Microglial direct contacts with synapses enhance synaptic activities in awake mice

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Abstract

Microglia are the primary immune cells in the central nervous system (CNS). They constitute 10 – 15% of all cells in the CNS. Microglia activate in injuries and diseases, changing their morphology, and having an impact on neurodegenerative disorders. In addition to their role in disease, recent studies showed that microglia are highly motile cells in the healthy brain, extending and retracting their processes. Moreover, they make direct contacts with synapses to monitor synaptic activity in an activity dependent manner. Recent evidence using embryonic zebrafish optic tectum showed that microglia also have direct contact on neuronal cell body to modify neuronal activity. In motor learning, microglia have an important role in promoting learning-related synapse formation through BDNF signaling. On the other hand, in the lever-push motor learning, the relationship between neuronal activity and individual movements becomes more consistent with motor learning.

These results lead my hypothesis that microglia could modify synaptic activity by their direct contacts to change local network activity during motor learning task. Therefore, in this study I aimed to investigate whether microglia play a substantial role of motor learning task or not, whether microglia can change populational neuronal

activities in primary motor cortex which is known as the most prominent motor output area, and whether microglia could change synaptic activity by their direct contacts or not.

In motor learning task, mice are trained to pull the lever to receive water as a reward. To investigate whether microglia can promote motor learning in this lever pull task experiment, double transgenic mice depleted microglia partially were used. In this microglia depletion mouse experiment, Iba1-tetracycline transactivator (Iba1-tTA) mouse and tetracycline operator-diphtheria toxin A (tetO-DTA) mouse were crossed. Using tetracycline-controllable gene expression system, Diphtheria toxin A (DTA) is expressed in mice which tTA is expressed if doxycycline (DOX) is withdrawn as a feed of these mice. I also used activated microglia mice which are injected Lipopolysaccharide (LPS) into peritoneal cavity to investigate whether this is only a property of resting microglia or not. I showed that success rate in control mice during motor learning increased more than that in microglia depleted mice and in LPS injected mice.

Next, I investigated whether neuronal activity in primary motor cortex are changed during motor learning task through microglial role, I measured Ca^{2+} transient as a neuronal activity using *in vivo* two-photon microscope imaging. I showed that microglia depletion mice (Dox off mice) and microglia activated mice (LPS injection mice) showed less synchronous activity in response to lever-pull movement compared with control mice

during motor learning. Moreover, I investigated populational neuronal activities in primary motor cortex during motor learning. I showed that the activity of layer 2/3 pyramidal neurons of the primary motor cortex in control mice showed stronger negative correlation between the correlation co-efficiency of the paired cells and the distance of the paired cells compared with that of Dox off mice and LPS injection mice during motor learning. It is demonstrated that activated microglia and microglia depletion suppress the synchronicity of local neuronal network activity. These results suggested that microglia play an important role in synchronizing network activity triggered by a motor learning task.

Finally, I confirmed whether microglial contact on synapse can induce a change of synaptic activity. I used AAV constracts into layer 5 primary motor cortex in Iba1-EGFP mice. This enabled me to visualize both microglia and synaptic activity in neuron simultaneously. In this experimental design, I showed local calcium responses in the postsynaptic spines increased significantly when microglial processes have contacts with spines. Activation of microglia by applying LPS inhibit spine activation with microglia contacts.

In conclusion, my result is that microglia enhance synaptic activity with their contact and this promotes the synchronized network activity during motor learning. When

microglia contact on synapse, it would enhance single neuronal activity which induce higher synchronicity in the functional subnetworks.

In discussion, there are two possibilities of connection between the synchronicity of neuronal network activity during motor learning task and enhancement of synaptic activity through microglial direct contact with synapses. One is that microglia have multiple processes and could simultaneously contact on synapses of various neurons within their territory to promote the synchronization of local network activity during motor learning task. The other possibility is to progress the synchronization of neuronal activities inside the subnetwork related to the motor learning. This is the new mechanisms of microglia have an important role of promoting the synchronized network activity by direct contact of synapses.

Thus, in the physiological state, microglia have an important function to promote synchronizing network activity and can be envisaged as key neuro modulators in the brain.

Introduction

Recently many findings about microglia indicate that microglia have an important role of homeostasis in the central nervous system (CNS). Although microglial functions have been studied extensively in the pathological state, their function in the physiological state remains unknown.

Microglia are the primary immune cells in the CNS. They constitute 10 – 15% of all cells in the CNS (Lawson et al., 1992). They are of mesodermal origin deriving from yolk-sac invasion of the CNS during the early embryonic stage (Ginhoux et al., 2010). Microglia activate in injuries and diseases, changing their morphology, and having an impact on neurodegenerative disorders.. In addition to their role in disease, recent studies showed that microglia are highly motile cells in the healthy brain, extending and retracting their processes (Nimmernjahn et al., 2005). Moreover, they make direct contacts with synapses to monitor synaptic activity in an activity dependent manner (Wake et al., 2009). Recent evidence using embryonic zebrafish optic tectum showed that microglia also have direct contact on neuronal cell body to modify neuronal activity (Li et al., 2012). On the other hand, in motor learning, microglia have an important role in promoting learning-related synapse formation through BDNF (Parkhurst et al., 2013).

Moreover, in the lever-push motor learning, the relationship between neuronal activity and individual movements becomes more consistent with motor learning (Peters et al., 2014). Many lines of evidence indicate that even in the physiological state, microglia have an important role.

These previous reports lead my hypothesis that microglia could modify synaptic activity by their direct contacts to change local network activity during motor learning task. Therefore, in this study I aimed to investigate whether microglia play a substantial role of motor learning task or not, whether microglia can change populational neuronal activities in primary motor cortex which is known as the most prominent motor output area (Masamizu et al., 2014), and whether microglia could change synaptic activity by their direct contacts or not.

Material and Methods

All animal experiments were approved by the Animal Research Committee of the National Institutes of Natural Sciences.

Animal

In all experiments, 6 – 12 month-old male mice were used. In motor learning task and *in vivo* two-photon Ca^{2+} imaging to visualize neuronal soma Ca^{2+} response, I used C57 bl/6 mouse. In microglia depletion mouse experiment using tetracycline-controllable gene expression system, I used double transgenic mouse which two kinds of single transgenic mice were crossed, one is Iba1-tetracycline transactivator (Iba1-tTA) mouse (Tanaka et al., 2012) and the other one is tetracycline operator-diphtheria toxin A (tetO-DTA) mouse (Stanger et al., 2007). Diphtheria toxin A (DTA) is expressed in mice which tTA is expressed if doxycycline (DOX) is withdrawn as a feed of these mice. DTA is expressed in mice under Dox 0.1 g/kg containing feed.

In the experiment of the interaction between microglia and synapse, I used Iba1-EGFP transgenic mouse. This mouse expresses EGFP under the control of the ionized Ca^{2+} binding adapter molecule 1 (Iba1) promoter. This promotor is specific in microglia and

macrophage (Hirasawa et al., 2005).

Motor learing task

To perform this task, I attach metal head plate to the mouse skull with dental cement. To habituate to this motor learning task device, mouse is inserted to the chamber with the head plate fixed. In 2 weeks, mouse is water-deprived for two days and is kept over 80% of its body weight. The task is lever-pull task for 14 consecutive days. When mouse pull the lever using right forelimb more than 600msec , mouse can receive 4 μl water as a reward from water pole. It is called success. On the other hand, if mouse pull the lever less than 600ms, mouse cannot receive water. It is called failure. Success rate is a number of success of all movements of lever pull using right forelimb. Each training is 1 hour per day for 14 days. After task, mouse is returned to the cage.

LPS injection

Microglia are activated using LPS injection into peritoneal cavity. LPS (1.0 mg / kg) is administered for 4 consecutive days (Chen et al., 2014). This causes microglia activation, which I confirmed by showing an increase of microglial soma area. In motor learning task experiments, LPS is administered for 14 consecutive days after microglia

activation in LPS injection for 4 consecutive days. In the experiment of the interaction between microglia and synapse, LPS is administered during imaging session.

Viral injection

In motor learning task and *in vivo* two-photon Ca^{2+} imaging to visualize neuronal soma Ca^{2+} response experiments, I used AAV1-hsyn-GCaMP6f for C57 bl/6 wild type mice or Iba1-tTA :: tetO-DTA mice. In the experiment of the interaction between microglia and synapse, I used AAV1 – CaMKⅡ – Cre, AAV1-Syn-FLEX-GCaMP6f, and AAV1-CAG-FLEX-tdTomato for Iba1-EGFP mice. During injection surgery, I used isoflurane inhalation as a general anesthesia. On the mouse skull, I made a 2.5 mm diameter craniotomy over the right forelimb area of primary motor cortex (circle centered at 1 mm lateral to bregma). Once I open the mouse skull, I keep the dura mater not removed. When I inject virus, I pulled glass pipette and front-loaded with virus solution. I injected 0.5 to 1 μl of AAV for each mice. After injection, I didn't remove the glass pipette soon and maintain it at least for 10 minutes, then withdraw it slowly. 2.5% agarose was covered on the site of the craniotomy. 4.5-mm diameter glass coverslip was placed on the agarose site of the craniotomy area with dental cement sealed on the edges.

Two-photon microscopy imaging

All images were acquired on a Zeiss LSM 7MP two photon microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY) with a 20X (1.0 N.A.) objective lenses. The laser intensity was 5-30 mW. A custom-made imaging chamber was used on the cranial window. Two photon imaging was performed operating at 950 nm wavelength. In *in vivo* two-photon Ca^{2+} imaging to visualize neuronal soma Ca^{2+} response experiments under motor learning task, the imaging plane was a depth of 200 to 300 μ m from the cortical surface which means Layer 2/3 on the primary motor cortex. The number of frames for each image session was 1000 frames. Frame rate was 0.39 second per frame. In the experiment of the interaction between microglia and synapse, the imaging plane was a depth of 20-100 μ m from the cortical surface which means Layer 1 on the primary motor cortex. The number of frames for each image session was 500 to 10000 frames. Frame rate was 0.24 to 0.39 second per frame. As an image processing tool, I used ImageJ (1.44k, US National Institutes of Health).

Brain fixation and immunohistochemistry

Animals were anesthetized and transcardially perfused with prepared 4% paraformaldehyde (PFA). Brains were removed, post-fixed for 48 hours in 4% PFA at

4 ℃ and then sectioned with vibratome (VT1000S; Leica, Tokyo, Japan) at 50 μ m thick coronal slices to investigate microglia morphology and microglia number. Sections were mouted with VECTASHIELD mounting medium (H-100, Funakoshi, Tokyo, Japan).

Statistics analysis

The data were expressed as mean ± SEM, with n indicating the number of data. Paired and unpaired Student's t-tests were performed to access statistical significance of the data.

Results

Part 1 : Microglia promote the synchronization of local network activity triggered by motor learning task

Success rate during motor learning over 14 consecutive days

In motor learning task, mice were trained for 14 consecutive days to use their right forelimb to pull the lever to receive water as a reward because of water restriction for 2 days (Figure1A). Mice can receive water as if they pull the lever and keep it more than 600ms. When mice cannot keep the lever over threshold more than 600ms, they don't get water. It is called failure (Figure1B). Mice were placed in the chamber for motor learning task. This motor learning task device can be set on the two photon microscope stage (Figure1C).

In previous paper, microglia have an important role in promoting learning – related synapse formation in motor learning (Parkhurst et al., 2013). To investigate whether microglia can promote motor learning in this lever pull task experiment, I used double transgenic mice depleted microglia partially. In this microglia depletion mice experiment, Iba1-tetracycline transactivator (Iba1-tTA) mouse (Tanaka et al., 2012) and tetracycline

operator-diphtheria toxin A (tetO-DTA) mouse (Stanger et al., 2007) were crossed. Using tetracycline-controllable gene expression system, Diphtheria toxin A (DTA) is expressed in mice which tTA is expressed if doxycycline (DOX) is withdrawn as a feed of these mice. The average density of microglia in Dox off mouse was significantly decreased compared with that in Dox on mouse (control mouse) (Figure 2A, 2B) (Dox on: n=3 mice, Dox off: n=3 mice, Statistics: P =0.0233, Error bar: SEM).

On the other hand, microglia can be activated by acute stimulus and chronic disease states (Ransohoff et al., 2009). It results in the deficiency of the microglial physiological role. To investigate whether activated microglia can also promote motor learning or not, microglia were activated using LPS injection into peritoneal cavity for 4 days injection (Chen et al., 2014). This resulted in microglia activation, which I confirm by showing that microglia cell soma area was significantly increased in LPS injected mice compared with that in saline injected mice (Figure3A, 3B) (Wilcoxon rank-sum test, control : n=3 mice, LPS : n=3 mice, P =0.036, error bar: SEM).

In motor learning task, I compared behavioral performance during motor learning in Dox on mice (control mice) with that in Dox off mice (microglia depleted mice). Motor learning phase is divided into two phases: early phase is day1 - 4 and late phase is day11 - 14. In early phase, there was no significant difference between Dox on and Dox off mice.

On the other hand, in late phase, there was significant difference between Dox on mice and Dox off mice (Figure 4A) (Success rate in early phase; 24.0 ± 9.4 % (n = 9) and 24.5 ± 13 %, (n = 7; p = 0.99), Success rate in late phase; 54.9 ± 6.1 % (n = 9) and 40.7 ± 12 % (n = 7, p = 0.041, two-way ANOVA with post hoc Tukey's test). Moreover, I compared behavioral performance during motor learning in control mice (resting microglia) and in LPS injected mice (activated microglia) comparing success rate. While there was no significant difference in early phase between control mice and LPS injected mice, there was significant difference in late phase between them (Figure4B) (Success rate in early phase; 26.7 ± 5.5 % in saline injected mice; n = 7, and 23.5 ± 8.3 % in LPS injected mice; n = 8, p = 0.90. Success rate in late phase; 54.1 ± 7.7 % in saline control mice, n = 7, and 37.6 ± 12 % in LPS injected mice, n = 8, $p = 7.8 \times 10^{-3}$, two-way ANOVA with post hoc Tukey's test). These results suggest that mice depleted of microglia or mice in which microglia are activated showed deficiency in the motor learning task. Resting microglia can promote lever pull motor learning task.

Microglia play an important role in synchronizing network activity triggered by a motor learning task

To investigate whether neuronal activity in primary motor cortex are changed during

motor learning task through microglial role, I measured Ca^{2+} transient as a neuronal activity using *in vivo* two-photon microscope imaging. To visualize neuronal activity, I used AAV1-hsyn-GCaMP6f for C57 bl/6 wild type mice or Iba1-tTA :: tetO-DTA mice in layer 2/3 of primary motor cortex. In this experiment, I focused on neuronal activities in layer 2/3 because this layer is the major input layer from deeper layer neurons and layer 2/3 neurons can produce motor cortex outputs (Weiler et al., 2008, Kaneko et al., 2000, Peters et al., 2014). I could visualize expressed neurons in two to three weeks after virus injection in Dox on mice and in Dox off mice (Figure5A). I measured neuronal Ca^{2+} responses in layer2/3 of primary motor cortex with lever trajectory in Dox on mice and in Dox off mice during motor learning (Figure5B). Because most of Ca^{2+} responses in Dox on mice seemed to be associated with lever-pull movement and less associations in Dox off mice, I measured synchronization rate in Dox on mice and Dox off mice. Synchronization rate is an average of fraction of neurons responding with Ca^{2+} activity triggered by the lever – pull task during imaging. Dox off mice showed less synchronous activity in response to lever-pull movement compared with Dox on mice during motor learning (Figure6) (Day 1: $p = 0.020$, day 5 : $p = 0.0085$, day 10 : $p = 0.021$, Dox on : n=5 mice, Dox off : n=5 mice, Unpaired *t*-test). These results suggest that triggered neuronal synchronization is reduced in microglia depleted mice.

I measured the correlation co-efficiency (C.C.) of paired neuron activities against their distance in Dox on mice and Dox off mice during motor learning task. Scatter plots of correlation coefficients of paired neuronal Ca^{2+} responses against a distance separating those two neurons in Dox on mice and Dox off mice during motor learning indicated that separated neurons were closer, there were higher correlated neuronal activities in Dox on mice, but there was no such relationship in Dox off mice (Figure 7). To quantify this relationship, I measured slop between C.C. of paired neuronal Ca^{2+} responses and a distance of those two neurons in Dox on mice and in Dox off mice during motor learning (Figure 8). There was a significant difference of slop in Dox on mice and in Dox off mice on day1 and day5. These results suggest that microglia within their territory could modify the spatial pattern of neuronal activity (day 1: $p = 0.017$, day 5 : $p = 0.026$, day 10 : $p = 0.72$, Dox on : n=5 mice, Dox off : n=5 mice, Unpaired t-test). Finally, I measured an average of correlation coefficients of paired neurons within 100μm in Dox on mice and in Dox off mice in day1, day5, and day10 (Figure 9). There is a significant difference of correlation coefficients within 100μm in Dox on mice and Dox off mice during motor learning. These results suggest that microglia within their territory could promote the synchronicity of local neuronal network activity (Dox on: n=5 mice, Dox off : n=5 mice, Statistics: Wilcoxon rank-sum test, day 1: $P = 0.000027$, day 5: $P = 0.000012$, day 10: P

= 0.030).

Next, to investigate whether this is only a property of resting microglia or not, I activated microglia using LPS injection into peritoneal cavity and measured Ca^{2+} transient as a neuronal activity using *in vivo* two-photon microscope, as was the same with Dox on and off experiments. I could visualize expressed neurons in two to three weeks after virus injection in control mice and in LPS injected mice (Figure10A). I measured neuronal Ca^{2+} responses in layer2/3 of primary motor cortex with lever trajectory in control mice and in LPS injected mice during motor learning (Figure10B). I measured synchronization rate in control mice and LPS injected mice. LPS injected mice showed less synchronous activity in response to lever-pull movement compared with control mice during motor learning (Figure11) (Day 1: $p = 0.031$, day 5 : $p = 0.019$, day 10 : $p = 0.00064$, Normal : n=5 mice, LPS : n=5 mice, Unpaired t-test). These results suggest that triggered neuronal synchronization is reduced in LPS injected mice.

I measured the C.C. of paired neuron activities against their distance in control mice and LPS injected mice during motor learning task. Scatter plots of correlation coefficients of paired neuronal Ca^{2+} responses against a distance separating those two neurons in control mice and LPS injected mice during motor learning indicated that separated neurons are closer, there are higher correlated neuronal activities in control mice, but there

was no such relationship in LPS injected mice (Figure 12). To quantify this relationship, I measured slop between C.C. of paired neuronal Ca^{2+} responses and a distance of those two neurons in control mice and in LPS injected mice during motor learning (Figure 13) (Day 1: p = 0.012, day 5 : p = 0.010, day 10 : p = 0.050, Normal : n=5 mice, LPS : n=5 mice, Unpaired *t*-test). There was a significant difference of slop in control mice and in LPS injected mice on day1 and day5. These results suggest that local network activity is reduced in LPS injected mice. Finally, I measured an average of correlation coefficients of paired neurons within 100μm in control mice and in LPS injected mice in day1, day5, and day10 (Figure 14). There was a significant difference of correlation coefficients within 100μm in control mice and LPS injected mice during motor learning. These results suggest activated microglia disrupt the synchronicity of local neuronal network activity. (Control: n=5 mice, LPS : n=5 mice, Statistics: Wilcoxon rank-sum test, day 1: P = 0.015, day 5: P = 0.040, day 10: P = 0.0013).

In part 1, These data demonstrate that high correlation co-efficiency of neuronal activity at all stages promotes increasing motor learning performance. In addition, microglia play an important role in synchronizing network activity triggered by a motor learning task.

In part 2 : Microglia directly make contact with synapses to enhance synaptic activity.

To investigate whether microglial contact on synapse can induce a change of synaptic activity in awake mice, I established the technique of observing small and microscopic structure, spine, with its activity measuring Ca^{2+} elevation and microglial process motility simultaneously and continuously *in vivo* using two photon Ca^{2+} imaging in awake mice. I used three AAV constracts, AAV1 - CaMKII - Cre, AAV1 - Syn - FLEX - GCaMP6f, and AAV1 - CAG -FLEX - tdTomato into layer 5 primary motor cortex in Iba1-EGFP mice. This enabled us to visualize both microglia and synaptic activity in neuron simultaneously in awake state. There was less neuronal activity in anesthetic state, but in awake state physiological neuronal activity was detected (Figure15).

Microglial process dynamics and spine activity before, during, and after contact on spine on time-lapse recording were observed (Figure16A). I measured Ca^{2+} traces of spine contacted by microglia and dendrite and localized Ca^{2+} response only in spine; it means a single synaptic response (Figure16B). Finally, I found that frequency of local Ca^{2+} responses significantly increased in spines during microglial contacts (Figure16C) (n=10 from 6 mice, Statistics: Paired *t*-test). This finding indicates that microglia directly contact on synapse to enhance the synaptic activity.

Next, to investigate whether this is only a property of resting microglia or not, I activated microglia using LPS injection into peritoneal cavity and measured spine activity. Images of time-lapse recording showing dynamics of activated microglial process and dendritic spine activity in LPS injected mice were acquired (Figure17A). Activated microglial process was approaching the spine, then contacted it, and finally was retracting. In the same way, I measured Ca^{2+} traces of spine and dendrite obtained (Figure17B). I found frequency of local Ca^{2+} responses did not increase in spines during contact with activated microglia (Figure17C) (n=5 from 3 mice, Statistics: Paired *t*-test). These results suggest that microglial modification of synaptic activity differs between the physiological states and pathological states and that microglia directly contact on synapses to enhance the synaptic activity.

In addition, I focused on the neuronal activities on the spines which microglia contact because microglial contact could influence with neuronal soma activities. To investigate these activities, I measured frequency of Ca^{2+} response from back-propagation, which corresponds neuronal soma activity, significantly increased in dendrite during microglial contact on spine compared with in dendrite which lacks the microglia contact. This result suggests that microglial contact with spines increases neuronal activity (n=17 from 18 mice, P = 0.0029, Statistics: Paired *t*-test).

Discussion

In this study, I established techniques for observing neuronal activity during motor learning and for simultaneously observing microglial process and synaptic activity *in vivo* using two photon Ca^{2+} imaging in awake mice. My findings are that microglia play an important role in synchronizing network activity triggered by a motor learning task and in the physiological state microglia enhance synaptic activity through direct contact with synapses.

There are two possibilities of connection between the synchronicity of neuronal network activity during motor learning task and enhancement of synaptic activity through microglial direct contact with synapses. One is that microglia have multiple processes and could simultaneously contact on synapses of various neurons within their territory to promote the synchronization of local network activity during motor learning task. In the previous report, microglia are extending and retracting their processes in the physiological CNS (Nimmerjahn et al., 2005), but it is still unknown whether these constant motions are changed or not changed during motor learning. This study showed that to maintain the synchronization of neuronal activities during motor learning was important to conduct motor learning. Just before motor learning, mice which had less

synchronization of neuronal activities in primary motor cortex didn't promote motor learning task better than mice which had more synchronization of neuronal activities. These results suggest that to conduct motor learning microglia in the physiological state must serve important functions. Because in microglia depleted mice or LPS injected mice their microglia are not in the physiological state, it is difficult to maintain the synchronization of neuronal activities. In the previous report, microglia play an important physiological role in motor learning by promoting learning-related synapse formation (Parkhurst et al., 2013). That means that microglia can change the morphology of spine during motor learning task through BDNF signaling. On the other hand, in this study microglia can change the function of synapse during motor learning task.

The other possibility of connection between the synchronicity of neuronal network activity during motor learning task and enhancement of synaptic activity through microglial direct contact with synapses is to progress the synchronization of neuronal activities inside the subnetwork related to the motor learning. This mechanism has three steps. First is that microglia enhance synaptic activity through direct contact with synapses. Second, neural excitability in the local network can increase by multiple enhancement of synaptic activities. This means that it is easy to activate populational neuronal activities within the local subnetwork once one single neuron is excited. Third,

the movement of pulling the lever can promote at least one single neuron in the local subnetwork of primary motor cortex. This activation can cause populational neuronal activities within the local subnetwork in high neuronal excitability condition. As I showed in the figure14, even in the day1 an average of correlation coefficients of paired neuronal Ca^{2+} responses within 100μm on an imaging area in control mice was higher than in LPS injected mice in primary motor cortex. These results suggest that there are local networks which are changing contantly and microglial direct contact with synapses can raise the whole excitability within these local network because microglia are distributed uniformly and the enhancement of synaptic activities are occurred within single microglia own territory

I showed in figure18 that Microglial contact with spines increases neuronal activity. I measured frequency of Ca^{2+} response from back-propagation, which corresponds neuronal soma activity, significantly increased in dendrite during microglial contact on spine compared with in dendrite which lacks the microglia contact. This result suggests that microglia have multiple processes and microglial contact with spines increases neuronal activity. This result can be connection between the synchronicity of neuronal network activity during motor learning task and the enhancement of synaptic activity through microglial direct contact with synapses.

In this research, I established techniques for observing neuronal activity during motor learning and for simultaneously observing microglial process and synaptic activity in vivo using two photon Ca^{2+} imaging in awake mice. Microglia play an important role in synchronizing network activity triggered by a motor learning task. In the physiological state, microglia enhance synaptic activity through direct contact with synapse. Thus, in the physiological state, microglia have an important function to promote synchronizing network activity and can be envisaged as key neuro modulators in the brain.

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Figure 1

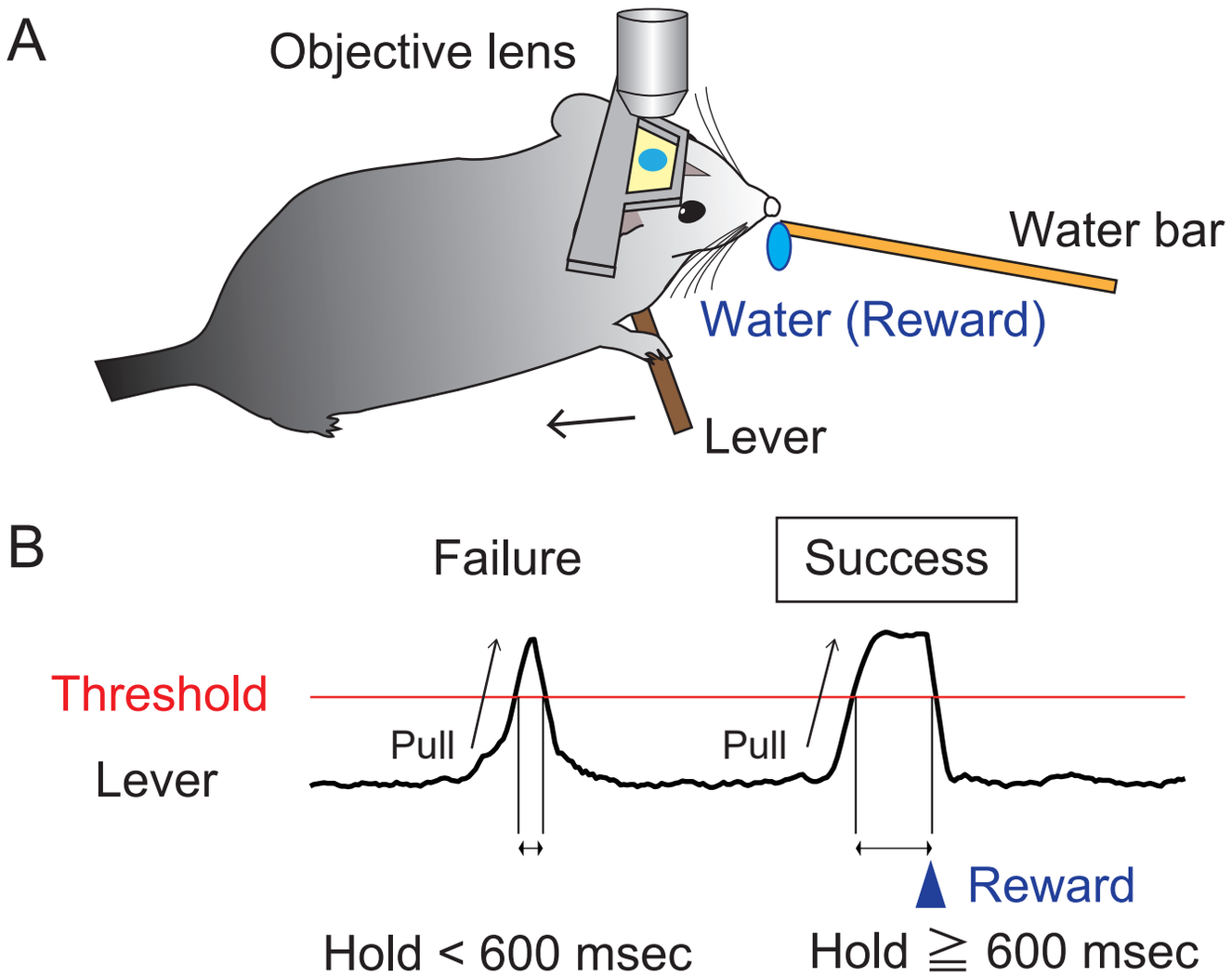

A
Objective lens
Water bar
Water (Reward)
Lever
B
Failure
Success
Threshold
Lever
Pull
Pull
Reward
Hold < 600 msec
Hold ≧ 600 msec
C
Head holder
Objective lens
Water bar
Lever
Armrest
Water (reward)

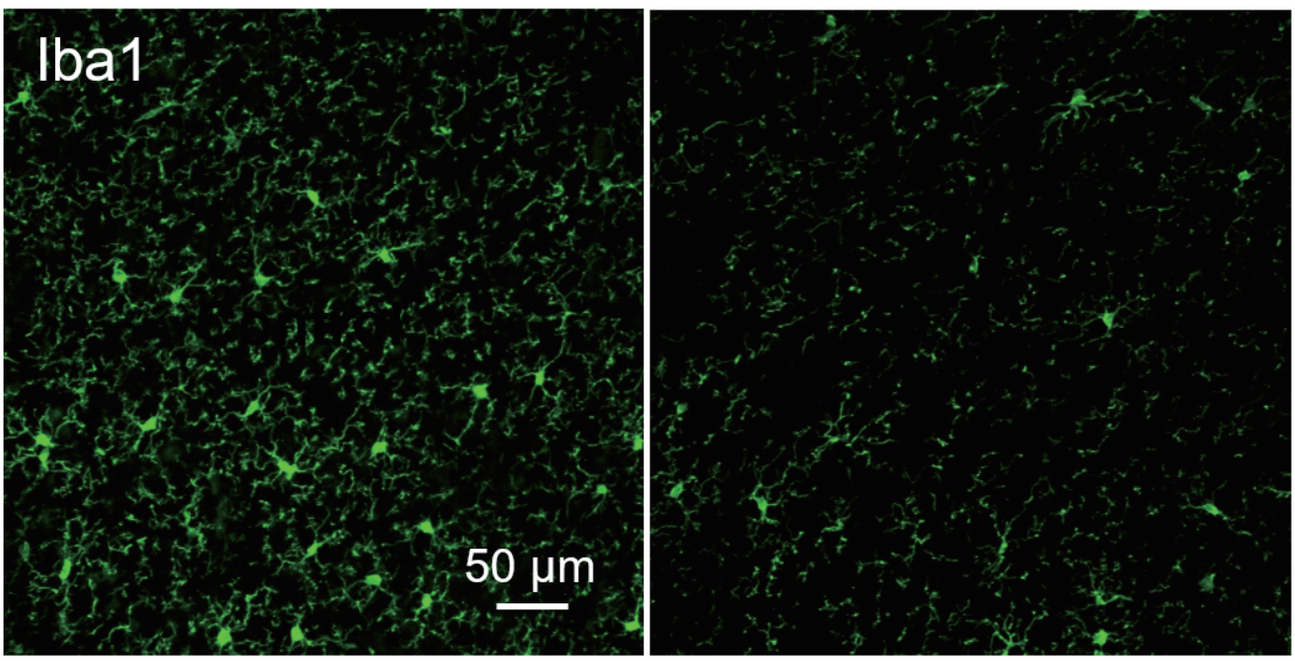
Figure 1

Motor learning task design

A. Behavioral schematic showing lever-pull motor learning task. To place objective lens on mouse head plate enables us to record two photon microscope image during motor learning task simultaneously.
B. Representative trace of lever movement in trial of failure (no reward) and success (reward). Black line shows lever movement trace; red line shows threshold of lever movement. Mouse receives water as a reward if mouse pulls the lever and keep it above a threshold for 600 msec. Blue arrowhead shows a timing of reward output from water bar.
C. Image of head-restrained mouse pulling a lever for water reward.

Figure 2

A


Iba1
50 μm
Dox on
Dox off

B

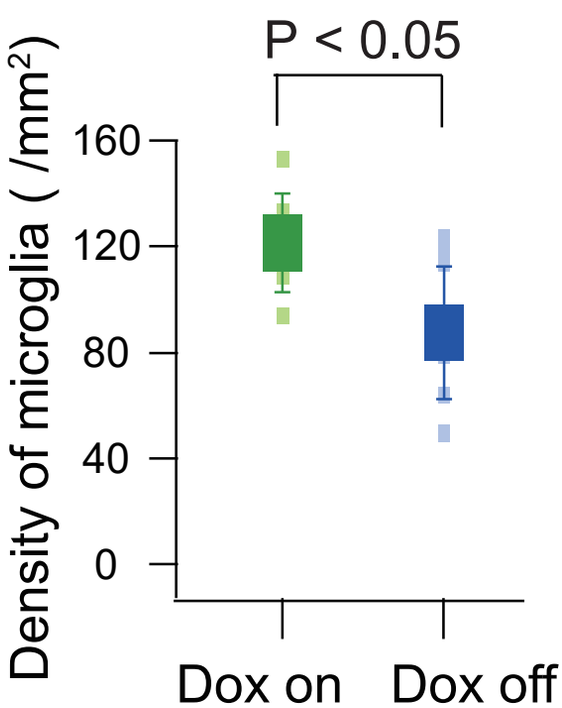

P < 0.05
Density of microglia (/mm2)
160
120
80
40
0
Dox on
Dox off

Figure 2

A. Representative images of immunohistochemical analysis of Iba1 expression in L2/3 of the primary motor cortex in Dox on mouse (control mouse; left) and Dox off mouse (microglia ablated mouse; right). Microglia are visualized using anti-Iba1 antibody.

B. Averaged density of microglia in Dox on mice and in Dox off mice, showing a significantly decrease in Dox off mice. *p = 0.0233 < 0.05, Wilcoxon rank-sum test (Dox on : n=3 mice, Dox off : n=3 mice, Statistics: P =0.0233 , Error bar: SEM)

Figure 3

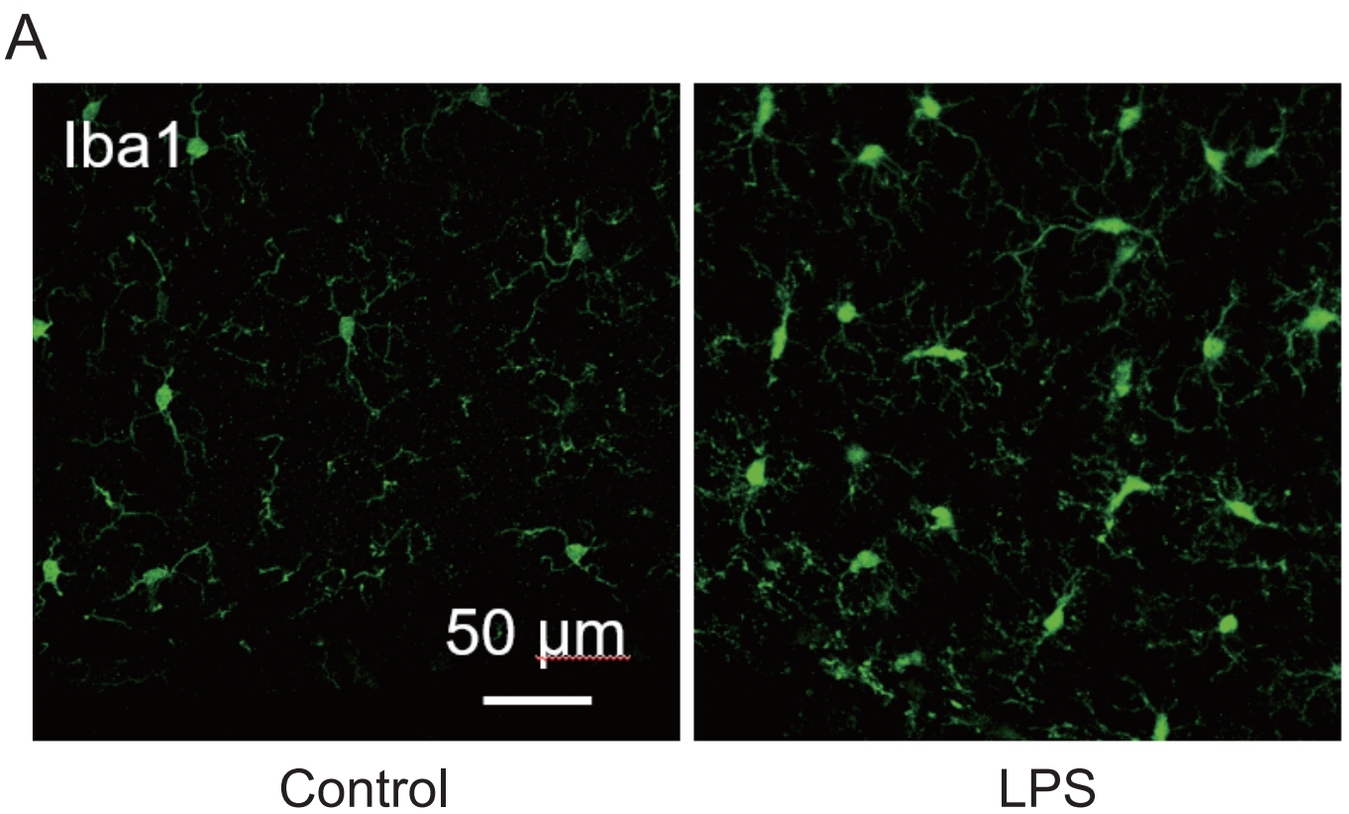

A
Iba1
50 µm
Control
LPS
B
*
Area of microglia cell body (µm²)
60
40
20
0
Control
LPS

Figure 3

A. Representative images of immunohistochemical analysis of Iba1 expression in L2/3 of the primary motor cortex in saline injected mouse (left) and in LPS injected mouse (right). Saline or LPS is injected into peritoneal cavity for four consecutive days.

B. Averaged area of microglia cell body in saline injected mice and in LPS injected mice. Microglial cell soma areas increase in LPS injected mice, which shows microglia activation. (Wilcoxon rank-sum test, control : n=3 mice, LPS : n=3 mice, P =0.036, error bar: SEM)

Figure 4

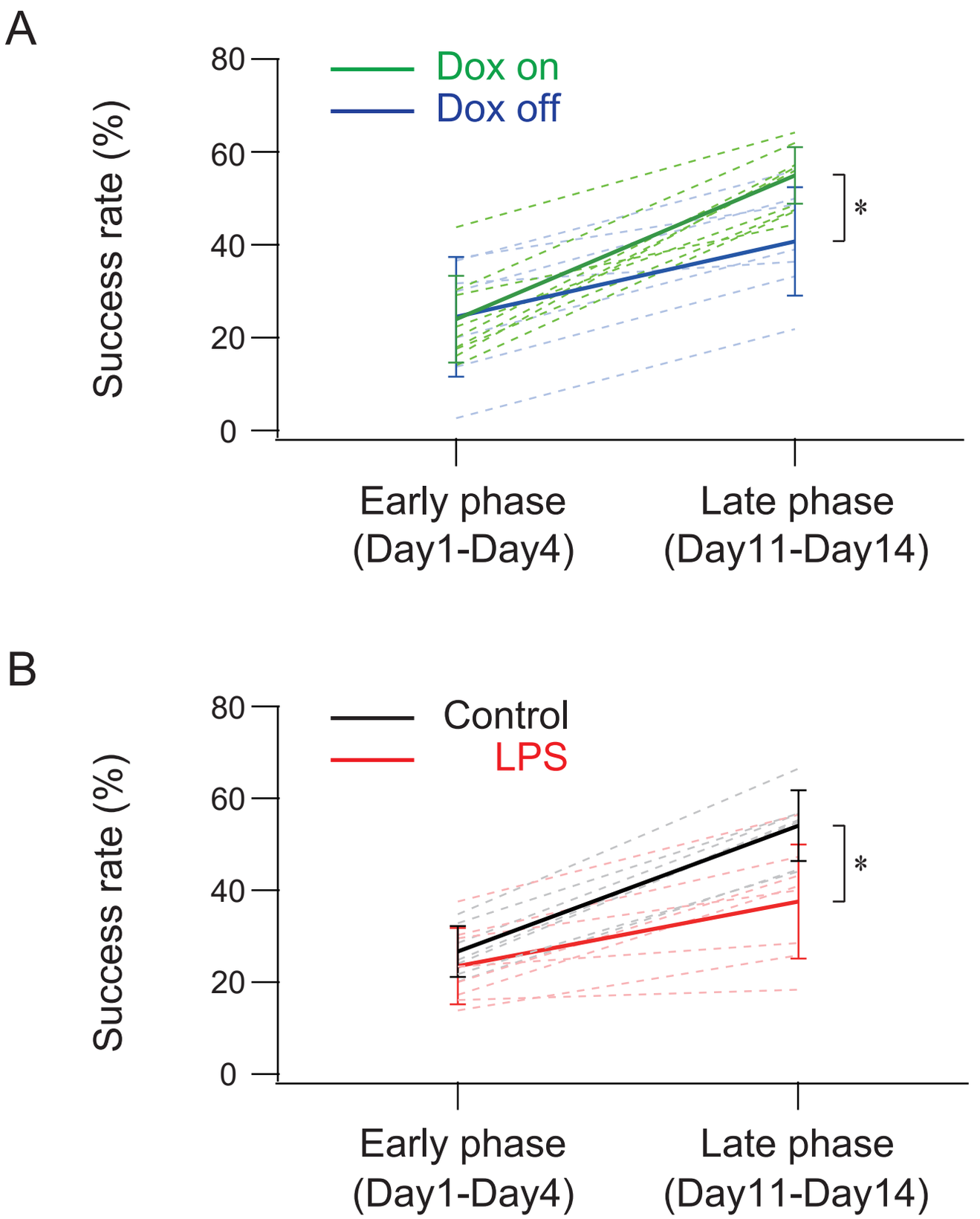

A
Success rate (%)
80
60
40
20
0
Dox on
Dox off
*
Early phase
(Day1-Day4)
Late phase
(Day11-Day14)
B
Success rate (%)
80
60
40
20
0
Control
LPS
*
Early phase
(Day1-Day4)
Late phase
(Day11-Day14)

Figure 4

A. Behavioral performance during motor learning in Dox on mice (green line) and Dox off mice (blue line) comparing success rate. Motor learning phase is divided into two phase, early phase and late phase. While there is no significant difference in early phase between Dox on and Dox off mice, there is significant difference in late phase between Dox on mice and Dox off mice. Thick green line represents the mean across Dox on mice. Thick blue line represents the mean across Dox off mice. Each thin green dotted line represents an individual Dox on mouse. Each thin blue dotted line represents an individual Dox off mouse. (Success rate in early phase; 24.0 ± 9.4 % (n = 9) and 24.5 ± 13 %, (n = 7; p = 0.99), Success rate in late phase; 54.9 ± 6.1 % (n = 9) and 40.7 ± 12 % (n = 7, p = 0.041, two-way ANOVA with post hoc Tukey's test)

B. Behavioral performance during motor learning in control mice (black line) and in LPS injected mice (red line) comparing success rate. While there is no significant difference in early phase between control mice and LPS injected mice, there is significant difference in late phase between them. Thick black line represents the mean across control mice. Thick red line represents the mean across LPS injected mice. Each thin gray dotted line represents an individual control mouse. Each thin red dotted line represents an individual LPS injected mouse. (Success rate in early phase; 26.7 ± 5.5 % in saline injected mice; n = 7, and 23.5 ± 8.3 % in LPS injected mice; n = 8, p = 0.90. Success rate in late phase; 54.1 ± 7.7 % in saline control mice, n = 7, and 37.6 ± 12 % in LPS injected mice, n = 8, p = 7.8×10^{-3}, two-way ANOVA with post hoc Tukey's test).

Figure 5 (Neuronal activity in Dox on mice and in Dox off mice)

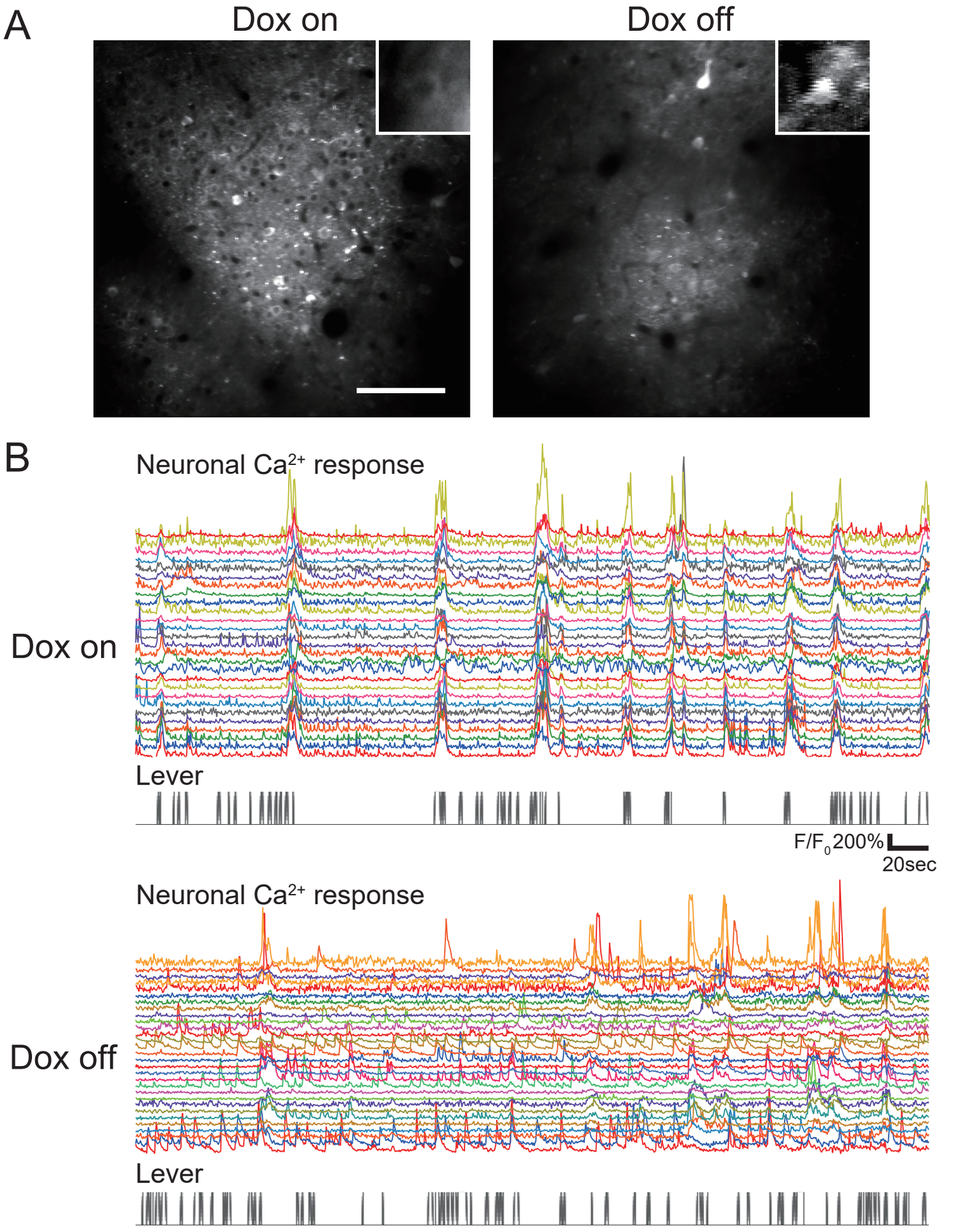


Figure 5

Neuronal activity in Dox on mice and in Dox off mice

A. Representative images of time-lapse recording of two photon Ca^{2+} imaging triggered by lever-pull task in layer2/3 of primary motor cortex in an awake mouse (Dox on mouse in left image and Dox off mouse in right mouse). Insets: magnified images of neuronal soma expressing GCaMP6f.

B. Representative traces of neuronal Ca^{2+} responses in layer2/3 of primary motor cortex and lever trajectory in Dox on mouse (top) and in Dox off mouse (bottom) during motor learning. Note that most of Ca^{2+} responses in Dox on mouse are associated with lever-pull movement, on the other hand, less associations in Dox off mouse.

Figure 6 (Synchronization rate in Dox on mice and Dox off mice)

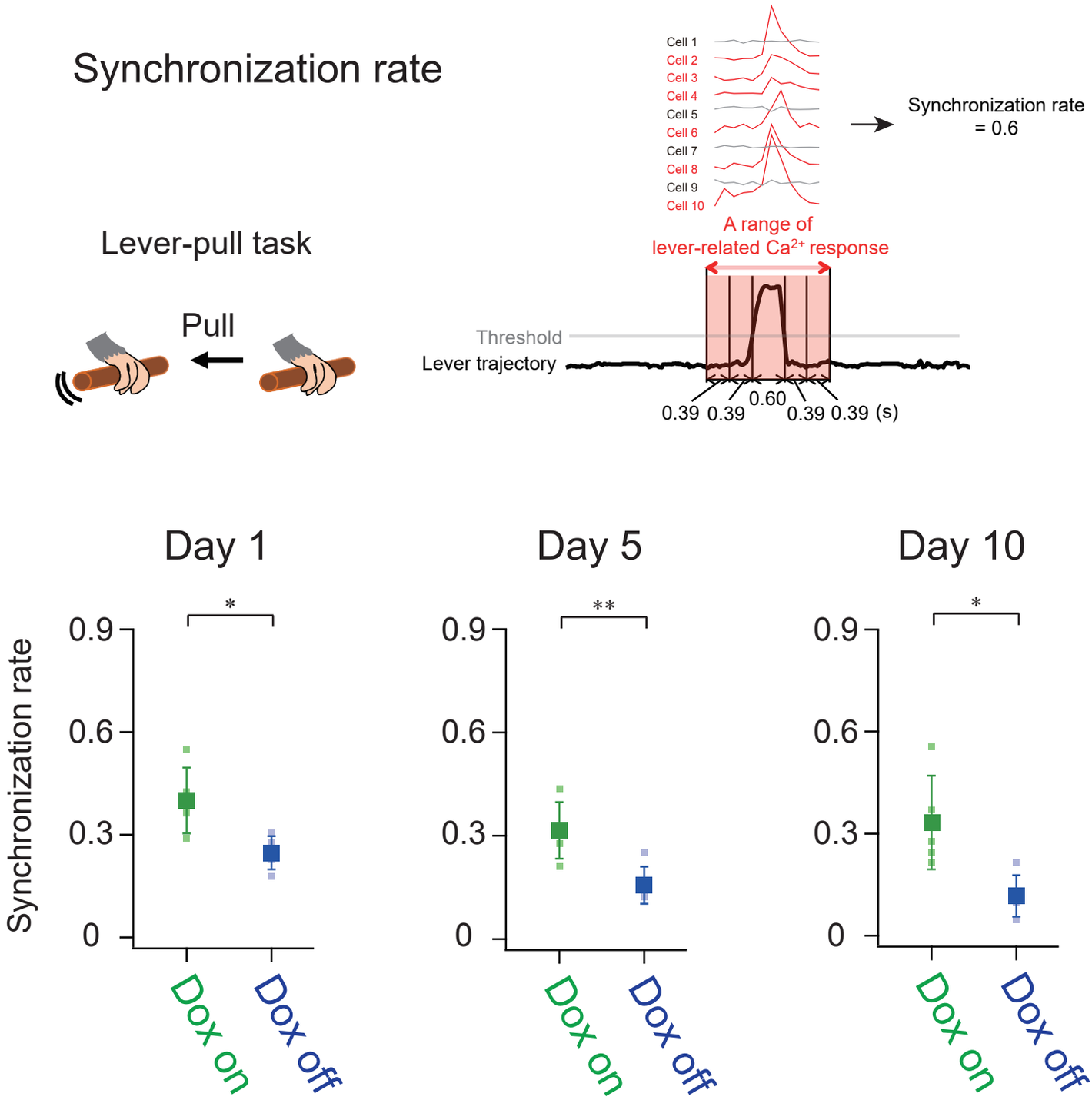


Figure 6

Synchronization rate in Dox on mice and Dox off mice

A. Synchronization rate in Dox on mice and in Dox off mice during motor learning in day1, day5, and day10. Note that Dox off mice show less synchronous activity in response to (associated with) lever-pull movement compared with Dox on mice during motor learning.

Figure 7 (Plot of correlation coefficients and a distance in Dox on mice and Dox off mice)

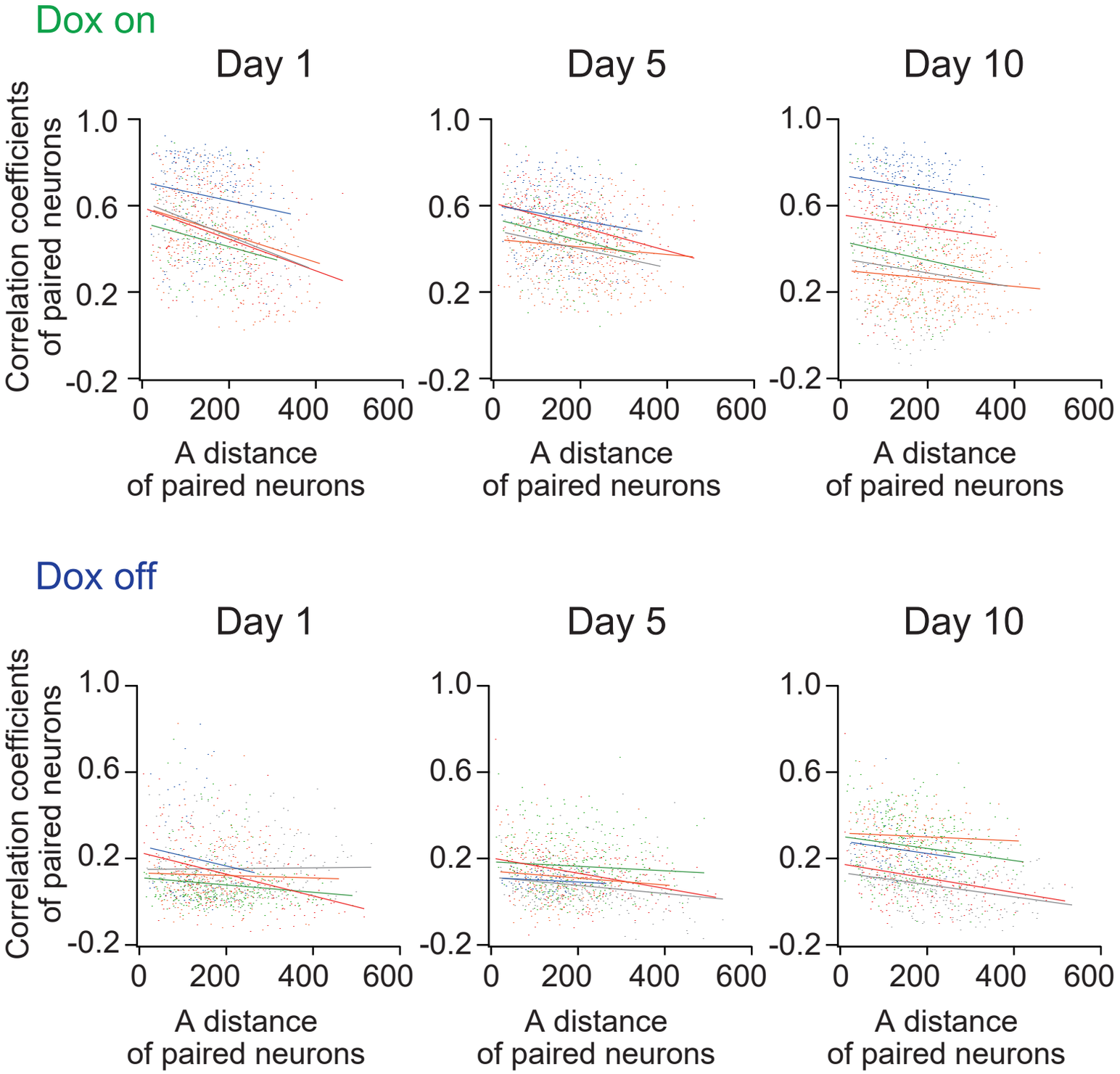


Figure 7

Correlation coefficients and distance of paired neurons in Dox on mice and in Dox off mice.

Scatter plots of correlation coefficients of paired neuronal Ca^{2+} responses (Y axis) against a distance separating those two neurons (X axis) in Dox on mice and Dox off mice during motor learning. Each line indicates linear fitting of those scatter plots from each mice. Note that separated neurons are closer, there are higher correlated neuronal activities in Dox on mice, but there is no such relationship in Dox off injected mice.

Figure 8 (Slope in Dox on mice and Dox off mice)

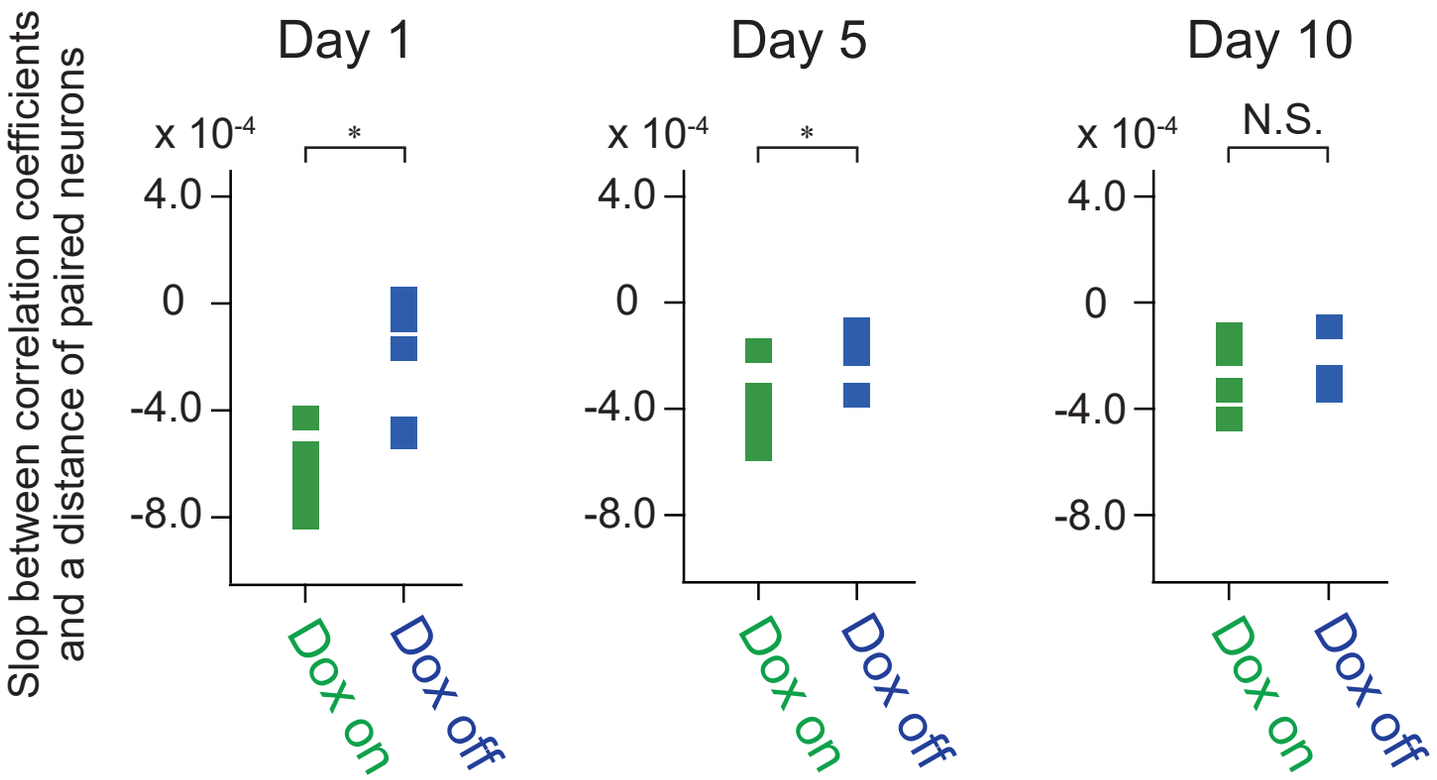


Figure 8

Slop between correlation coefficients and a distance of paired neurons in Dox on mice and Dox off mice.

Slop between correlation coefficients of paired neuronal Ca^{2+} responses and a distance of those two neurons in Dox on mice and in Dox off mice during motor learning. Note that there is a significant difference of slop in Dox on mice and in Dox off mice on day1 and day5. It suggests that microglia within their territory could modify the spatial pattern of neuronal activity. (day 1: $p = 0.017$, day 5 : $p = 0.026$, day 10 : $p = 0.72$, Dox on : n=5 mice, Dox off : n=5 mice, Unpaired t-test)

Figure 9 (Correlation coefficients in Dox on and Dox off)

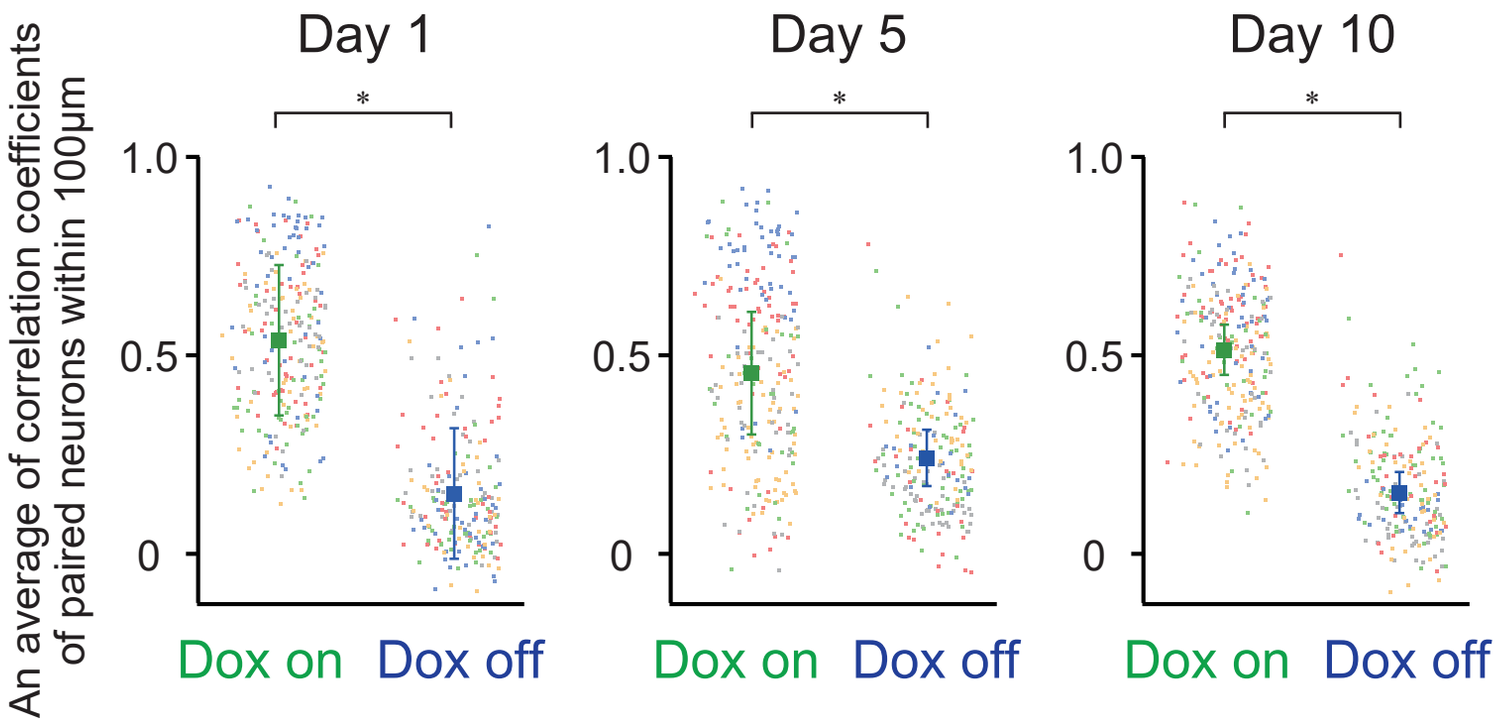


Figure 9

Correlation coefficients in Dox on mice and in Dox off mice.

An average of correlation coefficients of paired neurons within 100μm in Dox on mice and in Dox off mice in day1, day5, and day10. Note that there is a significant difference of correlation coefficients within 100μm in Dox on mice and Dox off mice during motor learning. It suggests microglia within their territory could promote the synchronicity of local neuronal network activity (C.C.: Correlation co-efficiency, Dox on: n=5 mice, Dox off : n=5 mice, Statistics: Wilcoxon rank-sum test, day 1: $P = 0.000027$, day 5: $P = 0.000012$, day 10: $P = 0.030$).

Figure 10 (Neuronal activity in control and in LPS injected mice)

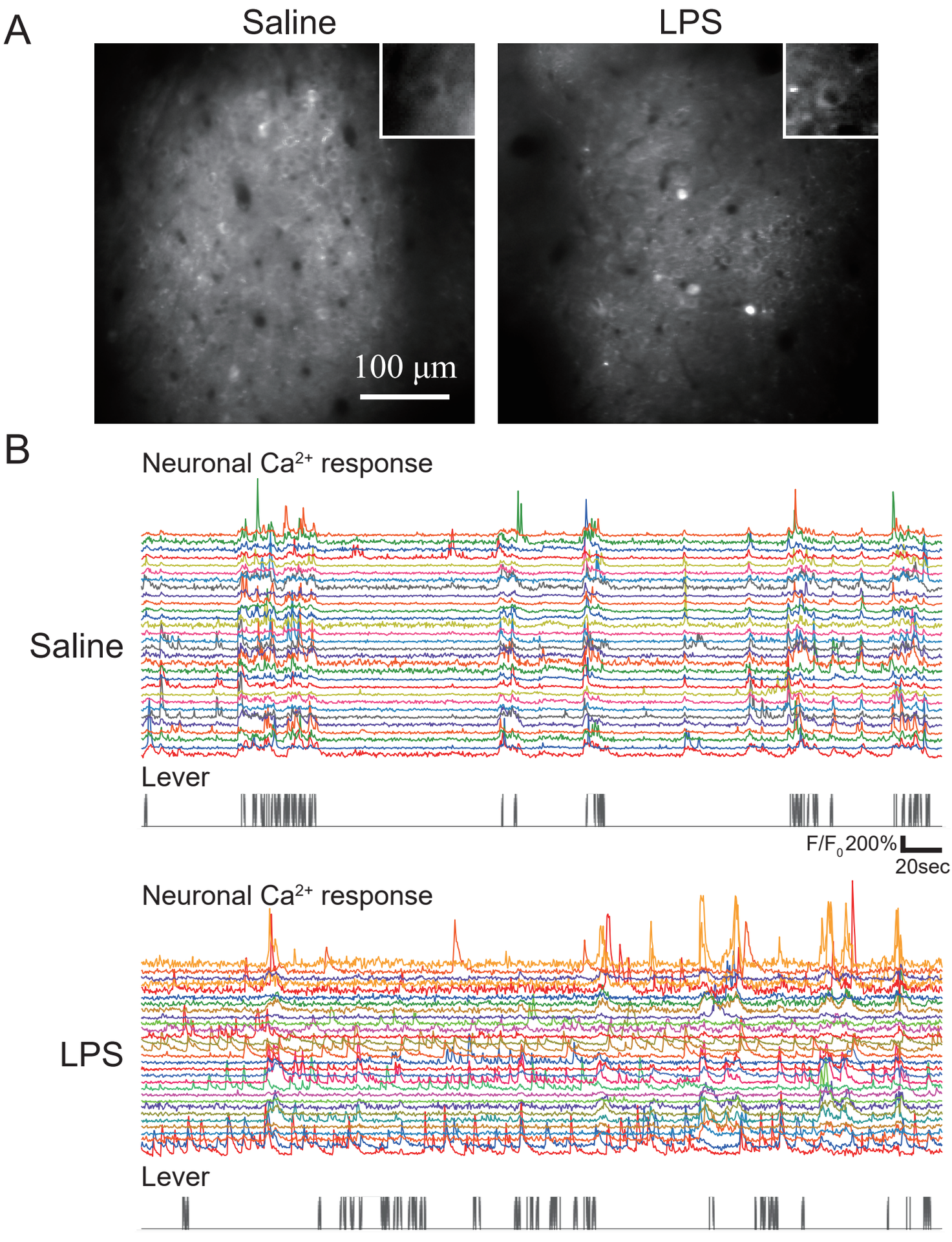


Figure 10

Neuronal activity in control mice and in LPS injected mice

A. Representative images of time-lapse recording of two photon Ca^{2+} imaging triggered by lever-pull task in layer2/3 of primary motor cortex in an awake mouse (saline injected mouse in left image and LPS injected mouse in right mouse). Insets: magnified images of neuronal soma expressing GCaMP6f.

B. Representative traces of neuronal Ca^{2+} responses in layer2/3 of primary motor cortex and lever trajectory in saline injected mouse (top) and LPS injected mouse (bottom) during motor learning. Note that most of Ca^{2+} responses in saline mouse are associated with lever-pull movement, on the other hand, less associations in LPS-injected mouse.

Figure 11 (Synchronization rate in control mice and LPS mice)

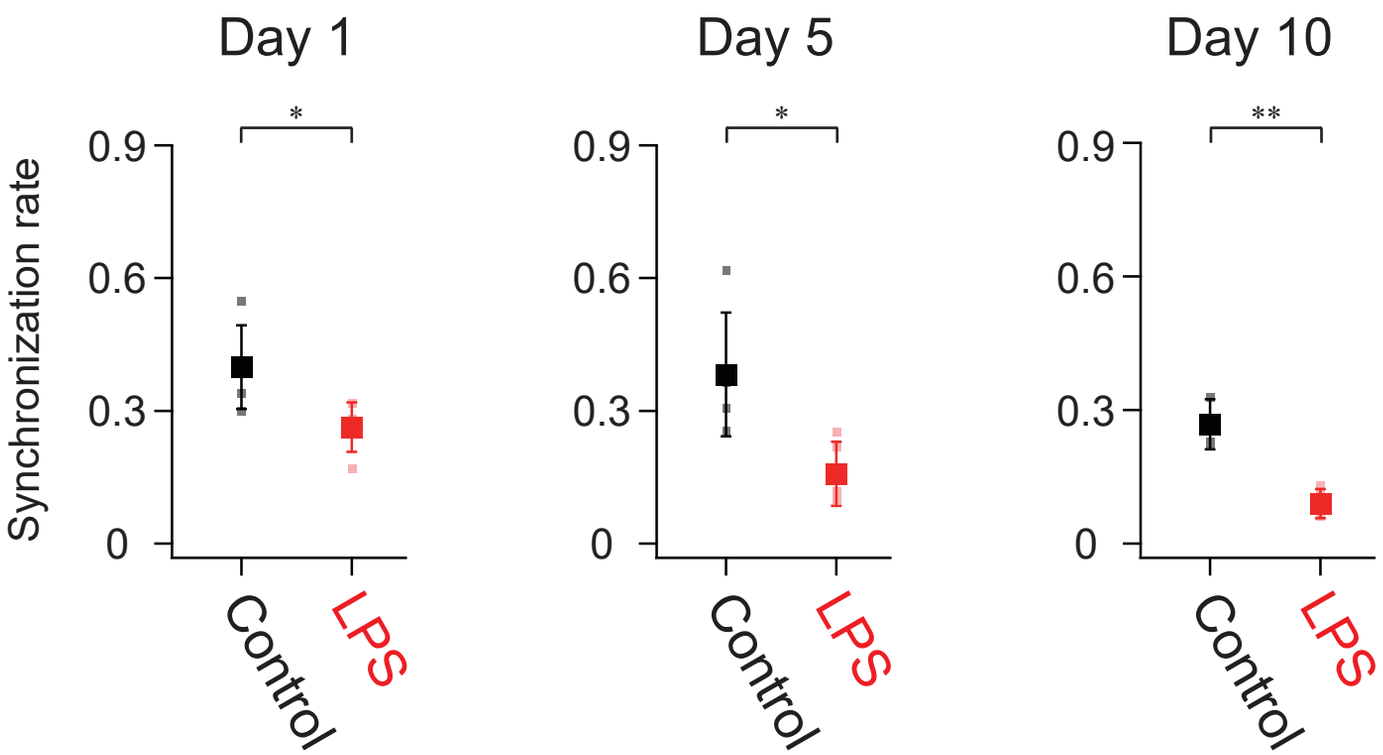


Figure 11

Synchronization rate in control mice and in LPS mice

Synchronization rate in control mice and in LPS mice during motor learning in day1, day5, and day10. Synchronization rate is defined as an average of fraction of total neurons responding with Ca^{2+} responses triggered by each lever-pull movement during imaging. Note that LPS mice show less synchronous activity in response to (associated with) lever-pull movement compared with control mice during motor learning (day 1: $p = 0.031$, day 5 : $p = 0.019$, day 10 : $p = 0.00064$, Normal : n=5 mice, LPS : n=5 mice, Unpaired t-test)

Figure 12 (Plot of correlation coefficients and a distance in control mice and LPS mice)

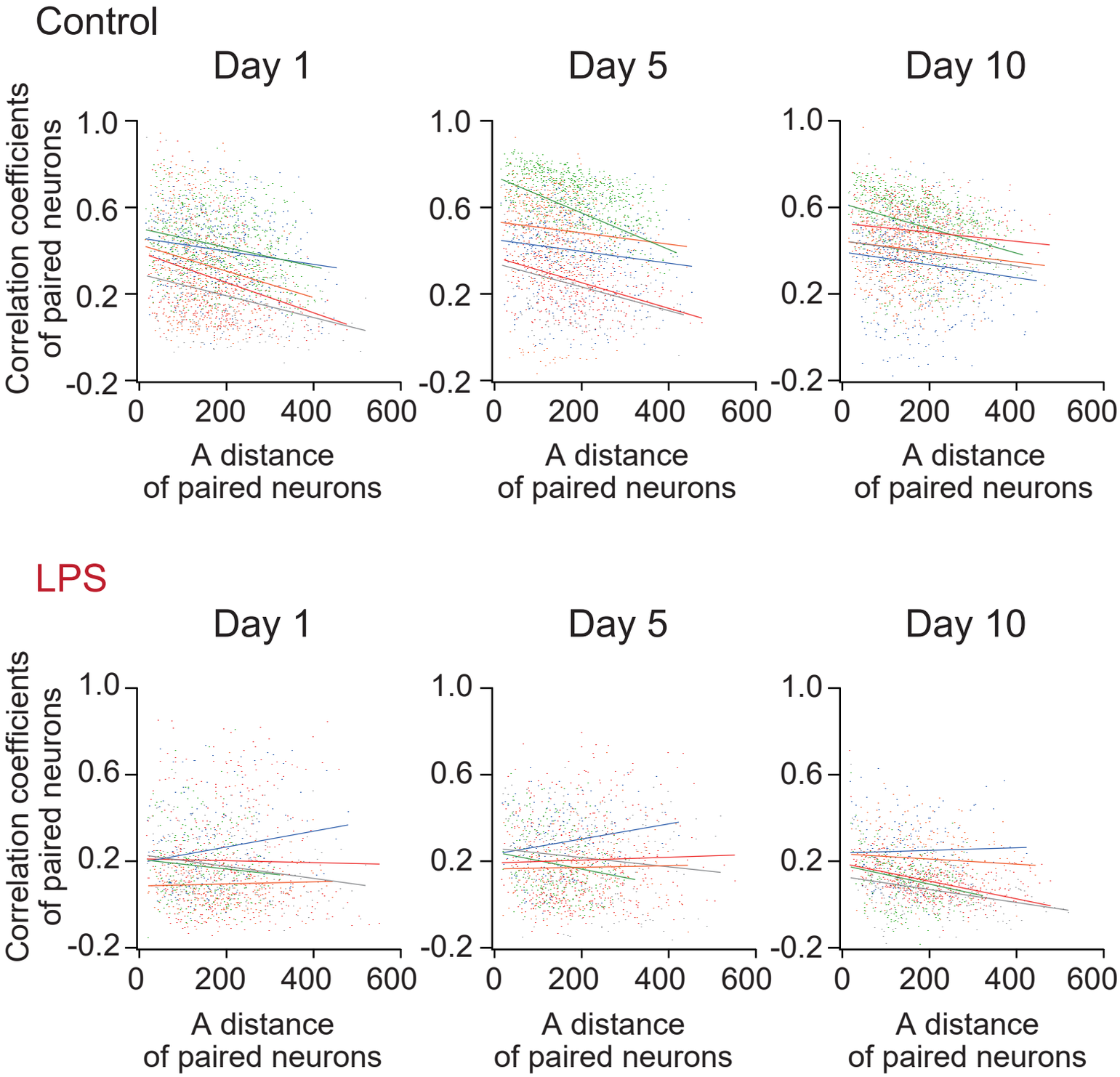


Figure 12

Correlation coefficients and distance of paired neurons in control mice and in LPS mice during motor learning.

Scatter plots of correlation coefficients of paired neuronal Ca^{2+} responses (Y axis) against a distance separating those two neurons (X axis) in control mice and LPS injected mice during motor learning. Each line indicates linear fitting of those scatter plots from each mice. Note that separated neurons are closer, there are higher correlated neuronal activities in control mice, but there is no such relationship in LPS injected mice.

Figure 13 (Slope in control mice and LPS mice)

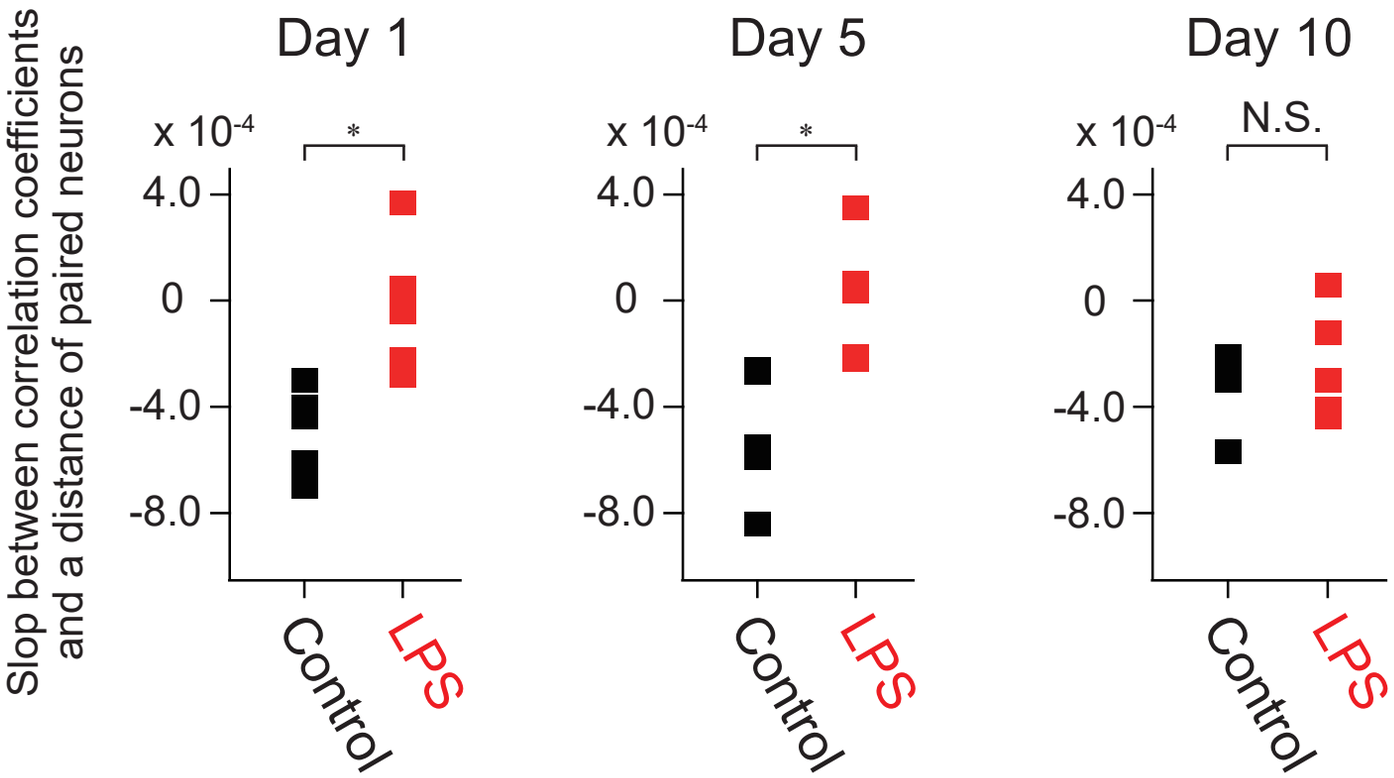


Figure 13

Slop between correlation coefficients and a distance of paired neurons in control mice and in LPS mice.

Slop between correlation coefficients of paired neuronal Ca^{2+} responses and a distance of those two neurons in control mice and in LPS mice during motor learning. Note that there is a significant difference of slop in saline injected mice and in LPS mice on day1 and day5. It suggests that microglia in physiological state could modify the spatial pattern of neuronal activity. (day 1: p = 0.012, day 5 : p = 0.010, day 10 : p = 0.050, Normal : n=5 mice, LPS : n=5 mice, Unpaired *t*-test)

Figure 14 (Correlation coefficients in control mice and LPS mice)

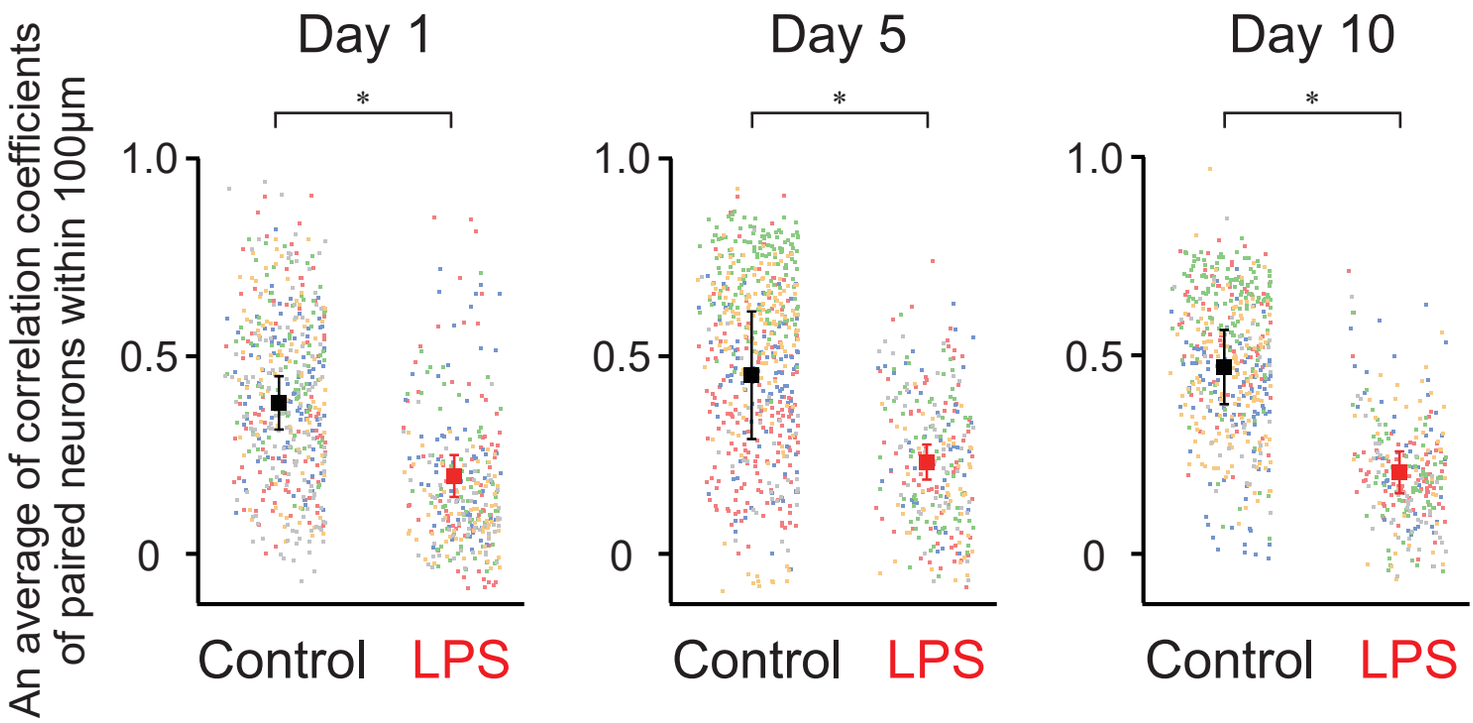


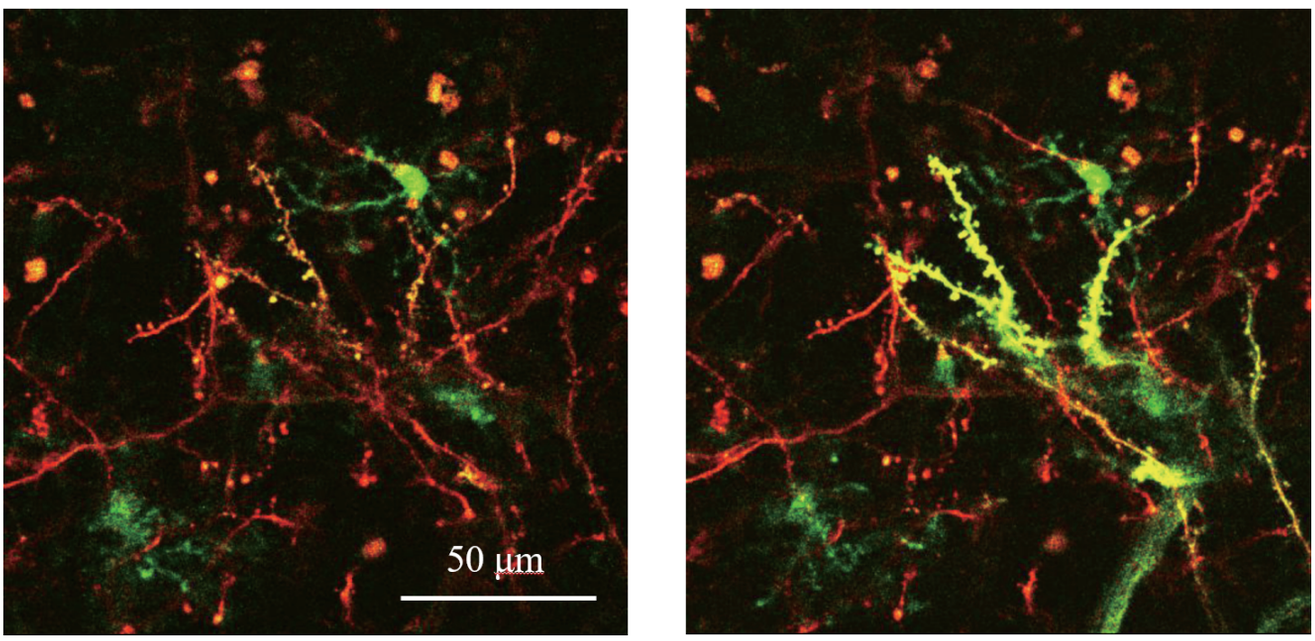
Figure 14

Correlation coefficients in control mice and in LPS mice.

An average of correlation coefficients of paired neuronal Ca^{2+} responses within 100μm on an imaging area in control mice and in LPS mice in day1, day5, and day10. Note that there is a significant difference of correlation coefficients within 100μm in control mice and LPS mice during motor learning. It suggests activated microglia disrupt the synchronicity of local neuronal network activity. (C.C.: Correlation co-efficiency, Control: n=5 mice, LPS : n=5 mice, Statistics: Wilcoxon rank-sum test, day 1: P = 0.015, day 5: P = 0.040, day 10: P = 0.0013)

Figure 15 (Neuronal activity in anesthetic mice and awake mice)

A



Anesthetic state

Awake state

Figure 15

Neuronal activity in anesthetic mice and in awake mice

Representative images of microglia and neuronal activity in layer1 of primary motor cortex. We use three AAV constracts, AAV1 - CaMKII - Cre, AAV1 - Syn - FLEX - GCaMP6f, and AAV1 - CAG -FLEX - tdTomato into layer 5 primary motor cortex in Iba1-EGFP mouse. This enabled us to visualize both microglia and synaptic activity in neuron simultaneously in awake state. Note that there is less neuronal activity in anesthetic state, but in awake state physiological neuronal activity is detected. We established the technique of observing small, tiny, and microscopic structure, spine, with its activity measuring Ca^{2+} elevation and microglial process motility simultaneously and continuously *in vivo* using two photon Ca^{2+} imaging in awake mice.

Figure 16 (Microglia contact on synapse to enhance synaptic activity)

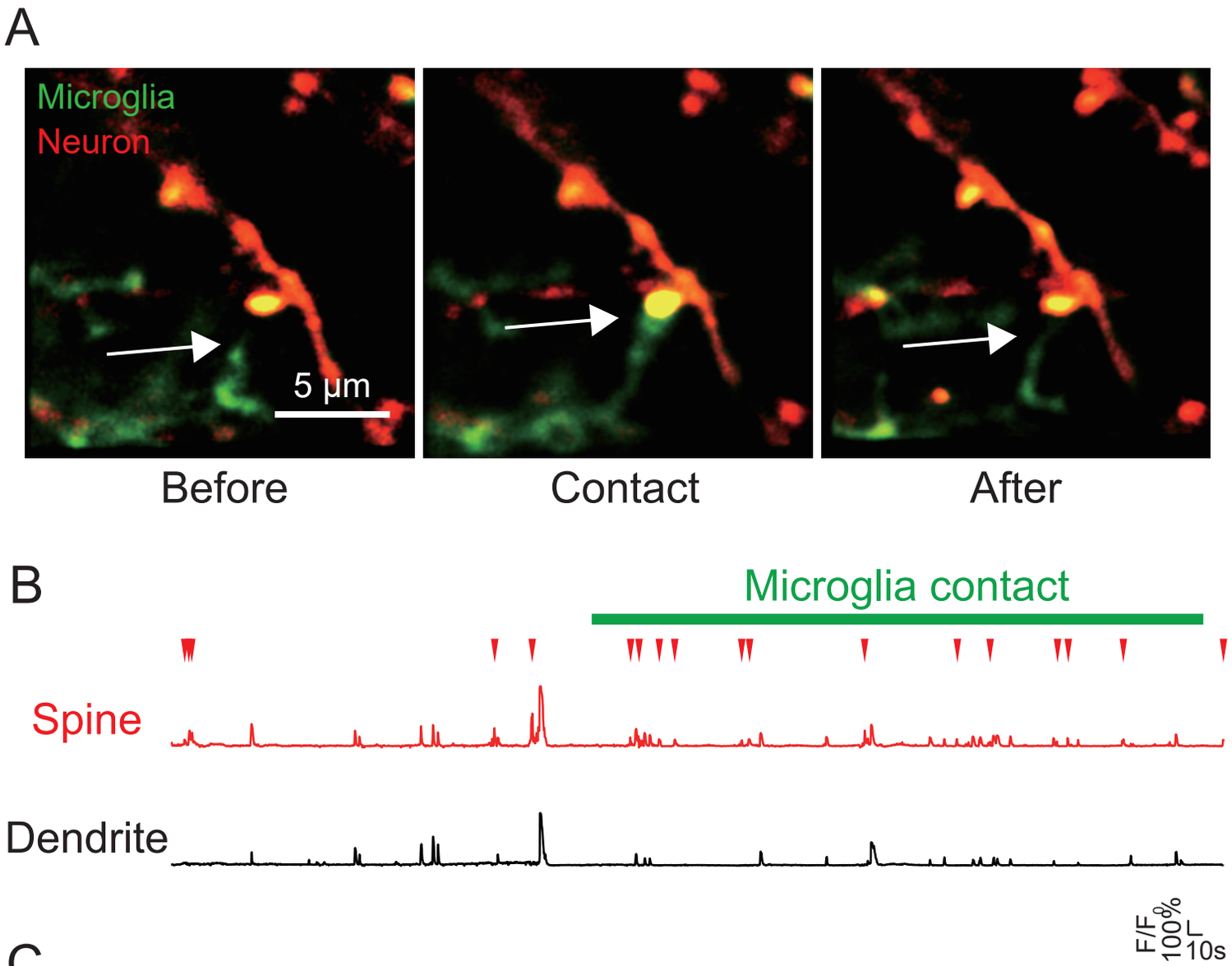


Figure 16

A. Representative images of time-lapse recording showing microglial process dynamics and spine activity before, during, and after contact on spine. White arrow shows a tip of microglial process.
B. Representative Ca^{2+} traces of spine contacted by microglia and dendrite. Red arrowheads show localized Ca^{2+} response only in spine; it is a single synaptic response. A green bar shows microglial contact time.
C. Frequency of local Ca^{2+} responses significantly increases in spines during microglial contacts. This finding indicates that microglia directly contact on synapse to enhance the synaptic activity. (n=10 from 6 mice, Statistics: Paired *t*-test)

Figure 17 (Activated microglia do not enhance synaptic activity)

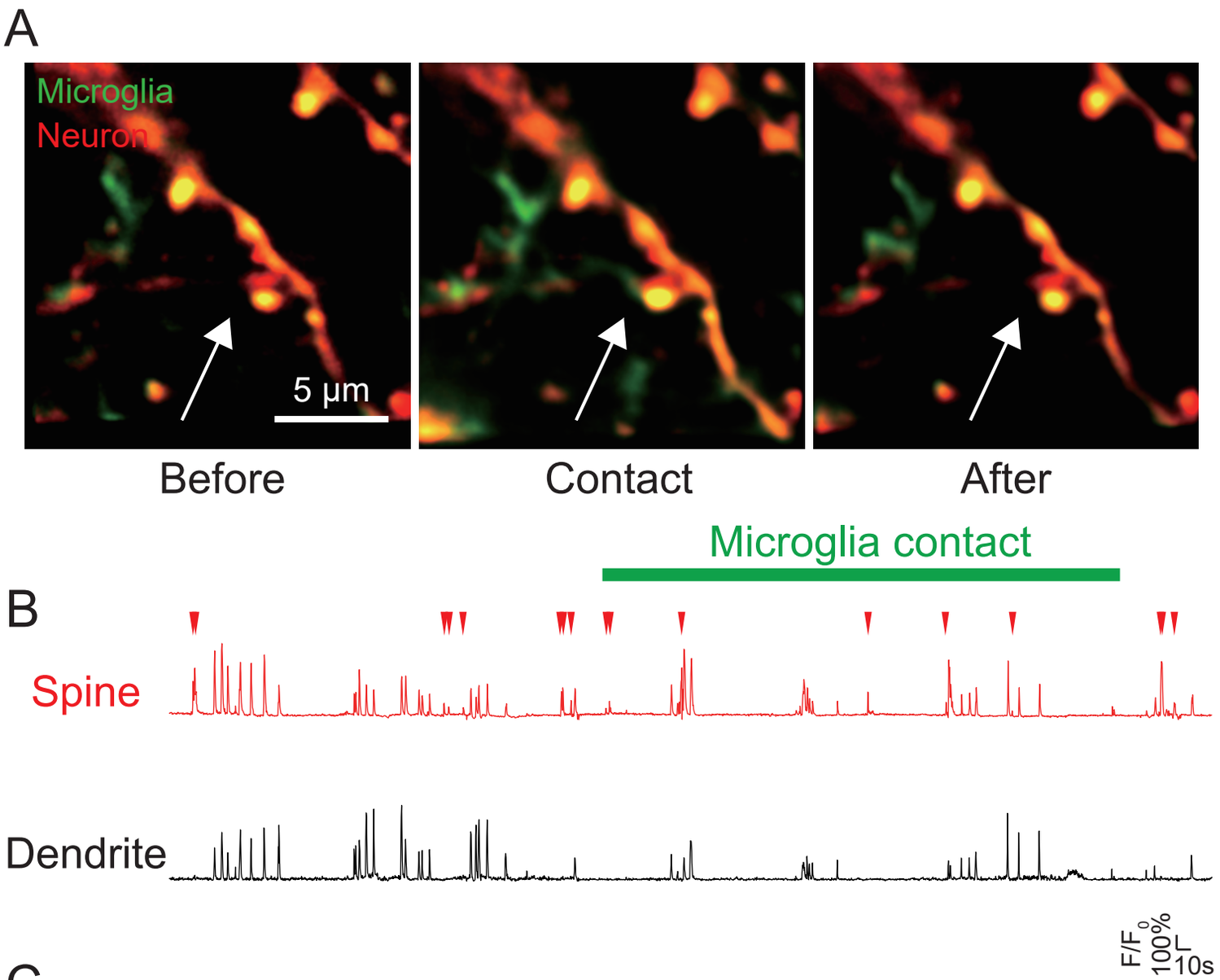


Figure 17

A. Representative images of time-lapse recording showing dynamics of activated microglial process and dendritic spine activity. Activated microglial process is approaching the spine (Before), then contacts it (Contact), finally is retracting (After). White arrow shows a tip of microglial process.

B. Representative Ca^{2+} traces of spine and dendrite obtained in A. Red arrowheads show localized Ca^{2+} response only in spine; it is single synaptic response. A green horizontal bar shows the microglial contact time.

C. Frequency of local Ca^{2+} responses do not increase in spines during contact with activated microglia. (n=5 from 3 mice, Statistics: Paired *t*-test)

Figure 18 (Microglial contact with spines increases neuronal activity)

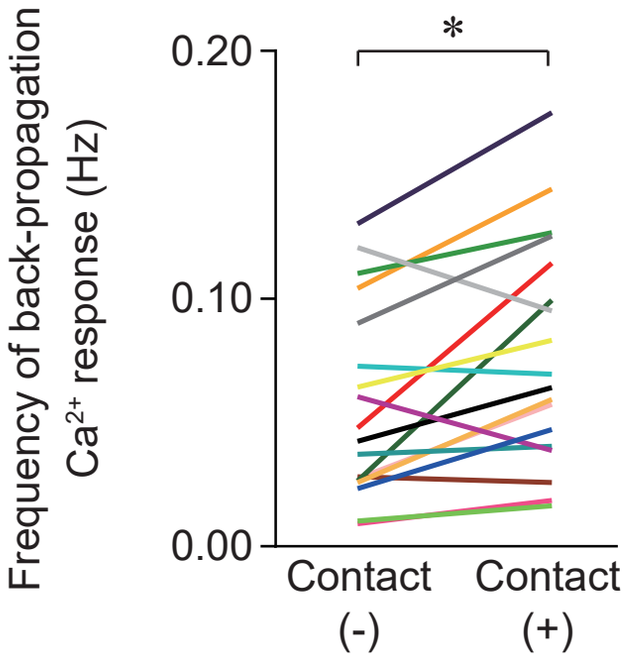


Figure 18

Microglial contact with spines increases neuronal activity

Frequency of Ca^{2+} response from back-propagation, which corresponds neuronal soma activity, significantly increases in dendrite during microglial contact on spine compared with in dendrite which lacks the microglia contact. It suggests microglial contact with spines increases neuronal activity. (n=17 from 18 mice, P = 0.0029, Statistics: Paired *t*-test)