROI.

Focal laser lesion

Focal laser injury was induced by targeting a circular region of interest ($6.5 \mu\text{m}$ diameter) within the mCherry-positive area at the center of the imaging field. The 920-nm laser was set to 55% power and operated in tornado scanning mode for 1 s, whereas for the experiments involving PMCA2 mice, 5 s of laser injury was given. Successful ablation was confirmed by the appearance of a localized auto-fluorescent sphere at the lesion site. Microglial responses to local injury were subsequently monitored (within a sec) via time-lapse two-photon imaging with a frame rate of 1 Hz.

Two-photon image processing and analysis

Two-photon images were analyzed to quantify microglial Ca^{2+} activity and motility. Images were first motion-corrected for in-plane motion using the ImageJ plugin termed flow registration. Then, images were denoised using a previously described algorithm [39] (<https://github.com/abrazhe/uCats>). ROIs encompassing the microglial somata, processes, and whole cells were manually delineated in ImageJ using freehand selection on the basis of mCherry fluorescence, and the registered GCaMP6m and mCherry signals were extracted from identical spatial regions.

To partially account for motion and area-related fluorescence changes, the corresponding GCaMP6m signal was corrected using the simultaneously acquired mCherry reference channel in analyses involving moving microglial processes. This followed the general rationale of two-channel motion-artifact correction, in which an activity-independent reference channel is used to estimate shared motion-related fluorescence changes in the activity-dependent channel [40]. Specifically, we generated log-transformed ratio images from the co-registered channels, and examined the residual GCaMP6m signal after removal of the mCherry-associated shared component. The resulting fluorescence traces were detrended by subtracting a second-order polynomial fit to remove slow baseline drift and were subsequently smoothed using a second-order Savitzky–Golay filter (7 frame window; 1 Hz acquisition). Baseline fluorescence was estimated by constructing a histogram of dF/F_0 values for each ROI and identifying the most populated region of the distribution. This region was iteratively expanded to include 50% of all the data points, and the mean of these values was used as the baseline estimate. The normalized, mCherry-corrected Ca^{2+} signal ($\Delta\text{F}/\text{F}_{\text{corr}}$) was then used for the quantitative GCaMP6m analyses reported throughout the manuscript.

Then, Ca^{2+} transients were detected using a two-threshold procedure. Candidate events were first identified when dF/F_0 exceeded baseline by more than $3 \times \text{SD}$, which was used to define event onset and offset. Among these candidate events, only those whose peak amplitude exceeded $3.5 \times \text{SD}$ above baseline were retained for further analysis, to reduce false positives due to noise. Then, quantitative parameters, including event frequency, peak amplitude, duration, rise time, decay time, and the integral of the curve (IC; the area under the dF/F_0 curve above threshold), were calculated. Event frequency was calculated consistently as detected events per minute. However, ROIs were defined at different spatial scales to address distinct biological questions.

To further confirm the presence of independent Ca^{2+} activity within individual processes, we also performed an event-based analysis using AQuA2 [18]. AQuA2 identifies active regions statistically, segments signal temporally into super-events, and then spatially into individual events. In the present analysis, single-channel GCaMP6m data were analyzed at 1 Hz with a spatial resolution of $0.497 \mu\text{m}/\text{pixel}$ (8-bit data). Stored processing settings included bleach correction, trend correction, and registration correction, with minimum event duration = 5 frames, minimum event size = 50 pixels, significance threshold = 3.5, minimum/maximum spatial scale = 3/7, moving-average window = 25 frames, and spatial smoothing = 0.5.

To correlate microglial motility with changes in Ca^{2+} activity, these data were quantified by analyzing changes in mCherry and GCaMP6m fluorescence intensity over time. To classify microglial structures as moving or non-moving, mCherry images were analyzed in 2-min windows (120 frames). For each window, a maximum-projection image was generated from the 120 frames and compared with the first frame of that window. Both images were normalized, and a manual threshold was set to generate binary mCherry masks. A 4-pixel (2×2) square array—with each pixel measuring $0.497 \mu\text{m}$, representing an area of approximately $0.99 \mu\text{m}^2$ —showing intensity changes above the threshold between consecutive 2-min windows was classified as a moving mCherry structure, whereas pixels remaining within the baseline mCherry mask without detectable change were classified as non-moving structures. Somata regions were excluded manually from all masks. Changes in Ca^{2+} activity were detected via the same method as described by subtracting the 2-min merged image from the baseline image, and those changes in GCaMP6m included changes in Ca^{2+} activity within the 2-min window. For all analyses, GCaMP6m signals were corrected using the simultaneously acquired mCherry channel before thresholding and

overlap analysis. Overlap between the Ca^{2+} mask and the moving or non-moving mCherry masks was then used to quantify Ca^{2+} activity associated with each structural compartment.

Imaris-based process tip tracking and correlation of Ca^{2+} and movement analysis

To quantify the relationship between microglial process motility and Ca^{2+} activity, the mCherry and GCaMP6m channels were first merged into a two-channel TIFF stack and imported into Imaris for tracking analysis. Tracking was performed on the merged time series so that structural movement identified in the mCherry channel could be directly linked to the corresponding GCaMP6m signal in the same spatial location.

Motile process tips were detected using the spots function in Imaris. Spot detection was performed with an estimated spot diameter of 1.5–2 μm , with background subtraction enabled, to enhance detection of fine motile protrusions. After automatic detection, spots were manually filtered using the Imaris quality index to remove spurious detections and retain only biologically relevant process tips. Spots were then linked across time using the autoregressive motion tracking algorithm, with a maximum linking distance of 2 μm and a maximum gap size of 3 frames.

For each tracked spot/track, Imaris-derived motility parameters were extracted, including speed, and the corresponding fluorescence intensity from mCherry and GCaMP6m were obtained for the same tracked structure. The mCherry-corrected GCaMP6m signal ($\Delta F/F_{\text{corr}}$) was then calculated as described above. The resulting track-level measurements were exported and pooled for downstream analysis. The continuous recording was divided into pre-laser and post-laser periods, and tracking-derived measurements were analyzed separately for each period.

To evaluate the relationship between motility and Ca^{2+} activity, the exported spot/track data were matched to their corresponding GCaMP6m measurements, and speed– Ca^{2+} correlations were calculated for each imaging session. These correlations were then used to assess whether faster-moving microglial process tips exhibited higher Ca^{2+} activity.

Statistics

Data are displayed as individual recording/FOV-level values, often overlaid on violin plots to show the distribution of the data. When numerical summary values are reported in the text or figure legends, they are presented as mean $\pm$ SEM unless otherwise indicated. Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). For Ca^{2+} and motility comparisons, lower-level measurements, including individual events, ROIs, or structures, were first summarized within each time-lapse recording/FOV, and the resulting recording/FOV-level values were used for statistical testing. All statistical tests were two-tailed. For comparisons between two independent groups, unpaired t-tests with Welch's correction were used unless a nonparametric test was more appropriate. For independent datasets with small sample size and non-Gaussian distributions, Mann–Whitney U tests were used. For paired comparisons from the same imaging field, time-lapse recording, FOV, imaging session, or animal, paired t-tests were used. Wilcoxon matched-pairs signed-rank tests were used for paired datasets when the distribution of paired differences did not support a parametric test. For comparisons across more than two matched conditions or post-lesion phases, repeated-measures one-way ANOVA with Geisser–Greenhouse correction was used when complete matched observations were available, followed by Sidak's multiple-comparisons test for planned comparisons. When outlier exclusion generated missing matched values, mixed-effects models using restricted maximum likelihood were used, followed by Sidak's multiple-comparisons test. For two-factor designs involving matched measurements within recordings/FOVs and independent experimental groups, two-way mixed-effects models were used when appropriate, followed by Sidak's multiple-comparisons test.

Correlation analyses were performed using Pearson correlation, and linear regression lines are shown for visualization of correlation plots. Potential outliers were identified in GraphPad Prism 10 using the ROUT method with $Q=1\%$. Outliers, when identified, are shown in the graphs as black triangles and were excluded before

statistical analysis, whereas remaining values are shown as black circles. For paired analyses, statistical testing was performed using complete matched pairs remaining after outlier exclusion unless a mixed-effects model was used to accommodate missing matched values. $P < 0.05$ was considered statistically significant.

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Authors' contributions Conceptualization: X.X., H.K.; Methodology: X.X., H.K., S.T., F.Z., and K.Z.; Investigation: S.T., F.Z., X.X., and H.K.; Resources: S.T., F.Z., A.C., Y.Z., A.B. and A.S.; Supervision: X.X., H.K.; Writing—original draft, review & editing: X.X., H.K. and S.T.

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Data availability All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Declarations

Ethics approval and consent to participate All animal procedures were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences.

Consent for publication Not applicable

Competing interests Authors declare that they have no competing interests.

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