

Aberrant cortical activity, functional connectivity, and neural assembly architecture after photothrombotic stroke in mice

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Abstract

Despite substantial recent progress in mapping the trajectory of network plasticity resulting from focal ischemic stroke, there is mixed evidence for changes in neuronal excitability and activity within the peri-infarct cortex of mice. Most of these data have been acquired from anesthetized animals, acute tissue slices, or immunoassays on extracted tissue, and thus may not reflect cortical activity dynamics in the intact cortex of an awake animal. Here, *in vivo* two-photon calcium imaging in awake, behaving mice was used to longitudinally track cortical activity, network functional connectivity, and neural assembly architecture for 2 months following photothrombotic stroke targeting the forelimb somatosensory cortex. This model is associated with sensorimotor recovery over the weeks following stroke, allowing us to relate network changes to behavior. Our data revealed spatially restricted but long-lasting alterations in somatosensory neural networks. Specifically, we demonstrate significant and long-lasting disruptions in neural assembly architecture concurrent with a deficit in functional connectivity between individual neurons. Reductions in neuronal spiking in peri-infarct cortex were transient but predictive of impairment in skilled locomotion measured in the tapered beam task. Notably, altered neural networks were highly localized, with assembly architecture and neural connectivity relatively unaltered a distance outside the peri-infarct cortex, even in regions demonstrated to be the site of functional remapping of the forelimb somatosensory representation in anaesthetized preparations. Thus, using longitudinal two-photon microscopy in awake animals, these data show a complex spatiotemporal relationship between peri-infarct neuronal network function and behavioral recovery that is more nuanced than functional remapping in response to strong sensory stimulation under anesthesia.

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This **important** study sheds light on several apparent discrepancies observed across animal studies examining neuroimaging biomarkers of functional recovery following focal ischemia. Using 2-photon imaging of calcium activity in awake mice, the authors show **solid** evidence that deficits in neuronal activity and functional connectivity after photothrombosis occur within a very small distance from the infarct (<750 microns) whereas these measures were relatively unaltered more distally, even those typically implicated with functional remapping of the forelimb representation in anaesthetized animals. These findings reveal a complex spatiotemporal relationship between perilesional neuronal network function and behavioral recovery that is more nuanced than previously reported, and motivates the need for better criteria for what is considered remapping.

Introduction

Investigations of the excitability of neurons in the peri-infarct cortex during recovery from ischemic stroke have yielded a complex and at times contradictory data set. Peri-infarct hyper-excitability was suggested by data from anesthetized rats that exhibited elevated spontaneous multiunit firing within peri-infarct regions 3-7 days after stroke [1]. Similarly, downregulated GABAergic inhibition in peri-infarct cortex [1–6], degeneration of parvalbumin- positive inhibitory interneurons [3,7], decreased paired pulse inhibition [8–10], increased NMDA receptor mediated excitation [11,12], and a downregulation of KCC2 [13–17] have suggested that the peri-infarct cortex is hyper-excitable during recovery, contributing to increased risk of epileptogenesis in the post stroke brain [13,18]. Conversely, several groups have reported that the sensory-evoked responsiveness of the peri-infarct cortex is diminished during recovery from focal photothrombotic stroke [19–25]. Even after months of recovery following forelimb (FL) representation targeted stroke, the remapped representation of the FL has been shown to display prolonged modes of activation with lower amplitude [19] and reduced temporal fidelity [23].

Almost all studies that have examined post-stroke cortical responsiveness have used surgical preparations with anesthetized animals. Anesthesia disrupts cortical activity dynamics and functional connectivity between cortical areas [26–30], and has been shown to reduce the magnitude and spread of cortical activation from sensory stimuli [31–39]. It further reduces the potential contribution of corollary discharge and reafference due to voluntary action in modulating sensory input and cortical responses [40]. It has also recently been shown that the propagation pattern of cortical activity differs between evoked and spontaneous activity, with spontaneous activity showing more complex trajectories and lower activity amplitudes [41].

Somatosensory responses are known to be modulated by the relevance of stimuli to behavior and task performance [42–47], and by corollary feedback during [48], or even before [49–51], the onset of movement. Together, these studies point to a complex, interconnected system that modulates the activity of somatosensory networks in the awake behaving animal that is either not present, or is altered, in studies of evoked cortical activity under the anesthetized state. While monitoring cortical activity in awake mobile animals presents methodological complexity, it avoids confounds associated with the anesthetized state and provides unique insight into evolving patterns of network activity that may be unique to the awake state.

To investigate neural activity patterns during stroke recovery while avoiding the potential confounds of anesthesia, the present work used two photon Ca^{2+} imaging within and distal to the peri-infarct region of a focal photothrombotic stroke lesioning the FL primary somatosensory cortex in mice. We used awake, freely behaving but head-fixed mice in a mobile homecage to longitudinally measure cortical activity, then used computational methods to assess functional connectivity and neural assembly architecture at baseline and each week for 2 months following stroke. Behavioral recovery from stroke was measured in the same animals on a tapered beam task and string pull task to determine the time course of sensorimotor deficits related to changes in cortical activity and network architecture. Our goal was to use longitudinal imaging and advanced computational analyses to define network changes in somatosensory cortex associated with sensorimotor recovery. We show that despite disturbances to the widefield topology and amplitude of the sensory-evoked FL map (acquired under anesthetic), it is only within the immediate peri-infarct region after FL targeted stroke that neural activity, functional connectivity, and neural assembly architecture are disrupted. We demonstrate that the peri-infarct somatosensory cortex displays a marked reduction in neural assembly number, an increase in neural assembly membership, and persistent alterations in assembly-assembly correlations, and that this occurs alongside transient deficits in both functional connectivity and neural activity. Notably, we show that reduced neuronal spiking is strongly correlated with a deficit in motor performance on the tapered beam task. Surprisingly, we also show that measurable deficits in neuronal activity, functional connectivity, and neural assembly architecture are absent within distal cortex as little as 750 μm from the stroke border, even in areas identified by regional imaging as locations of sensory-evoked FL representational remapping.

Results

The experimental timeline is illustrated in **Fig 1A**. Adult Thy1-GCaMP6S mice were implanted with chronic cranial windows and habituated on the floating homecage, tapered beam and string pull task (**Fig 1A-C**). The cFL and cHL somatosensory areas were mapped on the cortex using widefield Ca^{2+} imaging of stimulus-evoked activity (**Fig 1D**, 2A, see methods).

Regions of interest for longitudinal awake two-photon Ca^{2+} imaging of cellular activity (**Fig 1E**) were chosen based on pre-stroke widefield Ca^{2+} limb mapping (**Fig 2**) acquired under isoflurane anaesthesia. The first imaging region of interest was located at the boundary of the pre-stroke cFL and cHL somatosensory maps (termed “peri-infarct” region based on its proximal location to the photothrombotic infarct after stroke) and the second region as lateral to the pre-stroke cHL map (termed “distal” region due to its distance from the stroke boundary). These regions were imaged at baseline, and each week following photothrombotic stroke. Notably, these regions of interest incorporated the predicted areas of remapping for the limb associated somatosensory representations after focal cFL cortex stroke based on previous studies [25, 52]. Photothrombosis was directed to the cFL somatosensory representation identified by regional imaging. Resulting infarcts lesioned this region, and borders could be defined by a region of hyperfluorescence 1 week post-stroke (**Fig 1D**, Top). Within the peri-infarct imaging region, cellular dysmorphia and swelling was visually apparent in some cells during two photon imaging 1 week after stroke, but recovered over the 2 month post-stroke imaging timeframe (**Fig 1D**, Bottom). Morphological changes did not occur in the more distal imaging region lateral to the cHL.

Altered sensory-evoked widefield Ca^{2+} maps after stroke

Regional remapping occurs over the weeks following focal cortical stroke [25, 52]. Here, widefield Ca^{2+} imaging of the cranial window in isoflurane anaesthetized mice was performed during piezoelectric stimulation of the contralateral limbs prior to stroke, and at the 8 week post-stroke timepoint (**Fig 2A**, see methods), to define regional sites for cellular Ca^{2+} imaging and to

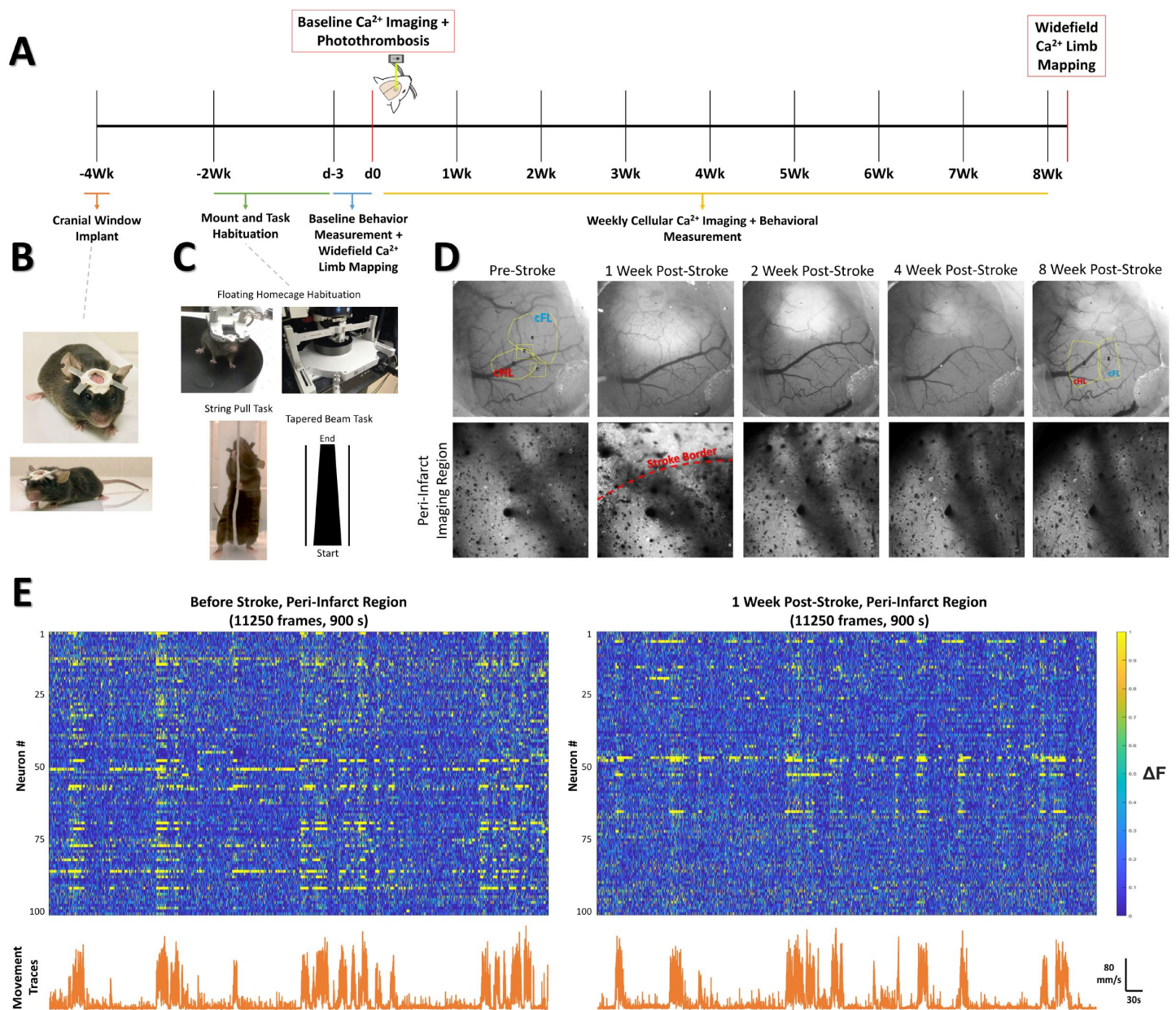


Fig 1.

Experimental timeline and methods.

(A) Timeline of experimental procedures, imaging times, and behavioral tests. (B) Glass cranial windows and headplates were implanted 2 weeks prior to beginning habituation protocols in Thy1-GCaMP6S mice. (C) Mice were habituated on the floating homecage, string pull and tapered beam for a period of 2 weeks prior to baseline measurements for behavior. Widefield Ca^{2+} response maps were used to determine locations for the imaging site located between the cFL and cHL sensory maps at baseline ("peri-infarct" imaging region) and imaging site lateral to the cHL at baseline ("distal" imaging region). For each Ca^{2+} imaging session, both imaging regions were imaged for a period of 15 minutes while simultaneously tracking animal movement within the mobile homecage. After baseline Ca^{2+} imaging, the somatosensory cFL map was targeted with photothrombosis. Behavioral testing on the string pull task and tapered beam task were performed weekly 1 day prior to the Ca^{2+} imaging session. After the final cellular Ca^{2+} imaging session at the 8 week time, stimulus-evoked widefield Ca^{2+} imaging was once again performed to determine the cFL and cHL somatosensory maps. (D) Representative widefield and two-photon calcium imaging at each weekly time. The area of hyper-fluorescence due to stroke damage is apparent, with its surface area decreasing over time. (E) Neuron Ca^{2+} traces (ΔF) for 100 neurons from one example animal pre-stroke (left panel) and 1 week post-stroke (right panel) in the peri-infarct somatosensory imaging region. Lower line graphs demonstrate the tracked movement (mm/s) within the floating homecage corresponding to the Ca^{2+} traces above.

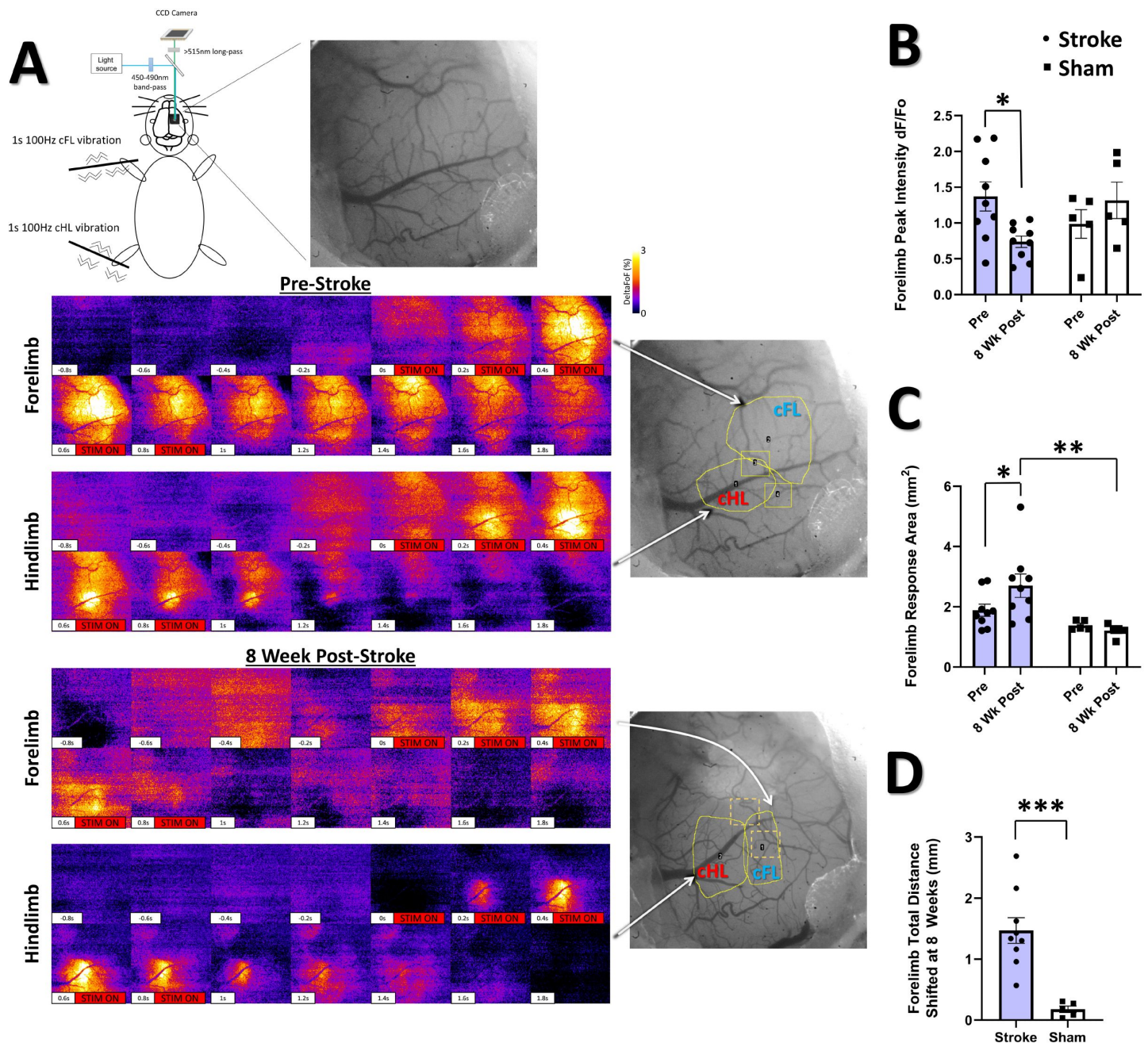


Fig 2.

Photostimulatory stroke results in forelimb representation remapping onto adjacent areas of cortex and altered sensory-evoked widefield Ca^{2+} response properties at 8 weeks post-stroke.

(A) Pseudocolored (%DeltaFoF) montages of representative cFL and cHL responses in S1 of an anesthetized animal pre-stroke and 8 week post-stroke resulting from oscillatory stimulation (1s, 100 Hz) of the cFL and cHL, respectively. At the 8 week post-stroke time, the cFL map has remapped posterior to its pre-stroke location into the area lateral to the cHL. (B) Mean peak forelimb Ca^{2+} transient response intensity (%DeltaFoF), measured from the thresholded cortical map area of the cFL. A significant interaction between time and stroke group was found, with *post-hoc* tests showing a significant decrease in the peak cFL response at 8-week post-stroke compared to pre-stroke ($P = 0.0143$). Mixed Effects Model, Time $F_{(1, 12)} = 0.8570$, $P = 0.3728$; Stroke Group $F_{(1, 12)} = 0.2092$, $P = 0.6556$. Interaction $F_{(1, 12)} = 8.632$, $P = 0.0124$. (C) Thresholded cFL response area (mm²). A significant main effect of group and significant interaction was found, with the stroke group showing a significant enlargement in the cFL response area at 8 weeks post-stroke compared to pre-stroke ($P = 0.0152$), and a greater response area at 8 weeks in the stroke group compared to sham ($P = 0.0036$). Mixed Effects Model, Time $F_{(1, 12)} = 2.273$, $P = 0.1575$. Group $F_{(1, 12)} = 7.328$, $P = 0.0191$. Interaction $F_{(1, 12)} = 5.372$, $P = 0.0389$. (D) The stroke group was found to have significantly greater total distance shifted for the forelimb map at the 8 week time compared to sham ($P = 0.0002$). Stroke $N = 9$, Sham $N = 5$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

monitor mesoscale changes to somatosensory evoked cortical representations. **Fig 2A** shows representative montages from a stroke animal illustrating the cortical cFL and cHL Ca^{2+} responses to 1s, 100Hz limb stimulation of the contralateral limbs at the pre-stroke and 8 week post-stroke timepoints. The location and magnitude of the cortical responses changes drastically between timepoints, with the cFL remapping posterior to its pre-stroke location into the area lateral to the cHL map at 8 weeks post-stroke. We observed a significant decrease in the cFL evoked Ca^{2+} response amplitude in the stroke group at 8 weeks post-stroke compared with the pre-stroke timepoint (**Fig 2B**). This is in agreement with past studies [19–25], and suggests that cFL targeted stroke reduces forelimb evoked activity across the somatosensory cortex in anaesthetized animals even at late time points 2 months after stroke. We observed a concurrent increase in cFL evoked response area in the stroke group 8 weeks post-stroke (**Fig 2C**), suggesting broader regional activation in response to cFL stimulation with significant posterior remapping of the FL representation (**Fig 2D**). There were no group differences between stroke and sham groups in cHL evoked intensity, area, or map position (data not shown).

Impaired performance on tapered beam but not string pull after focal somatosensory stroke

Post-stroke sensorimotor impairment was measured on the tapered beam task and string pull task. In the tapered beam, errors (slips off the beam), indicative of post-stroke impairment, were tracked as the number of left (affected) side slips, right (non-affected) side slips, and the distance to first slip (**Fig 3A**). We observed a significant interaction between stroke and time after stroke in the number of left side (contralesional) slips (**Fig 3B**), with *post-hoc* tests confirming a significant increase in left side slips at the 1 week timepoint for stroke animals compared the pre-stroke timepoint ($P = 0.0125$) and in stroke animals compared to sham ($P = 0.0435$). Consistent with the unilateral nature of the photothrombotic damage to the cortex in this study, no main effect or interaction was seen in the number of right side slips (**Fig 3C**). A statistical trend ($P = .0979$) towards a main effect of group was observed in the normalized distance to first slip (**Fig 3D**). These results are consistent with a transient deficit in motor behavior on the tapered beam during the first week after stroke induced by focal cortical lesion to the sensorimotor cortex [53,54], as opposed to more sustained deficits observed in models of middle cerebral artery occlusion [55,56]. In contrast to previous studies investigating string pull after stroke [57], we did not detect a significant effect of photothrombosis on any kinematic parameters during string pulling (Fig S1).

Firing rate of neurons in the peri-infarct cortex correlates with performance on the tapered beam task

The firing rate of cortical somatosensory neurons in our awake behaving animals (**Fig 4A**) was calculated using a custom Matlab algorithm to identify ‘significant’ calcium transients [58,59] and the mean firing rate of the sampled neural population during periods of movement and periods of rest in the mobile homecage. The normalized average firing rate in the peri-infarct region during recovery during movement and rest is illustrated in **Figures 4B** and **4C**, respectively. A significant interaction between stroke group and recovery time on firing rate was detected at rest (**Fig 4C**), though a qualitative (but not statistically significant) reduction in firing rate at 1 week was also apparent during movement (**Fig 4B**). Notably, normalized firing rate at 1 week post-stroke during movement and at rest were significantly negatively correlated with the number of left slips in the tapered beam at the same 1 week timepoint ($R^2 = 0.5326$, $P = 0.002$ and $R^2 = 0.4346$, $P = 0.0075$, respectively) (**Fig 4D** and **4E**). No main effects or significant interactions were observed in the distal imaging region, though a statistical trend ($P = 0.0667$) towards a main effect of group was observed during animal movement (**Fig 4F**). Normalized firing rate during movement at 1 week within the distal region was not significantly correlated with left slips on the tapered beam at the same timepoint ($R^2 = 0.0637$, $P = 0.3286$) (**Fig 4H**). A

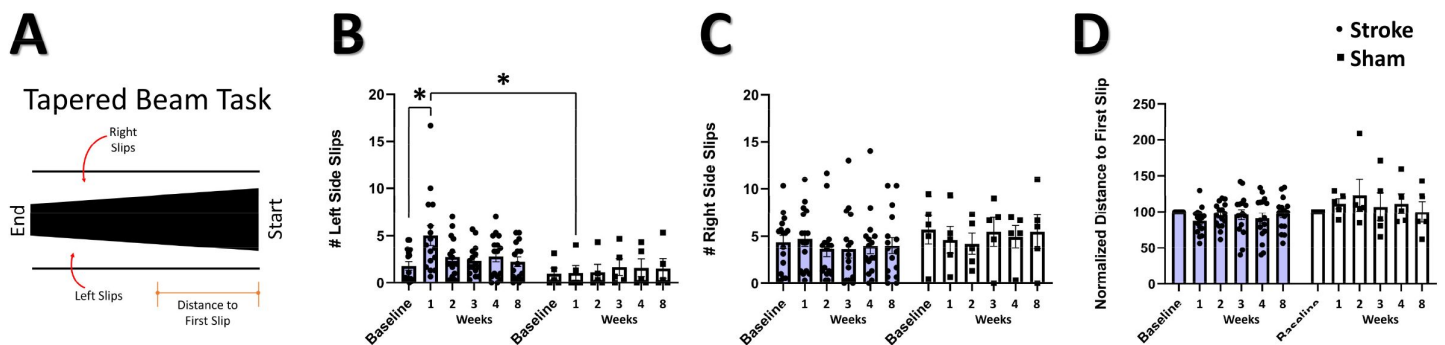


Fig 3.

Impaired performance on tapered beam task after stroke.

(A) Illustration of the tapered beam test with the three elements measured; left (contralesional) side slips, right side slips, and distance to first slip. (B) Mean number of left side slips. A significant interaction between time and stroke group was observed, with *post-hoc* tests showing a greater number of left side slips at 1 week in the stroke group relative to the sham group ($P = 0.0435$), and in the stroke group at 1 week relative to baseline ($P = 0.0125$). Mixed Effects Model, Time $F_{(2.007, 38.13)} = 2.178$, $P = 0.1270$; Stroke Group $F_{(1, 19)} = 2.260$; $P = 0.1492$. Interaction $F_{(5, 95)} = 2.914$, $P = 0.0172$. (C) Mean number of right-side slips. No main effects or interactions were observed. (D) Normalized distance to first slip (as % of baseline). A trend towards a main effect of group was observed, with no trends found in *post-hoc* tests. Mixed Effects Model, Time $F_{(3.135, 59.56)} = 0.6447$, $P = 0.5960$; Stroke Group $F_{(1, 19)} = 3.030$, $P = 0.0979$; Interaction $F_{(4, 76)} = 1.242$, $P = 0.4596$. Stroke $N = 16$, Sham $N = 5$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

statistical trend suggesting a negative correlation between normalized firing rate at rest and left slips was observed in the distal cortex ($R^2 = 0.2320$, $P = 0.0503$) (Fig 4I). These results run contrary to previous reports of increased multi-unit activity as early as 3-7 days after focal photothrombotic stroke in the peri-infarct cortex [1], and indicate decreased neuronal spiking 1 week after stroke in regions immediately adjacent the infarct that is strongly related to sensorimotor impairment. While the majority of neuronal activity occurred during movement, correlations between firing rate and animal movement were low and did not vary between stroke and sham groups (Fig S2).

Altered functional connectivity in the peri-infarct somatosensory cortex

Studies in humans and animals using mesoscale imaging methods indicate that stroke disrupts functional connectivity between widespread cortical regions [26,60–70]. However, few studies have examined functional connectivity at the level of local neural populations after focal stroke [71,72], and none have examined the primary somatosensory cortex. To define functional connectivity in local neural networks within the peri-infarct somatosensory cortex, we plotted the functional connectivity of the neural population in terms of the strength of their cell-cell correlations (Fig 5) and quantified the properties of these connections (Fig 6). We observed a significant loss of functional connectivity as early as 1 week after stroke within the peri-infarct region. This was visually apparent in the stroke group functional connectivity plots at the 1 week timepoint (Fig 5A and 5B) compared to the same location in sham animals (Fig 5E and 5F).

Notably, no profound loss of average functional connectivity is visually apparent in the distal region map in the stroke (Fig 5C and 5D) or sham (Fig 5G and 5H) groups. Within the peri-infarct region, we observed a statistical trend ($P = .0551$) towards a main effect of stroke in the normalized average number of all significant functional connections per neuron (Fig 6A) and a main effect of stroke group on strong connections ($r > .03$) per neuron (Fig 6B). Significant connections were reduced by stroke, and *post-hoc* tests confirmed fewer significant connections 1 week after stroke relative to sham controls. No main effects or interactions between stroke and time were identified in connectivity or significant connections in distal regions of interest (Fig 6D and 6E, respectively), highlighting the regional specificity of these network changes relative to the infarct and the original forelimb functional representation. The alterations in significant connections between neurons in peri-infarct parallel a persistent deficit in functional connectivity lasting up to 30 days that was identified within the primary and supplementary motor cortex of mice using *in vivo* imaging after focal motor cortex stroke [71,72]. Surprisingly, network density, defined as the ratio of measured significant functional network connections to the number of total possible network connections if all neurons were interconnected, was not decreased in either peri-infarct or distal regions in our study (Fig 6C and 6F, respectively). This is in contrast with previous research in the primary and supplementary motor cortex after focal motor cortex stroke that has demonstrated deficits in network density lasting up to 3 weeks post-stroke [72].

Neural assembly structure is disrupted in the peri-infarct region after stroke

The neural assembly hypothesis defines coordinated activity in groups of neurons as the basis for the representation of external and internal stimuli within the brain [73–75]. Likewise, perturbations within the structure and function of assemblies may signify aberrant processing of stimuli within neural networks, and is believed to play a significant role in the dysfunction observed in brain damage and neuropsychiatric diseases [74]. Although the use of sophisticated methods for detecting neural assemblies in large populations of neurons has gained increasing

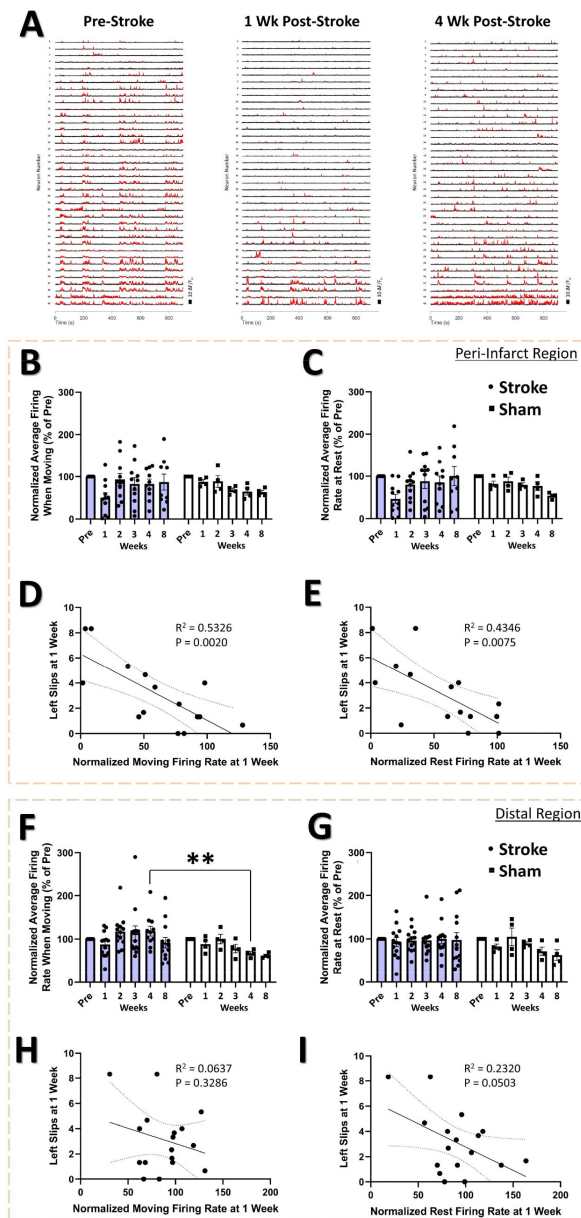


Fig 4.

Firing rate of neurons in the peri-infarct cortex correlates with performance on the tapered beam task.

(A) Example Ca^{2+} traces for 40 example neurons at each time point from the peri-infarct imaging region for one example animal in the stroke group. Significant Ca^{2+} transients are highlighted by red segments. A decrease in the number of Ca^{2+} transients is visually apparent at 1 week post-stroke. (B) In the peri-infarct imaging region, no main effects or interaction was seen in the normalized average firing rate during animal movement, however *post-hoc* analysis indicates a trend towards decreased normalized average firing rate at 1 week in the stroke group relative to sham ($P = 0.0593$). Mixed Effects Model, Time $F_{(2.448, 29.99)} = 0.9487$, $P = 0.4144$; Stroke Group $F_{(1, 13)} = 0.04256$, $P = 0.8397$; Interaction $F_{(4, 49)} = 1.852$, $P = 0.1339$. A significant interaction was seen in the normalized average firing rate at rest (C), with *post-hoc* tests showing a trend towards a decrease in firing rate at rest between the stroke and sham groups at the 1 week post time ($P = 0.0793$, Mixed Effects Model, Time $F_{(2.240, 27.44)} = 1.048$, $P = 0.3712$; Stroke Group $F_{(1, 13)} = 0.006936$, $P = 0.9349$; Interaction $F_{(4, 49)} = 2.604$, $P = 0.0471$). Normalized firing rate 1 week post-stroke was negatively correlated with the number of left slips in the tapered beam at the same 1 week time during both movement ($R^2 = 0.5326$, $P = 0.002$) (D) and rest ($R^2 = 0.4346$, $P = 0.0075$) (E). In the distal imaging region, no main effects or interaction were found in the normalized average firing rate for movement (F) or at rest (G). No correlation was observed between the normalized moving firing rate at 1 week in the distal region and the number of left slips on the tapered beam ($R^2 = 0.0637$, $P = 0.3286$) (H). A statistical trend was observed towards a negative correlation between normalized firing rate at rest at 1 week with left slips on the tapered beam at the same 1 week time ($R^2 = 0.2320$, $P = 0.0503$) (I). Peri-infarct region Stroke $N = 11$, Sham $N = 4$. Distal region Stroke $N = 13$, Sham $N = 4$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

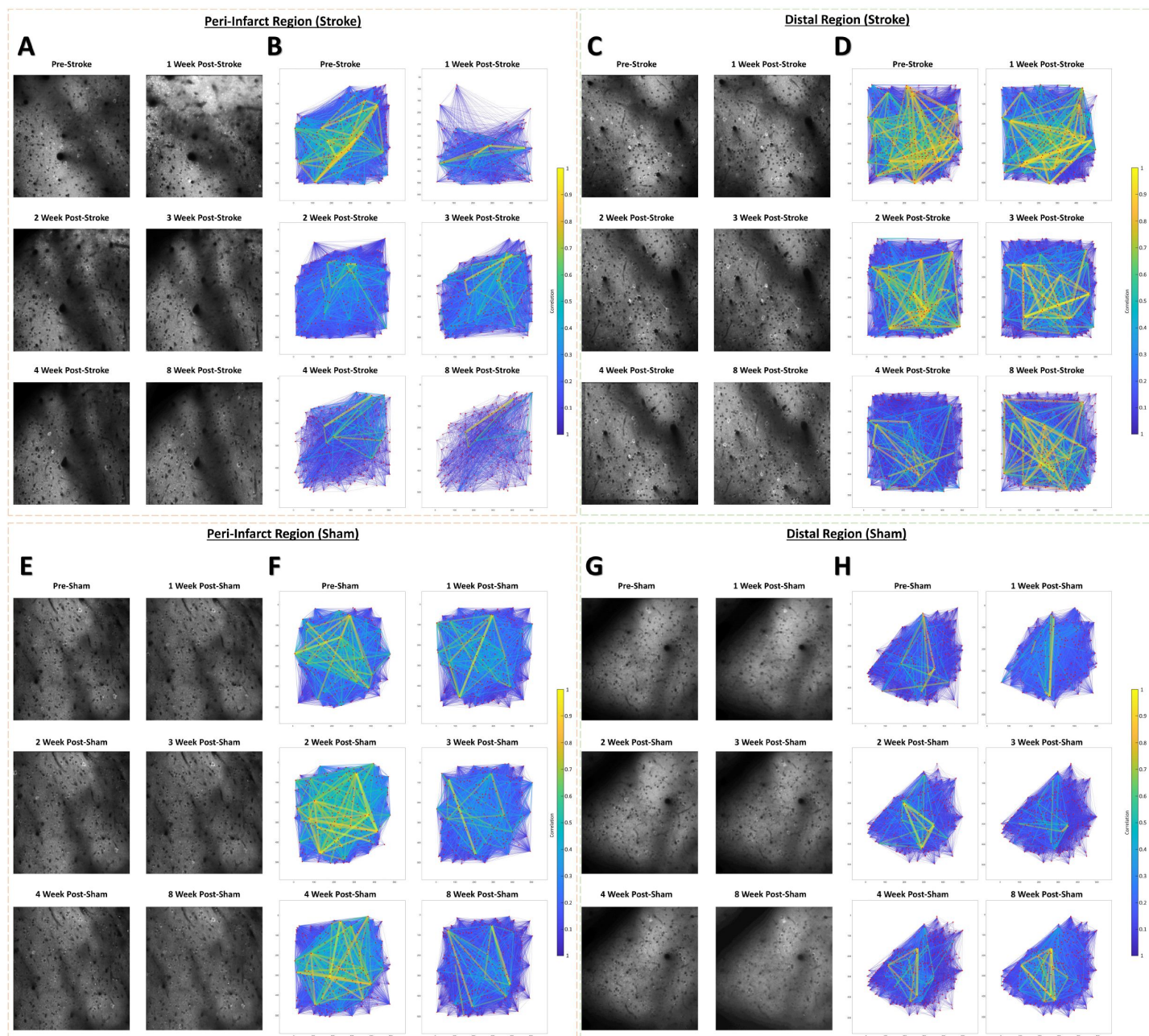


Fig 5.

Example cellular GCaMP6S Ca^{2+} fluorescence images and population functional connectivity plots.

(A-D) Ca^{2+} fluorescence images and functional connectivity plots from the peri-infarct imaging region and the distal imaging region for one example stroke group animal. Population functional connectivity plots demonstrate neurons as red dots, and the functional connection strength as lines with color and line width determined by the strength of the correlation between neurons. Notable cortical damage is visible in the upper portion of the 1 week post-stroke Ca^{2+} fluorescence image in the peri-infarct imaging region (A), which notably improves over the 2-4 week times. A notable loss of functional connections and loss in the number of connections with strong correlation (>0.3) is visible in the functional connectivity plots at 1 week post-stroke in the peri-infarct imaging region (B). In the distal imaging region post-stroke, less structural change is visible in the cellular Ca^{2+} fluorescence images (C), and less change to functional connectivity of the population over time (D). (E-H) Ca^{2+} fluorescence images and functional connectivity plots from the peri-infarct imaging region and the distal imaging region for one example sham group animal. Less structural changes within the cellular Ca^{2+} fluorescence images and in the functional connectivity plots are visually apparent relative to the example stroke animal.

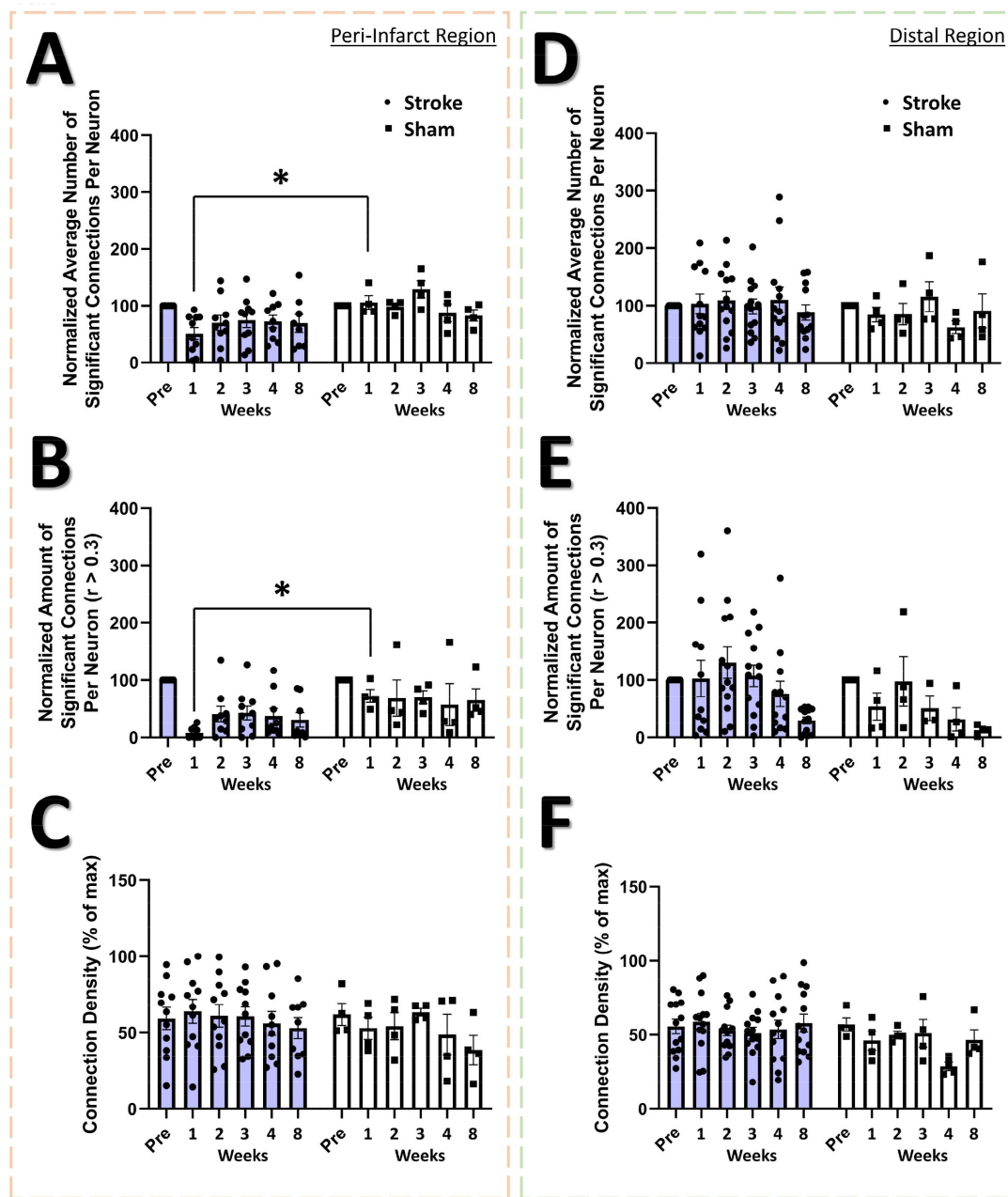


Fig 6.

Altered functional connectivity in the peri-infarct somatosensory cortex.

In the peri-infarct imaging region, a trend towards a main effect of group was found in the normalized average number of significant connections per neuron (A), with *post-hoc* tests showing a significant decrease at the 1 week post time in the stroke group relative to sham ($P = 0.0440$, Mixed Effects Model, Time $F_{(4.132, 47.11)} = 2.217$, $P = 0.1498$; Stroke Group $F_{(1, 13)} = 4.505$, $P = 0.0551$; Interaction $F_{(4, 45)} = 1.585$, $P = 0.1947$). A significant main effect of group was found in the normalized number of significant connections per neurons with correlation greater than 0.3 (B), with *post-hoc* tests indicating a significant decrease at the 1 week post time in the stroke group relative to sham ($P = 0.0363$). (Time $F_{(2.102, 22.60)} = 0.3885$, $P = 0.6925$. Group $F_{(1, 13)} = 6.423$, $P = 0.0262$. Interaction $F_{(4, 43)} = 0.6512$, $P = 0.6292$). (C) No main effects or interactions are seen in the connection density for the peri-infarct imaging region. (Time $F_{(3.251, 40.31)} = 0.3546$, $P = 0.8015$. Group $F_{(1, 13)} = 1.257$, $P = 0.2824$. Interaction $F_{(5, 62)} = 1.146$, $P = 0.3462$). In the distal imaging region, no significant main effects or interactions are seen in the normalized number of significant connections per neuron (D) (Time $F_{(3.173, 46.32)} = 0.5674$, $P = 0.6487$. Group $F_{(1, 15)} = 0.3172$, $P = 0.5816$. Interaction $F_{(5, 73)} = 1.525$, $P = 0.1925$), normalized amount of significant connections with correlation greater than 0.3 (E) (Time $F_{(2.228, 32.86)} = 2.196$, $P = 0.1224$. Group $F_{(1, 15)} = 1.036$, $P = 0.3249$. Interaction $F_{(4, 59)} = 0.7713$, $P = 0.5482$), or in the connection density (F) (Time $F_{(3.467, 52.01)} = 1.299$, $P = 0.2841$. Group $F_{(1, 15)} = 2.062$, $P = 0.1715$. Interaction $F_{(5, 75)} = 1.251$, $P = 0.2942$). Peri-infarct region Stroke $N = 11$, Sham $N = 4$. Distal region Stroke $N = 13$, Sham $N = 4$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

popularity with the advent of large population recordings using Ca^{2+} imaging [76], no previous studies have determined the longitudinal effects of stroke on the architecture of neural assemblies in cortex. To determine if focal stroke to the cFL cortex affects the properties of neural assemblies, we used a PCA-Promax procedure [58,59,76] for neural assembly detection to determine assembly size, population membership, assembly quantity, and overlap in assembly membership. The PCA-Promax method for determining neural assemblies allows neurons to participate in more than a single assembly [58,59], a property necessary to examine the ability of neural assemblies to dynamically form with varying members of the population for only brief periods of time [58,59,76]. **Fig 7** shows representative cellular Ca^{2+} fluorescence images with neural assemblies color coded and overlaid for each timepoint for the peri-infarct imaging region and for the distal imaging region for an example stroke and sham animal (**Fig 7A** and **7B**, respectively). We observed a significant main effect of stroke group on the number of assemblies (**Fig. 8A**) after stroke in the peri-infarct imaging region, representing an overall reduction in assemblies. *Post-hoc* comparisons confirmed significantly reduced number of assemblies 1 and 4 weeks after stroke relative to sham controls (**Fig 8A**). A significant main effect on the normalized number of neurons per assembly was detected, reflecting increased membership in each assembly that peaked 1 week after stroke (**Fig 8B**). We observed a statistical trend ($P = 0.0729$) suggesting a higher average percent of the population per assembly (**Fig 8C**). These data suggest that after stroke, neurons in peri-infarct cortex are functionally grouped into few distinct assemblies, with more neurons per assembly and more of the local network in the same assembly. For the distal imaging region, no differences in neural assembly properties were observed between the stroke and sham groups (**Fig 8D-E**).

Correlation between neural assembly activations is increased after stroke

If differential activation of neural assemblies in the naïve brain relates to representation of different aspects of the external world [58,73,75,77], then changes in the relationship between the activation patterns of assemblies may predict disrupted processing of sensory information within neural networks and how these networks differentially represent external stimuli. An increase in assembly size and reduction in assembly number might reflect a mechanism to replace lost function and increase somatic sensitivity, but may come at the cost of reduced stimulus specificity/fidelity within the network. The activation magnitude of assemblies can be correlated between assemblies over the recording period (**Fig 9A**). A significant main effect of stroke was detected, reflecting an increase in the average correlation coefficient for significantly correlated assembly-assembly pairs after stroke in the peri-infarct, but not distal cortex, when compared to sham (**Fig 9B**, 9C). This increased tendency for functionally connected assemblies to be co-active after stroke may reduce the signal to noise ratio for stimulus representation within the network and may reduce stimulus specificity within peri-infarct cortex. While assembly activations were often correlated with movement or rest periods (**Fig S3**), and 60% of assemblies in both the peri-infarct and distal regions were significantly correlated with animal movement at all time points (S3C and S3E, respectively), stroke did not change the average correlation between assembly activation and animal movement in either the peri-infarct (S3B **Fig**) or distal region (S3D **Fig**).

Discussion

Here, we used longitudinal two photon calcium imaging of awake, head-fixed mice in the mobile homecage to examine how focal photothrombotic stroke to the forelimb sensorimotor cortex alters the activity and connectivity of neurons adjacent and distal to the infarct. Consistent with previous studies using intrinsic optical signal imaging, mesoscale imaging of regional calcium responses (reflecting bulk neuronal spiking in that region) showed that targeted stroke to the

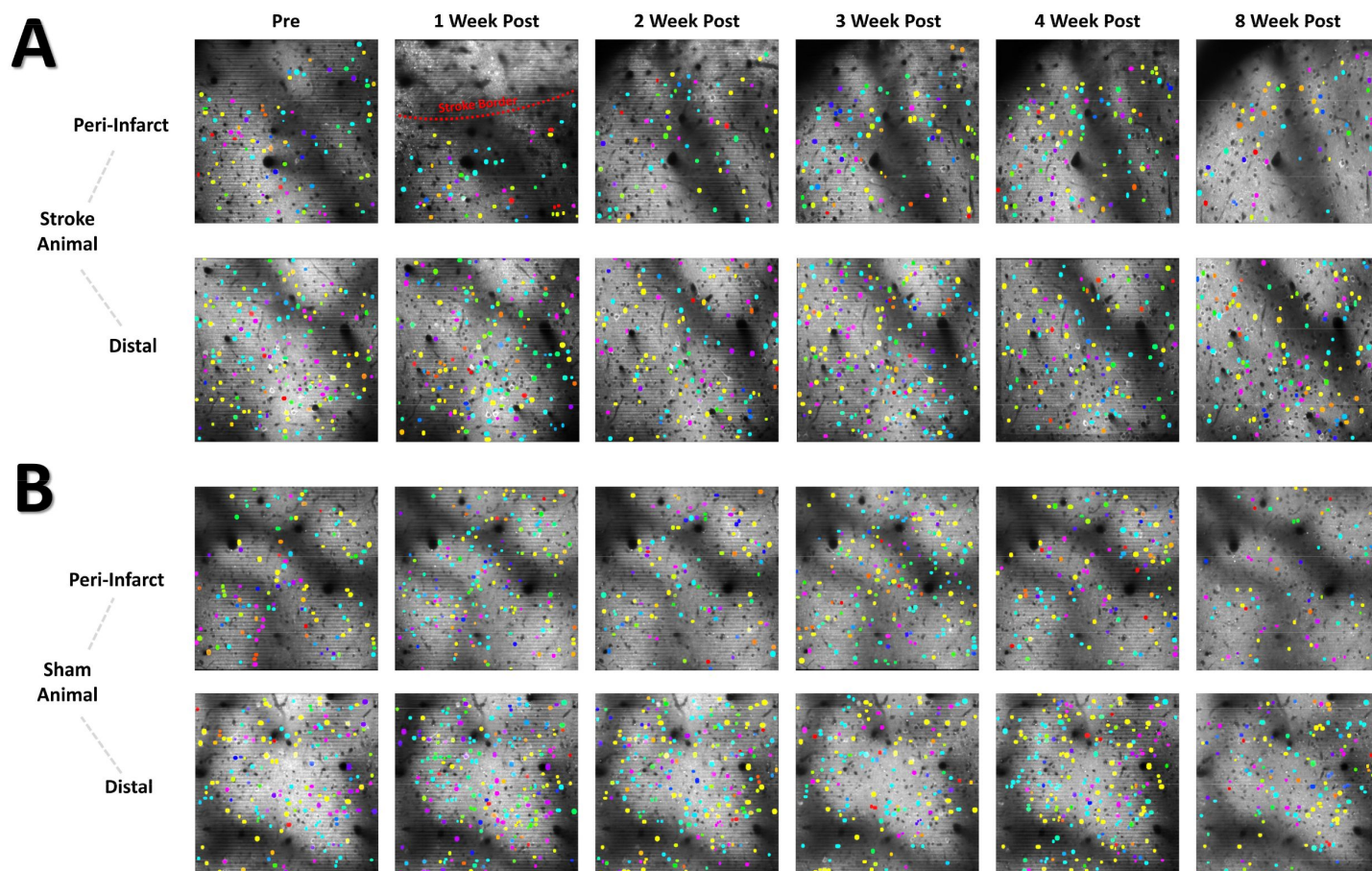


Fig 7.

Color coded neural assembly plots depict altered neural assembly architecture after stroke in the peri-infarct region.

(A) Representative cellular Ca^{2+} fluorescence images with neural assemblies color coded and overlaid for each time depict a loss in the number of neural assemblies after stroke in the peri-infarct region, and a concurrent increase in the number of neurons for each remaining assembly. (B) Representative sham animal displays no visible change in the number of assemblies or number of neurons per assembly.

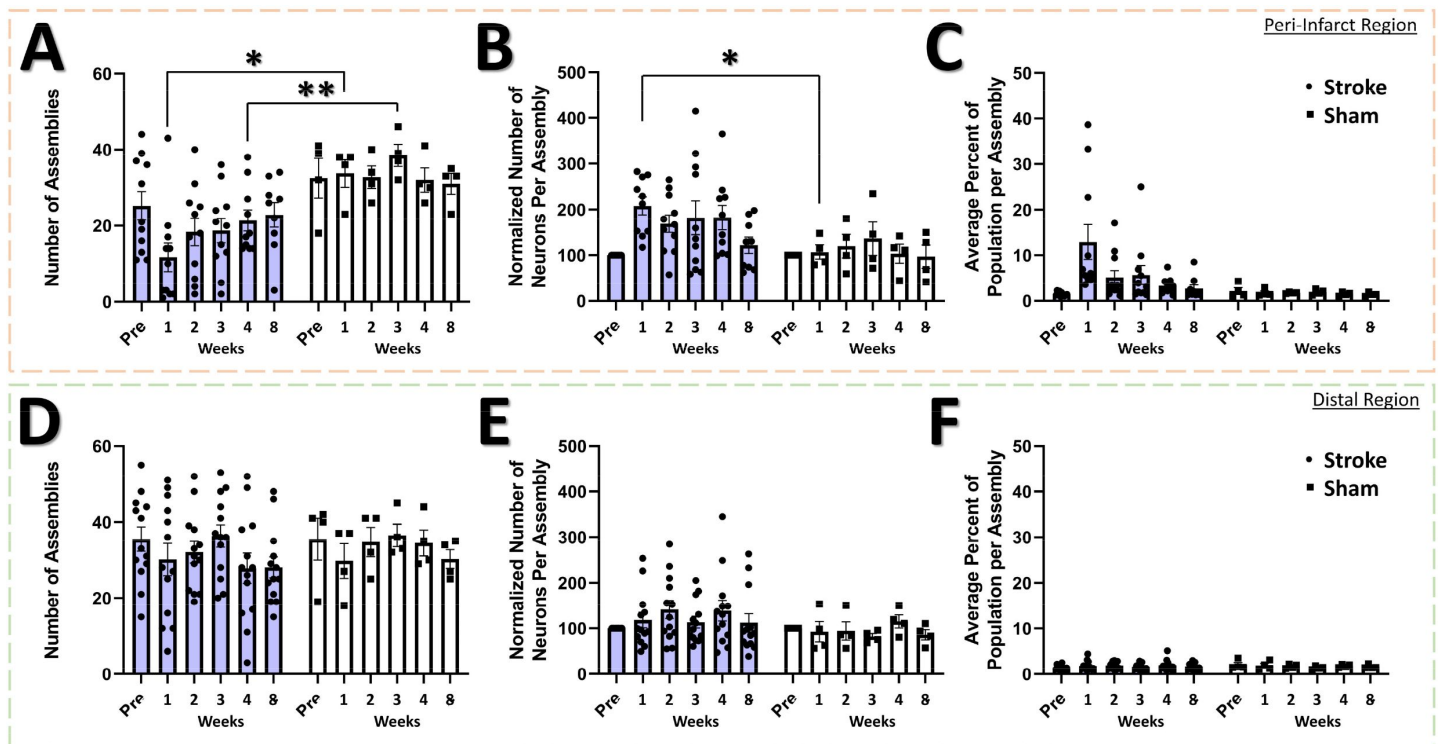


Fig 8.

Neural assembly structure is disrupted in the peri-infarct region after stroke.

In the peri-infarct imaging region, a significant main effect of group was found for the number of assemblies (**A**) (Time $F_{(2.883, 35.75)} = 0.8231$, $P = 0.4857$. Group $F_{(1, 13)} = 11.90$, $P = 0.0043$. Interaction $F_{(5, 62)} = 1.496$, $P = 0.2040$), with *post-hoc* tests showing a significant decrease in the number of assemblies at the 1 week and 3 week times for the stroke group compared to sham ($P = 0.0114$ & 0.0058 , respectively). A statistical trend is also observed at the 2 week time for the stroke group compared to sham ($P = 0.0589$). A trend towards a main effect of group was found in the normalized number of neurons per assembly (**B**, Mixed Effects Model, Time $F_{(2.361, 28.80)} = 2.185$, $P = 0.1234$; Stroke Group $F_{(1, 13)} = 3.694$, $P = 0.0768$; Interaction $F_{(5, 61)} = 1.202$, $P = 0.3192$), with *post-hoc* tests showing a significant increase in the normalized number of neurons per assembly at 1 week for the stroke group compared to sham ($P = 0.0124$). A trend towards a main effect of group was found in the average percent of the population per assembly (**C**, Time $F_{(1.711, 21.22)} = 1.738$, $P = 0.2023$; Stroke Group $F_{(1, 13)} = 3.807$, $P = 0.0729$. Interaction $F_{(5, 62)} = 1.765$, $P = 0.1333$), with *post-hoc* tests showing a trend towards an increase in the stroke group relative to sham at the 1 week post time ($P = 0.0863$). No main effects or interaction was observed in the distal imaging region for number of assemblies (**D**), normalized number of neurons per assembly (**E**), or average percent of the population per assembly (**F**). Peri-infarct region Stroke $N = 11$, Sham $N = 4$. Distal region Stroke $N = 13$, Sham $N = 4$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

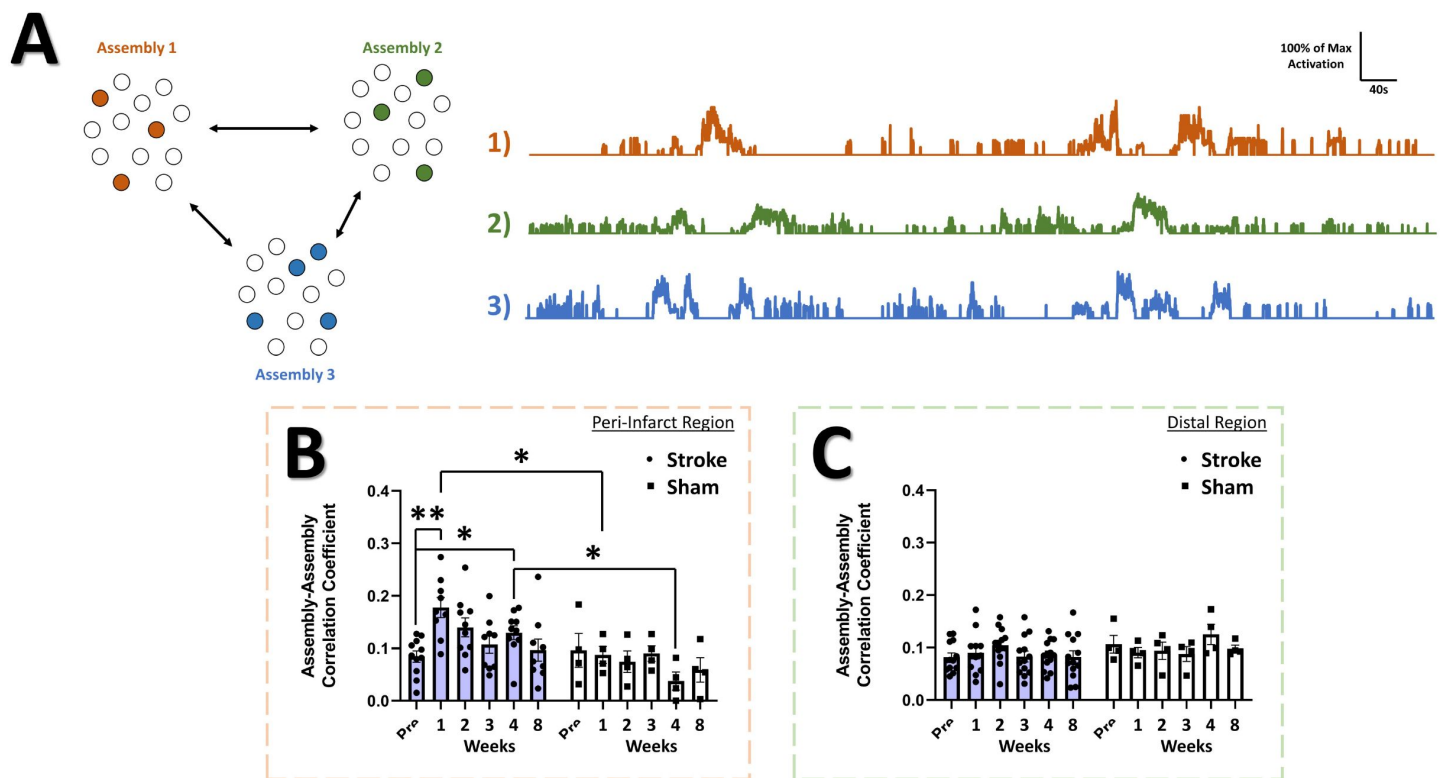


Fig 9.

Correlation among neural assemblies is increased in the peri-infarct imaging region after stroke.

(A) Illustration of three example color coded neural assemblies from a neural population and their corresponding time series activations that were correlated. In the peri-infarct imaging region, a significant main effect of group was found in the correlation coefficient for significantly correlated assembly-assembly pairs (B, Mixed Effects Model, Time $F_{(3.718, 42.39)} = 2.014$, $P = 0.1141$; Stroke Group $F_{(1, 13)} = 9.155$, $P = 0.0097$. Interaction $F_{(5, 57)} = 2.348$, $P = 0.0524$), with *post-hoc* tests indicating a significantly higher correlation at 2 week ($P = 0.029$) and 4 weeks ($P = 0.0253$) in the stroke group relative to sham, and at 1 week ($P = 0.0084$) and 4 week ($P = 0.0261$) in the stroke group compared to pre-stroke. In the distal imaging region, no significant main effects or interaction was found for the correlation coefficient for significantly correlated assembly-assembly pairs (C). Peri-infarct region Stroke $N = 11$, Sham $N = 4$. Distal region Stroke $N = 13$, Sham $N = 4$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

somatosensory cFL representation completely disrupts the sensory evoked forelimb representation in the infarcted region. Functional remapping of this representation occurred over 8 weeks after injury, with new sensory evoked spiking found primarily in a region lateral to the cHL representation. Mesoscale calcium imaging allows direct measurement of aggregate neuronal spiking, and remapped cortex exhibited reduced amplitudes of sensory-evoked activity with a broader, less defined cortical map relative to sham. Longitudinal two-photon calcium imaging in awake animals was used to probe single neuron and local network changes adjacent the infarct and in this distal remapped region. This imaging revealed a decrease in firing rate at 1 week post-stroke of neurons only in the peri-infarct regions that was significantly negatively correlated with the number of errors made with the stroke-affected limbs on the tapered beam task. Peri-infarct cortical networks also exhibited a reduction in the number of functional connections per neuron and a sustained disruption in neural assembly structure, including a reduction in the number of assemblies and an increased recruitment of neurons into functional assemblies. Elevated correlation between assemblies within the peri-infarct region peaked 1 week after stroke and was sustained throughout recovery. Notably, distal networks in the region associated with functional remapping in anaesthetized preparations was largely undisturbed.

Cortical plasticity after stroke

Plasticity within and between cortical regions contributes to partial recovery of function and is proportional to both the extent of damage, as well as the form and quantity of rehabilitative therapy post-stroke [78,79]. A critical period of highest plasticity begins shortly after the onset of stroke, is greatest during the first few weeks, and progressively diminishes over the weeks to months after stroke [19,80–84]. Functional recovery after stroke is thought to depend largely on the adaptive plasticity of surviving neurons that reinforce existing connections and/or replace the function of lost networks [25,52,85–87]. This neuronal plasticity leads to somatosensory and motor functional remapping to adjacent areas of the cortex and altered topographical organization, as observed here. The driver for this process has largely been ascribed to a complex cascade of intra- and extra-cellular signaling that ultimately leads to plastic re-organization of the microarchitecture and function of surviving peri-infarct tissue [52,78,82,86,88–90]. Consistent with our findings, structural and functional remodeling is dependent on the distance from the stroke core, with closer tissue undergoing greater re-organization than more distant tissue (for review, see [52]).

Previous research examining the region at the border between the cFL and cHL somatosensory maps has shown this region to be a primary site for functional remapping after cFL directed photothrombotic stroke, resulting in a region of cFL and cHL map functional overlap [25]. Within this overlapping area, neurons have been shown to lose limb selectivity 1 month post-stroke [25]. This is followed by the acquisition of more selective responses 2 months post-stroke and is associated with reduced regional overlap between cFL and cHL functional maps [25]. Notably, this functional plasticity at the cellular level was assessed using strong vibrotactile stimulation of the limbs in anaesthetized animals. Our findings using longitudinal imaging in awake animals show an initial reduction in firing rate at 1 week post-stroke within this region that was predictive of functional impairment in the tapered beam task. This transient reduction may be associated with reduced or dysfunctional thalamic connectivity [91–93] and reduced transmission of signals from hypo-excitability thalamo-cortical projections [94].

Importantly, the strong negative correlation we observed between the normalized firing rate of the neural population within the peri-infarct cortex and the number of errors on the affected side, as well as the quick recovery of firing rate and tapered beam performance, suggests this peri-infarct cortex activity within the peri-infarct region contributes to the impairment and recovery. This common timescale of neuronal and functional recovery also coincides with angiogenesis and re-establishment of vascular support for peri-infarct tissue [81,95–98].

Altered network connectivity after stroke

A highly distributed network of neural circuits forms the basis of information flow in the mammalian brain [99,100]. Many disease states are thought to result in disturbances in neural dynamics and connectivity of these networks due to a process of randomization that affects the network nodes and connections, leading to degraded functional performance of the networks [100,101]. Most studies looking at the functional connectivity of cortical networks after stroke have focused on connectivity between cortical regions and few have looked at functional connectivity within neural populations at the single neuron level within a region of cortex. Our finding of a decreased number of functional connections per neuron in the peri-infarct somatosensory cortex are consistent with recent research that demonstrated a decrease in the total number of functional connections for both inhibitory cells [71] and excitatory cells [71,72] in the motor and premotor cortex approximately 1 week after focal sensorimotor stroke.

Reductions in strong functional connections between neurons in our experiments peaked 1 week after stroke, with only partial recovery over the next 7 weeks. These changes were restricted to peri-infarct cortex, and were not observed in regions associated with functional remapping in response to vibrotactile stimulation under anesthesia. As the cumulative total number of functional connections within a neural network is dependent on the number of cells measured from the population, it is possible that a decrease in the number of connections at early timepoints in both studies may either reflect a loss of neurons and/or the functional silencing of neurons within the imaged areas. Unlike Bechay et al. [72], connection density (as % of max possible connections) did not decrease in either the peri-infarct somatosensory cortex or distal somatosensory cortex after stroke in our study. Thus, we observed a significant reduction in the number of strong functional connections between neurons, but not a reduction in the density of connections. It is notable that the peak deficit in peri-infarct functional connectivity in our study occurs simultaneously with reduced neuronal firing and sensorimotor impairment. Early disruption has been reported in anaesthetized animals, and our data in awake animals demonstrates that early network changes may be a key contributor to dysfunction, and a restoration of spiking in peri-infarct regions is related to recovery [19–25].

Altered network dynamics after stroke

According to the cell assembly hypothesis, transient synchronous activation of distributed groups of neurons organize into “neural assemblies” that underly the representation of both external and internal stimuli in the brain [73]. These assemblies have been demonstrated in the mammalian [102–111] and zebrafish [58,112–114] brain, and are often similar in presentation between spontaneous and sensory evoked activations [58,102,107,115,116]. Indeed, the similarity between spontaneous assemblies and sensory evoked assemblies tends to increase during development [102], potentially illustrating the “sculpting” of neural circuits towards commonly encountered sensory stimuli. To date, however, no study has examined changes to the architecture of neural assemblies on the level of single cells within neural networks of the somatosensory cortex in the post-stroke brain. Our data shows a transient decrease in the number of assemblies and an increase in the number of neurons per assembly for the peri-infarct cortex. If assemblies within peri-infarct somatosensory cortex contribute to differential processing of distinct elements of sensory experience, a loss in the number of assemblies may contribute to a loss in sensory range, as is observed in human stroke patients [117–121]. Although there have been calculations on the typical size of cell assemblies in several regions of the brain as a percentage of the network population (for review, see [73]), whether there is an optimal number of assemblies or optimal membership size of a neural assembly for a particular neural population is currently unknown. It is possible that a decrease in the number of assemblies and an increase in the membership of neurons in remaining assemblies may signify inefficient or ineffective forms of sensory processing. Likewise, it has been shown that electrical stimulation of single somatosensory neurons is sufficient to bias animal behavior towards a desired behavioral

response [122,123], thereby suggesting that a sparse neural code may be sufficient for sensation. It has also been argued that minimizing correlation within the neural population serves to reduce representational redundancy and improves representational efficiency [124,125], thereby suggesting that fewer assemblies, with each containing more members, may be an inefficient form of representational coding for sensory information. Furthermore, we observed a persistent increase in the correlation between the activations of assemblies in the peri-infarct cortex that peaks 1 week post-stroke. Co-activation of multiple assemblies simultaneously may decrease the signal-to-noise ratio of the representational information that each specific assembly holds. It may further reduce efficient information transfer within and between cortical networks, and may relate to prolonged modes of somatosensory activation [19] and reduced fidelity in response to sensory stimuli observed in mouse models of focal FL stroke [23].

Consistent with previous research [25], we show that at the 8 week timepoint after cFL photothrombotic stroke the cFL representation, identified by mechanical limb stimulation under anaesthesia, is shifted posterior from its pre-stroke location into the area lateral to the cHL map. Notably, our distal imaging region was directly within this remapped cFL area. Despite this, we found little change in the normalized firing rate in either moving or resting states in this region. This is in contrast to past research in anesthetized animals that indicated an increase in sensory-evoked activity within this area lateral to the cHL representation 1-2 months after focal cFL stroke [25]. Moreover, there was no indication that the cFL remapping into this distal area lateral to the cHL map resulted in any detectable change to the level of population correlation, functional connectivity, assembly architecture or assembly activations as compared to sham.

Notably, sensory-evoked activity for regional mapping was evoked via strong vibrotactile (1s, 100Hz) limb stimulation. The combination of anesthetic and strong stimulation may unmask connectivity in distal regions during anesthesia that is not apparent during more naturalistic stimulation during awake locomotion or rest. Nonetheless, these results support a limited spatial distance over which the peri-infarct area exhibits network functional deficits. These results are also consistent with a spatial gradient of plasticity factors that are generally enhanced with closer proximity to the infarct core [82,86,88,89].

In summary, this work demonstrates that the peri-infarct region near to the stroke core displays decreased neuronal activity 1 week after stroke that is predictive of poor performance on the tapered beam task. Moreover, local neuronal networks adjacent the stroke exhibited reductions in functional connectivity and sustained alterations in the structure of neuronal assemblies, suggesting that focal injury leads to long lasting alterations in functional connectivity between neurons in local neuronal networks. Interestingly, altered connectivity and neuronal assembly structure was not detected in more distal cortical regions, even though those regions were associated with functional remapping of somatosensory-evoked activity in response to vibrotactile limb stimulation under anaesthesia. The persistence of these distal networks during recovery highlights the importance of awake imaging and naturalistic stimuli to fully define functional network changes during recovery from nervous system injury. The restriction of these network changes to peri-infarct cortex could reflect the small penumbra associated with photothrombotic stroke, and future studies could make use of stroke models with larger penumbral regions, such as the middle cerebral artery occlusion model. Larger injuries induce more sustained sensorimotor impairment, and the relationship between neuronal firing, connectivity, and neuronal assemblies could be further probed relative to recovery or sustained impairment in these models.

Animals

Three to nine month old Thy1-GCaMP6S mice (Strain GP4.3, Jax Labs) (N=16 stroke (average age: 5.4 months), 5 sham (average age: 6 months)) were used in this study. Mice were group housed in standard laboratory cages in a temperature-controlled room (23°C), maintained on a 12 hr light/dark cycle, and given standard laboratory diet and water *ad libitum*. Cages were randomly assigned to stroke or sham groups such that stroke and sham mice did not co-habitate the same cages. Animal weight was monitored daily during the entirety of the experiment. All experiments were approved by the University of Alberta's Health Sciences Animal Care and Use Committee and adhered to the guidelines set by the Canadian Council for Animal Care. Animals were euthanized through decapitation under deep urethane anesthesia following the end of the final imaging session.

Chronic cranial window implantation

Mice were implanted with a chronic cranial window [126] four weeks prior to the first imaging time point (Fig 1B). A surgical plane of anesthesia was achieved with 1.5% isoflurane. Body temperature was measured using a rectal probe and maintained at 37±0.5°C. Mice were administered 0.15 mL of saline subcutaneously to maintain hydration levels. Dexamethasone (2 µg/g) was given subcutaneously to prevent cortical swelling and hemorrhaging during the craniotomy procedure. The skull was exposed by midline scalp incision and the skin retracted.

The skull was gently scraped with a scalpel to remove the periosteum. Grooves were scraped into the skull surface to improve adhesion of dental cement to the skull. A 4 x 4 mm region of the skull overlying the right hemisphere somatosensory region was thinned to 25-50% of original thickness using a high-speed dental drill (~1-5mm lateral, +2 to -2 mm posterior to bregma). A dental drill was used to progressively thin the overlying skull until the bone could be removed with forceps, leaving the dura intact. The exposed cortical surface was bathed in sterile saline solution. A 5mm diameter coverslip was held in place over the craniotomy and its edges attached to the skull using cyanoacrylate glue. A metal headplate was positioned and secured to the skull using dental cement. Animals were injected subcutaneously with buprenorphine (1.0 mg/kg), removed from the isoflurane, and allowed to recovery in a temperature-controlled recovery cage. Mice were returned to their home cage once recovered and monitored daily for weight and post-surgical signs for the duration of the chronic experiment. Mice were allowed to recover for a period of two weeks after window implantation prior to beginning behavior and task habituation (Fig 1C). Animals were excluded at the 2 week post-implantation timepoint if their cranial window became cloudy or non-imageable.

Tapered beam task habituation, testing and analysis

Methods for tapered beam habituation and automated recording and analysis have been described previously [53], with minor modification as follows. Nesting material from the homecage was placed inside of a dark box at the narrow end of the beam to motivate animals to cross the beam. For the first three sessions, mice were placed at the wide end of the beam and allowed to freely explore the beam for a period of 2 minutes, after which the experimenter placed a sheet of paper behind the animal as it crossed the beam to block its return path to the wide end and motivate it to cross to the narrow end with dark box. Once the mouse had reached the dark box with bedding material at the narrow end, they were given 60 seconds within the dark box to associate crossing the beam with reaching the safety of the dark box. The dark box was transported to their respective cage for the animal to further associate reaching the dark box with a safe transition to their cage. On subsequent days, mice were continuously run through the tapered beam for a period of 2 mins each, with a return to their cage after each crossing. On each of the 3 days prior to the first Ca²⁺ imaging session, mice were tested with three crossings of the

beam per day to determine baseline crossing performance. Left and right side slips were captured automatically by Raspberry Pi computer attached to touch sensors on the tapered beam, and Python scripts run to determine number of slips for each side and distance to first slip.

Performance on the 9 baseline trials were average to determine a singular average baseline performance level. Mice were tested with three trials on each day prior to each weekly post-imaging timepoint to measure changes in performance.

String pull task habituation, testing and analysis

Methods for string pull habituation and testing have previously been described [127, 128], with modification as follows. For habituation, mice were individually placed in a transparent rectangular cage without bedding and allowed to freely explore for a period of 5 minutes. 20 strings with variable length were hung over the edge of the cage. Half of the strings were baited with chocolate flavored sucrose pellets. Mice were removed from the apparatus once all of the strings had been pulled into the cage, or once 20 minutes had elapsed. On subsequent days 2 and 3, mice were again placed in the cage with 20 strings to pull. Following this 3-day habituation, from days 4-14 mice were habituated in an alternate transparent string pull box with high sides and transparent front face for video recording. Within this second habituation box, mice received 3 trials with baited strings hung facing the recording camera. The session was terminated when the animal had pulled all three strings or once 20 minutes had elapsed, and the apparatus prepared for the next mouse. On the last day prior to the first Ca^{2+} imaging session, 3 trials of string pull from each mouse were recorded with the use of a GoPro Hero 7 Black (60 fps, 1920×1080 pixels). Mice were re-tested with this same 3 string protocol the day prior to each of their weekly imaging timepoints. Video recordings were analyzed using a semi-automated Matlab string pull package [128]. Reach and withdraw movement scaling for the left and right paw was calculated by running a Pearson's correlation between the series of Euclidian distances that the paw travelled during each of the reach or withdraw movements during the string pull and correlating with the peak speed of the paw during each of the respective reaches or withdraws [129]. A value of 1 indicates a strong linear relationship between longer reach/withdraw paw movement distance and greater peak speed that the paw obtained. Paw reach distance was calculated as the average distance that the paw travelled during each reach movement. Path circuitry is used as a measure of how direct of a path a reach or withdraw movement takes between its starting and end points and is calculated as the total distance travelled divided by the Euclidian distance between the start and end points [129]. The greater the path circuitry value is above 1, the more the path of the paw deviated from the ideal direct path between the start and end points of the reach/withdraw. A bimanual correlation coefficient was calculated by first detrending the timeseries of Y-axis paw position to remove general changes in the posture of the animal over the course of the string pull attempt, then running a Pearson's correlation between the Y-axis paw position measurements for the right and left paw.

Mobile floating homecage habituation and measurement of movement parameters

Methods for floating homecage (Neurotar, Finland) habituation have previously been described [130], with modification as follows. Two weeks after cranial window implantation, each mouse was handled for 5 minutes 3 times per day for the first 2 days to habituate them to handling. For days 3-4, each mouse was handled and repeatedly wrapped and released with a soft cloth for a period of 5 mins 3 times per day in order to habituate to the wrapping procedure. Mice were also given 1 period of 5 minutes each per day to freely explore the floating homecage without being head-restrained. For days 5-8, twice per day the animals were head-restrained in the floating homecage and allowed to move around the floating homecage for a period of 15 minutes with the room lights off before being returned to their cage. For days 8-14, twice per day the mice were head-restrained in the floating homecage for 25 minutes with the floating homecage attached to the microscope in the same conditions in which the animals would be imaged on day 15 onwards.

On day 14 (one day prior to their first imaging session) baseline performance on the string pull and tapered beam tasks was measured prior to their last habituation in the floating homecage. Animal movement within the homecage during each Ca^{2+} imaging sessions was tracked to determine animal speed and position. Animal speed was thresholded at 30mm/s to determine periods of time in which the animal was moving and periods of rest were determined as speed below 30mm/s.

Identification of forelimb and hindlimb somatosensory cortex representations using

widefield Ca^{2+} imaging of sensory-evoked responses

On day 10 of habituation, mice were anesthetized with 1.25% isoflurane after they had completed all daily task training and floating homecage habituation. Mice were head-fixed on a stereotaxic frame with continuous isoflurane anesthesia. Body temperature was measured using a rectal probe and maintained at $37 \pm 0.5^\circ\text{C}$. The cortical surface was illuminated with a xenon lamp (excitation band-pass filtered at 450–490nm). Fluorescent emissions were long-pass filtered at 515nm and captured in 12-bit format by a Dalsa Pantera 1M60 camera mounted on a Leica SP5 confocal microscope using a 2.5X objective. The depth of focus was set at 200 μm below the cortical surface. Custom-made piezoceramic mechanical bending actuators were used to elicit oscillatory limb stimulation during widefield Ca^{2+} imaging. The piezo bending actuators comprised a piezo element (Piezo Systems # Q220-A4-203YB) attached to an electrically insulated metal shaft holding a metal U-bend at its end. The palm of the mouse paw was placed within the U-bend, and the U-bend bent to shape to lightly secure the palm. The metal U-bend made contact across a vertical rectangular area of approximately 3x1mm on the palmar and dorsal surface of the hand. Stimulators were driven with square-wave signals from an A-M Systems Model 2100 Isolated Pulse Stimulator. Stimulation alternated between contralateral forelimb (cFL) and contralateral hindlimb (cHL) for up to 40 trials of stimulation of each limb.

Placement of actuators was on the glabrous skin of the forepaw or hindpaw, with consistent alignment relative to the flexion of wrist and ankle. Images were captured for 5.0s at 10 Hz (1s before and 4s after stimulus onset; interstimulus interval = 20s). The trials for each limb were averaged in ImageJ software (NIH). 10 imaging frames (1s) after stimulus onset were averaged and divided by the 10 baseline frames 1s before stimulus onset to generate a response map for each limb. Response maps were thresholded at 95% maximal response to determine limb associated response boundaries, merged, and overlaid on an image of surface vasculature to delineate the cFL and cHL somatosensory areas.

Calcium imaging

On the day following testing for behavioral performance on the string pull and tapered beam tasks, animals were head-fixed within the mobile homecage and Ca^{2+} imaging was performed. Awake in-vivo two-photon imaging was performed using a Leica SP5 MP confocal microscope equipped with a Ti:Sapphire Coherent Chameleon Vision II laser tuned to 940 nm for GCaMP6S excitation. A Leica HCX PL APO L 20x 1.0NA water immersion objective was used. Images were acquired using Leica LAS AF using no averaging, a zoom of 1.7x and a frame-rate of 25Hz. Images were acquired at 512x512 pixels over an area of 434x434 μm , yielding a resolution of 0.848 μm per pixel. Two imaging regions were chosen for each animal, with one corresponding to the half-way point between the pre-stroke cFL and cHL somatosensory maps previously determined with widefield Ca^{2+} imaging (the “peri-infarct” imaging region), and the other an imaging region located just lateral to the cHL sensory map (the “distal” imaging region). These regions can be seen in [Fig 1D](#) and [Fig 2A](#). Each imaging region was recorded for 15 minutes while simultaneously tracking the animal’s movement and position within the mobile homecage. Imaging depth was set between 100–180 μm below the cortical surface. There were no differences between the stroke group and sham group for movement within the mobile homecage, though both groups displayed

an equivalent decrease in the amount of time they spent moving within the 15 minute recording sessions over the course of the 8 week experimental timeline, potentially reflecting continuing habituation over this period.

Photothrombotic stroke and sham procedure

After baseline cellular Ca^{2+} imaging, mice were anesthetized using 1.25% isoflurane and head-fixed on a stereotaxic frame with continuous isoflurane anesthesia. Body temperature was measured using a rectal probe and maintained at $37 \pm 0.5^\circ\text{C}$. The mapped cFL area was used as a guide for a targeted photothrombosis procedure. Briefly, a 2mm diameter hole was punched in black electrical tape and attached onto the glass window to block stray light from illuminating cortical areas outside of the desired region. Rose Bengal (a photosensitive dye) was dissolved in 0.01M sterile phosphate buffered saline (Sigma) and injected intraperitoneal (30mg/kg). The cFL cortical area visible through the punched electrical tape was illuminated using a collimated beam of green laser light (532 nm, 17mW) for 20 minutes to photoactivate the Rose Bengal and cause a focal ischemic lesion. Sham controls were treated in the same manner as stroke, however illumination of the laser was omitted.

Two-photon image processing and analysis of neuronal activity

Timeseries images (15 minutes per imaging region, 22500 frames per recording, 25fps) were group averaged in groups of 2, then motion corrected using the TurboReg plugin in Fiji with translation only registration [131]. Z-projections within Fiji of the average and standard deviation were used to define neuronal ROI through manual tracing. Using custom written scripts in Matlab 2020b, ROIs were imported and converted into a format suitable for the Ca^{2+} imaging toolbox used for subsequent steps [59]. Neuropil for each ROI was determined by expanding an annular donut around each ROI to calculate the neuropil $\Delta\text{F}/\text{F}_0$ surrounding each ROI. During computation of neuron ROI $\Delta\text{F}/\text{F}_0$, fluorescence was corrected for neuropil contamination using the formula ($F_{\text{corrected}} = F_{\text{raw}} - \alpha * F_{\text{neuropil}}$) where α was set to 0.4 based on previous studies suggesting that α values between 0.3-0.5 were optimal [132], and with F_{neuropil} determined by the peri-somatic donut neuropil fluorescence closely associated with each neuron. A smoothed estimate of the baseline fluorescence was calculated by taking a 30s running average of the 8th percentile of the raw fluorescence, which was then subtracted from the raw fluorescence to remove baseline drift.

Ca^{2+} trace deconvolution to determine neuronal transients

To determine significant fluorescent Ca^{2+} transients from the $\Delta\text{F}/\text{F}_0$ of each neuron, a dynamic threshold implementing a Bayesian odds ratio estimation framework for noise estimation was used to determine transients that met the condition of being greater than 98% of the confidence interval for the calculated fluorescent baseline noise, and were compatible with a tau of $\sim 1.8\text{s}$ for GCaMP6S [58,59]. Determination of firing was performed on the neuronal $\Delta\text{F}/\text{F}_0$ traces, as implemented in [58,59], in order to define the Ca^{2+} transient rate and to generate raster plots. This information was used to determine neuronal activity “firing rate” as shown in Fig 4.

Neuron-neuron correlation analysis and functional connectivity

Using custom written scripts in Matlab, Pearson product-moment correlation coefficients were calculated between the z-scored Ca^{2+} traces derived from each of the neuronal ROIs in a given optical section. Distance between neuron pairs was calculated from the Euclidean distance between the central points of the neuron ROIs of the image frame. Functional connectivity plots (Fig 5) were generated by plotting neuron ROI centroids as red dots, and lines between them with line weight and color determined by the strength of the correlation between them.

Stationary variable block bootstrapping (5000 iterations) was performed as a statistical test for the significance of each pairwise correlation and only correlations that were greater than the 99th percentile of the bootstrap were deemed statistically significant and plotted. Average number of significant connections per neuron was calculated by dividing the number of functional connections that met the bootstrapping threshold by the total number of neurons in the population. Connection density was calculated as percent of max by taking the total number of significant functional connections and dividing by the total potential number of functional connections if every neuron within the population was functionally connected with all other neurons.

Determination of neural assemblies and their activity patterns

To evaluate the co-activity patterns of neurons that form putative neural assemblies, a PCA-Promax procedure was applied as previously described [58, 59], with a zMax threshold manually selected by the first clear minimum in the distribution of z-scored maximal ROI loadings. Notably, the PCA-Promax procedure relaxes the PCA orthogonality condition using a PROMAX oblique rotation of the PC axes [133], such that assemblies can contain neurons found within other assemblies. Due to this relaxation of the PCA orthogonality condition, assemblies with high overlap whose dot product exceeded 0.6 were merged [133]. All assemblies were compared to surrogate control datasets, and only those assemblies whose members were significantly correlated and synchronous were kept ($p < 0.05$). To determine the co-activity of neurons within assemblies, a matching index was calculated [58, 59, 134, 135]. The matching index quantifies the proportion of neurons within the assembly that are co-activated simultaneously over the timeseries, with a maximal value of 1 indicating perfect overlap in assembly member activation. The significance of each assembly activation over the timeseries was determined by comparing the activation events to a probability distribution based on the size of the assembly, the size of the total population of N ROIs, and with a threshold p -value of <0.05 considered to be a statistically significant activation. For cross-correlation of assembly activations with speed (Fig S3) and assembly-assembly correlations (Fig 9), the matching index was first multiplied by the Boolean timeseries of individual assembly activation significance to generate matching index timeseries of significant assembly activations only (presented in Fig S3 and Fig 9 as percent of maximal assembly activation). ROIs of assembly members were color coded according to their assembly membership, and overlaid on an averaged fluorescence image to generate assembly plots (Fig 7).

Statistical analysis

Multivariate comparisons were made using a mixed-effects model for repeated measures based on a restricted maximum likelihood generalized linear mixed model as implemented in Graphpad Prism 9.0.0, with Bonferroni-Sidak corrections used for *post-hoc* comparisons.

Bonferroni-Sidak *post-hoc* testing was used to compare the means of the stroke vs. the sham group at each timepoint, as well as to identify within group differences by comparing different timepoints within each group. Normalized data was analyzed using multivariate mixed-effects models based on a restricted maximum likelihood generalized linear mixed model, with the “pre” timepoint removed from calculation of main effects, interaction, and *post-hoc* statistical testing. Grubb’s test was used to remove data outliers with alpha set to 0.05. All zero time-lag cross-correlations were computed using Pearson’s r . A stationary bootstrapping procedure was used as a test of the significance of the calculated Pearson’s r of each pairwise timeseries comparison.

Within the stationary bootstrapping procedure, 5000 iterations were run and an average block length of 23 frames (1.84s) was used based on previous studies indicating ~1.8s as the decay time for GCaMP6S Ca^{2+} sensor within neurons [136, 137]. Pairwise r values greater than the 99th percentile of the stationary bootstrap were deemed significant. Non-significant pairwise r values

were excluded from analyses of average correlation values. For all statistical comparisons, a p value of ≤ 0.05 was considered statistically significant. A p value of ≤ 0.10 was considered a statistical trend. Data are expressed as the mean $\pm$ SEM.

Author Contributions

MVB and IRW designed all experiments. MVB performed experiments and analyzed data. MVB and IRW wrote the manuscript together.

Competing Interests

The authors declare no competing interests.

Data Availability

All data generated or analyzed during this study will be made publicly available on OSF (<https://osf.io/wam9j/> )

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Reviewer #1 (Public Review):

Summary: This impressive study by Bandet and Winship uses 2-photon imaging in awake-behaving mice to examine long-term changes in neural activity and functional connectivity after focal ischemic stroke. The authors discover that there are long-lasting perturbations in neural activity and functional connectivity, specifically within peri-infarct cortex but not more distant cortical regions. Overall I thought the study provided important new findings that were supported by compelling data.

Strengths: This is a technically challenging study to perform and the authors show high-quality data. The manuscript was well-written, and the figures were clearly presented. I really like the analytic tools they applied, which were rigorous and provided some novel insights regarding neural activity patterns during movement or rest. The discovery of long-lasting impairments in neural activity/functional connectivity is an important one as it is important for future stroke studies to recognize what problems need to be rectified in the post-stroke brain, even many weeks after injury. They also suggest there is a much more nuanced relationship between macroscopic changes in somatosensory maps and single-cell activity. Overall, I think this is a well-executed study whose primary conclusions were justified by the data presented.

Weaknesses: I found very little in the way of weaknesses. The statistics were notably conservative and are appropriate.

Reviewer #2 (Public Review):

This study investigates the excitability of neurons in the peri-infarct cortex during recovery from ischemic stroke. The excitability of neurons in the peri-infarct cortex during stroke recovery has produced contradictory findings: some studies suggest hyper-excitability to direct-brain stimulation, while others indicate diminished responsiveness to physical stimuli. However, most studies have used anesthetized animals, which can disrupt cortical activity and functional connectivity. The present study used two-photon Ca^{2+} imaging after focal photothrombotic stroke to examine neural activity patterns in awake mice. The authors found reduced neuronal spiking in the peri-infarct cortex that was strongly correlated with motor performance deficits. Additionally, the authors found disruptions in neural activation, functional connectivity, and assembly architecture in the immediate peri-infarct region but not in the distal cortex regions.

The findings of this study are very important as they show that there is no measurable change in terms of neuronal activation and reorganization in distal regions of remapped cortical response areas after stroke.

However, cortical response areas are calculated using a threshold of 95% peak activity within a trial. The threshold is presumably used to discriminate between the sensory-evoked response and collateral activation / less "relevant" response (noise). Since the peak intensity is lower after stroke, the "response" area is larger - lower main signal results in less noise exclusion. Predictably, areas that show a higher response before stroke than after are excluded from the response area before the stroke and included after.

We suggest a reinterpretation of the findings: much of the non-remapped areas are included when using a within-trial threshold as a criterion, and the absence of increased neuronal activation and reorganization is evidence for this claim. The take-home message of this study should be that we need a much better criterion for what we consider remapping.