In vivo neuronal and astrocytic activation in somatosensory cortex by acupuncture stimuli

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Abstract

Acupuncture is a medical treatment that has been widely practiced in China for over 3000 years, yet the neural mechanisms of acupuncture are not fully understood. We hypothesized that neurons and astrocytes act independently and synergistically under acupuncture stimulation. To investigate this, we used two-photon in vivo calcium recording to observe the effects of acupuncture stimulation at ST36 (Zusanli) in mice. Acupuncture stimulation in peripheral acupoints potentiated calcium signals of pyramidal neurons and astrocytes in the somatosensory cortex and resulted in late-onset calcium transients in astrocytes. Chemogenetic inhibition of neurons augmented the astrocytic activity. These findings suggest that acupuncture activates neuronal and astrocytic activity in the somatosensory cortex and provide evidence for the involvement of both neurons and astrocytes in acupuncture treatment.

Key Words: acupuncture; astrocyte; chemogenetic; neuron; N-methyl-D-aspartate receptor; somatosensory cortex; transient receptor potential A1; two-photon in vivo imaging

Introduction

Acupuncture is a technique that has been practiced for millennia (Linde et al., 2005; Witt et al., 2005; Haake et al., 2007; Walker et al., 2010). The clinical benefit of acupuncture for pain management has been recognized by accumulating clinical evidence (Witt et al., 2005; Hinman et al., 2014; Jensen and Finnerup, 2014; Scarborough and Smith, 2018). Although the practice is currently used worldwide, its mechanism is unknown. Previous studies in mouse models have demonstrated that the release of neuromodulators adenosine triphosphate and dopamine, mediated by ascending and vagus nerves, plays a role in anti-nociceptive and anti-inflammatory actions of acupuncture (Goldman et al., 2010; Torres-Rosas et al., 2014; Park and Namgung, 2018). It remains unknown whether neurons or astrocytes play the central role in the neural mechanism of acupuncture, or whether they work together to achieve the therapeutic effect.

The primary somatosensory cortex (S1) processes both spatial and graded inputs from the spinal cord (Liu et al., 2018). Human imaging data has suggested that S1 plays a role in the top-down modulation of nociceptive inputs from the spinal cord (Liu et al., 2018). Human imaging data has suggested that S1 plays a role in the top-down modulation of nociceptive

Materials and Methods

Animals

Male C57BL/6J mice (n = 60, 4 weeks old, specific pathogen-free) were purchased from Guangdong Medical Laboratory Animal Center (license No. SCXK(2019)0003). Mice were group-housed under a 12-hour light/dark cycle and were provided with water and food ad libitum. All experimental protocols were preapproved by the Ethics Committee of Experimental Animals of Jinan University (approval No. 20193939) on February 27, 2019 in accordance with Institutional Animal Care and Use Committee guidelines for animal research. All experiments were designed and reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020).

Stereotaxic viral injection

In this experiment, the following adeno-associated virus (AAV) vectors were administered: AAV-hsyn-GCaMP6s (150 nL per site without dilution, titer: > 1 × 1010 genome copies per mL) for neuronal calcium activity recording; AAV-gfaABC1D-1ck-GaMMP6f (250 nL per site without dilution, titer: 1.2 × 1011 genome copies per mL) for astrocytic calcium activity recording, and a 2:1 mixture of AAV-gfaABC1D-1ck-GaMMP6f and AAV-CaMKIIa-hM4D(Gi)-mCherry.
In vivo two-photon imaging

In vivo two-photon imaging was performed as previously described (Holm et al., 2012). To record calcium activity from the somatosensory cortex of each mouse, the mouse head was fixed onto a solid metal plate using a customized scaffold that was fixed on the skull using dental cements. S1 was localized (0.5 mm anterior and 1.5 mm lateral to the Bregma) using a stereotaxic apparatus (RWD). All the scalp and skull tissues were removed to create the imaging window (2 mm radius), which was covered with a glass coverslip. The imaging session was performed at 24 hours after the craniotomy surgery. The mouse received acupuncture needle stimulation (as described below) under a two-photon microscope (Zeiss, Jena, Germany, LSM780) with a 20x water immersion objective using a 920 nm laser for green fluorescent-calcium proteins (GCaMPs) excitation. The calcium signals were captured from neurons and astrocytes in layer V (500 to 600 μm below the pia) using a frame rate of 2 Hz with 4x digital zoom, 0.75 μm interval, 512 × 512 pixels. Acquired images were corrected for spatial distortions using the TurboReg package of ImageJ v1.52a (National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012). Time-series images were processed in ImageJ (Figure 1A and B). The calcium activity of each neuron was noted by manually delineating regions of interest, from which the mean intensity of calcium values (F) was extracted. Data were then normalized as ΔF/F₀, with the calculation (F – F₀)/F₀, where F₀ (basal value) was the averaged F values during the first 30 seconds of each recording session. A calcium transient was defined as when the ΔF/F₀ value exceeded the baseline level by at least three folds of standard deviation of population ΔF/F₀. The total integrated calcium was calculated as the time-integration of all ΔF/F₀ values during the recording period. In some recording sessions, clozapine-N-oxide (CNO, Cambridge, MA, USA, Cat# 266011, RRID: AB_266011) or HC030031 (A1 cation channel inhibitor) was injected at 30 minutes before Ca²⁺ imaging (and acupuncture stimulation). MK801 (N-methyl-D-aspartate receptor (NMDAR) antagonist; 0.1 mg/kg, MedChem Express, Princeton, NJ, USA) or HC030031 (A1 cation channel receptor antagonist) was injected at 30 minutes before Ca²⁺ imaging (and acupuncture stimulation). CNO and MK801 were dissolved directly onto the dura matter via the transcranial imaging window.

Manual acupuncture stimulation

Acupuncture stimulation was performed during all calcium imaging sessions. We chose ST36 (Zusanli) as the major stimulation acupoint as it is widely used in relieving neuropathic pain (Kim et al., 2018). ST36 is located 4 mm below and 1–2 mm lateral to the midpoint of the knee in mice. A second acupoint, GB34 (Yanglingquan) located at the depression anterior and inferior to the fibular head was also used. The non-acupoint control site was located 2 mm outside of the ST36. An acupuncture needle (0.35 mm × 25 mm, Wkato, Suzhou, China) was slowly inserted vertically into the acupoint with a depth of 2 mm. Acute acupuncture recording sessions lasted for 3 minutes, during which three consecutive acupuncture sessions (20-second duration for each, with the needle twisted twice per second) were applied with a 30-second interval between the two sessions. For chronic recordings, 20-minute persistent needle stimulation was performed.

Immunofluorescence staining

After calcium imaging, mice were intraperitoneally anesthetized using 1.25% Avertin and perfused with 4% paraformaldehyde. The whole brain tissue was immersed in 4% paraformaldehyde overnight, and then dehydrated with 30% sucrose. A glass tissue was sectioned into coronal slices (40 μm thickness) using a sliding microtome (Leica, Wetzlar, Germany). Brain slices were washed in phosphate buffered saline and were blocked for 2 hours in 2% bovine serum albumin. The primary antibodies (mouse anti-NeuN, 1:500, Synaptic Systems, Goettingen, Germany, Cat# 266011; rabbit anti-mouse Olig2, 1:500, Millipore, Temecula, CA, USA, Cat# AB58620, RRID: AB_19137; mouse anti-mouse Parvalbumin (PV), 1:500, Millipore, Cat# AB2117401; rabbit anti-mouse GABA, 1:500, ABCAM, Cat# AB109186, RRID: AB_10861310; rabbit anti-mouse c-fos, 1:1000, Cell Signaling Technologies, Inc., Danvers, MA, USA, Cat# 2250S, RRID: AB_2250S) in 0.2% Triton X-100 in blocking solution were applied to the slices and incubated at 4°C for 48 hours. After phosphate buffered saline washes, slices were then incubated with the appropriate fluorophore-conjugated secondary antibody (goat anti-mouse conjugated with Alexa Fluor 594, 1:1000, Jackson ImmunoResearch, Cat# 711-585-152, RRID: AB_2340621) for 2 hours at room temperature. Images were acquired using a confocal microscope (Zeiss).

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). All data were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Parametric data were compared using one-way analysis of variance followed by Bonferroni’s multiple comparison tests. Non-parametric data were analyzed by Mann-Whitney U test.

Results

Acupuncture induces calcium transients in S1 neurons

We used two-photon microscopy in mice transfected with an AAV vector expressing fluorescent calcium indicator GCaMP6s (Additional Figure 1), and recorded the calcium activity of layer 5 pyramidal neurons (LSPNs) while the mouse received manual acupuncture stimulation at the hindlimb acupoint ST36 or GB34 (Figure 2A).

For each mouse, we compared calcium activity within the same field of view under different scenarios: non-acupoint stimulus on the contralateral side of field of view; puncture at ST36 on contralateral side or ipsilateral side; or ST36 puncture under general anesthesia (Figure 2K and L). These results indicate that ST36 acupuncture stimulation at the contralateral side remarkably activated calcium transients, which were synchronized with each acupuncture course (Figure 2C and D, with gray shades). Acupuncture at ST36 on the ipsilateral side also induced calcium activation of LSPNs, but with lower intensity compared with that induced by contralateral side puncture (Figure 2E). The induced calcium potentiation in ST36 was silenced on each side by general anesthesia (Figure 2F and G) and was not observed in the primary motor cortex (Figure 2H and I). These results indicate that S1 activation was induced by acupuncture stimulation, and did not represent artifacts such as from body movements. Needle stimulation at a second acupoint (GB34) also induced higher neuronal calcium activity compared with that at the non-acupoint control (Figure 2J). Furthermore, the calcium potentiation was largely abolished by the pre-infusion of MK801, which is a potent antagonist for NMDAR, suggesting that the induced calcium activity was mainly dependent on glutamatergic transmission (Figure 2K and L).

Acupuncture activates calcium transients in astrocytes

We next investigated whether acupuncture stimulation affects astrocytes. Recent in vivo studies have suggested that neuronal excitation may alter the calcium signals in astrocytes (Stobart et al., 2018), and that astrocytic calcium transients may be involved in astrocytic-glutamatergic transmission. To monitor in vivo calcium activity of astrocytes, mice were transfectected with GCaMP6s into S1 under a high fibular glutamatergic acid protein (gAICD) with astrocyte-preferential expression (Additional Figure 2) (Yu et al., 2020). Using two-photon calcium recording, we compared the activity of astrocytes between non-acupoint stimulation and acupuncture stimulation (Figure 3B). Consistent with the findings in neurons, acupuncture treatment led to stronger calcium transients in astrocytes compared with non-acupoint stimulation (Figure 3C and D). The astrocytic calcium signals were remarkably suppressed after MK801 application (Figure 3E and G), suggesting its dependence on glutamatergic transmission. To further clarify the mechanism, we administered HC030031 to S1 during the in vivo recording to block the receptor potential A1 cation channel, which is the major calcium transporter in astrocytes (Shigemori et al., 2011). The application of HC030031 remarkably abolished the astrocytic calcium activity after ST36 acupuncture (Figure 3F and G). These data collectively suggested the potentiation of astrocytic calcium activity by acupuncture.

Astrocytic calcium activity is mediated by neurons

The abolishment of astrocytic calcium activity by NMDAR blockade (Figure 3E) suggested that neuronal activation might be the driving force of astrocytic calcium transients. We then compared the kinetics of calcium spikes between neurons and astrocytes under identical acupuncture treatment. Astrocytic calcium transients showed a slow-onset pattern. Specifically, the neuronal calcium transients were synchronized with each acupuncture course (Figure 3D, with gray shades). The latencies of both the initiation and zenith of astrocytic calcium spikes were over 10 seconds longer than those of neurons (Figure 3D). The different time latency suggested a possible regulatory role of neuronal excitation in driving astrocytic calcium activity.

To investigate this, we transfected inhibitory designer receptors exclusively activated by designer drugs mH4D1 into S1 LSPNs, along with the astrocytic calcium indicator GCaMP6s (Figures 4A and B). After clozapine-N-oxide administration, PN activity was remarkably suppressed (Figure 4B). We then recorded the calcium activity of astrocytes upon acupuncture stimulation.
Clozapine-N-oxide treatment induced a stronger calcium response compared with the response in the saline control group (Figure 4C and D). These results seemed to indicate: (1) the rise of neuronal and astrocytic calcium transients upon acupuncture is independently regulated; and (2) neuronal activation probably promotes the decay of the astrocytic calcium response, rather than the presumed potentiation role.

Next, we performed a persistent acupuncture treatment to further examine the kinetics of both neuronal and astrocytic calcium activity. Compared with the spontaneous activity under resting state, the 20-minute acupuncture stimulation induced a tonic-like excitation of calcium activity in neurons (Figure 4E).

In astrocytes, the major calcium peak occurred shortly after the onset of acupuncture followed by multiple lower peaks (Figure 4F).

Figure 2 | Acupuncture induces calcium transients in S1 neurons. (A) Top, schematic diagram showing the acupoints ST36 (Zusanli) and GB34 (Yonglingquan), and non-acupoint in adjacent sites as the sham control group; bottom, transsection sites (arrow) of GCaMP6s in S1. Scale bar: 500 μm. (B) Pseudo-colored images reflecting calcium intensity of neurons in selected field of view from S1 under resting state and during acupuncture. Scale bar: 50 μm. (C–K) Time-series records of normalized calcium values (in ΔF/F) during the period of repeated acupuncture stimuli (grey shaded box). The temporal scale (x-axis) was presented in seconds. Three acupuncture courses (20 seconds each) were sequentially applied, with a 30-second resting interval between two treatments. In each group, calcium activities from representative fields of view were normalized and averaged for plotting. Fields of view numbers: n = 9 for C–D, n = 5 for E, n = 10 for F. Animal numbers: n = 3 each. (I) Comparison of total integrated astrocytic calcium activity during the acupuncture course. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance. Group effect: F = 20.10, P < 0.001. Bonferroni’s multiple comparison test: **P < 0.01. cNS: Needle stimulation on the contralateral side; GCaMP6s: green fluorescent-calcium protein 6s; iNS: needle stimulation on the ipsilateral side; M1: primary motor cortex; NS: needle stimulation; S1: primary somatosensory cortex.

Figure 3 | Acupuncture activates calcium transients in astrocytes. (A) Transfection sites of AAV-gfaABC1D-GCaMP6s in S1 were colocalized with astrocyte marker S100B (white arrowheads, red, stained by Alexa Fluor 594). Scale bars: 50 μm. (B) Pseudo-colored images for the intensity of astrocytic calcium spikes in selected fields of view during the resting state and NS. Scale bars: 50 μm. (C–F) Time-series recordings of normalized calcium values (in ΔF/F) during the acupuncture sessions (grey shaded box). The temporal scale (x-axis) was presented in seconds. The protocol of acupuncture was the same as in Figure 2. In each group, the calcium activity from different fields of view was recorded for data plotting. Field of view numbers: n = 9 for C–D, n = 5 for E, n = 10 for F. Animal numbers: n = 3 each. (G) Comparison of total integrated astrocytic calcium activity. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance. Group effect: F = 50.929, P < 0.001. Bonferroni’s multiple comparison test: **P = 0.002. (H) Comparison of kinetics of calcium spikes between neurons and astrocytes from the recordings under cNS stimulation on ST36 of awake mice. cNS: Needle stimulation on the contralateral side; GCaMP6s: green fluorescent-calcium protein 6s; iNS: needle stimulation on the ipsilateral side; M1: primary motor cortex; NS: needle stimulation; S1: primary somatosensory cortex.

Figure 4 | Astrocytic calcium activity is mediated by neurons. (A) Transfection of hM4Di into excitatory neurons of S1 (arrow) and transfection of gfaABC1D-GCaMP6s into astrocytes. Scale bar: 500 μm. (B) Co-staining (arrows) of c-Fos (blue) and hM4Di (red) in S1. Mice were anesthetized CNO, followed by astrocytic calcium imaging as in C, and were immediately sacrificed for brain sectioning and immunofluorescence assay. Scale bar: 50 μm. Right, CNO treatment significantly suppressed the activity of transfected neurons (Mann-Whitney U test, *P = 0.0012). (C) Time-series recordings of normalized astrocytic calcium signals during acupuncture treatment. Grey shades indicate the acupuncture period. Approximately 15–20 cells from four animals were plotted. (D) Total integrated astrocytic calcium activity was potentiated by CNO treatment (Mann-Whitney U test, **P = 0.0022). (E) Left, time-series recordings of normalized neuronal calcium activity under resting state or during 20-minute continuous manual acupuncture on ST36. A total of 18 fields of view from four animals were plotted in each group. Right, the total neuronal calcium activity was elevated under acupuncture (Mann-Whitney U test, *P = 0.0293). (F) Left, time-series recordings of normalized astrocytic calcium activity under resting state or during the acupuncture on ST36. A total of 18 fields of view from four animals were plotted in each group. Right, the total astrocytic calcium activity was elevated during acupuncture (Mann-Whitney U test, *P = 0.0303). The temporal scale (x-axis) of c-fos was presented in seconds. Data are expressed as mean ± SEM. The experiments were repeated twice. Acup: During the acupuncture on ST36; CNO: clozapine-N-oxide; DAPI: 4’,6-diamidino-2-phenylindole; GCaMP6s: green fluorescent-calcium protein 6s; S1: primary somatosensory cortex; Spon: resting state.
Discussion

By comparing awake mice with those under anesthesia, the present study provided evidence that acupuncture induced strong calcium signaling for both verum acupuncture points (ST36 and GB34) and sham acupoint sites. We also performed a similar experiment in which astrocytes in the somatosensory cortex expressed calcium indicator. The present study is the first to show that neurons and astrocytes produced comparable activity under acupuncture stimulation, indicating that acupuncture utilizes both neurons and astrocytes to deliver its sensory effect.

We would like to address a few other interesting findings in the data. First, sham acupuncture is not a minor content, as it has been used to argue for a placebo effect, thus denying the scientific basis not only for sham acupuncture but also for verum acupuncture. Our results are the first to show in vivo neuronal and astrocytic communication in response to acupuncture, and to demonstrate that sham acupuncture has cellular effects. The results are in agreement with previous studies (Jin et al., 2015; Song et al., 2005; Haake et al., 2005).

Second, there was strong activity in somatosensory cortex (S1) but no response in motor cortex (M1), indicating the specificity of acupuncture. Third, there was essentially no latency observed in calcium transients in S1 induced by acupuncture at both the beam and acupuncture points, confirming fast neuronal transmission. Fourth, anesthesia inhibited all ST36-S1 responses, indicating the central nervous system has disabled the signaling.

Our findings demonstrated the activation of calcium transients in both cortical neurons and astrocytes during acupuncture stimulation, providing the first in vivo and real-time evidence for neural activation induced by acupuncture. The present study is the first to show a calcium transient in astrocytes which is consistent with the current knowledge of astrocytes as the regulator and architect of neuronal processing (Eroglu and Barres, 2010; Panatier et al., 2015). Our results demonstrated the activation of astrocytic calcium transients in S1 cortex following acupuncture stimulation, indicating the potential role of astrocytes in mediating the mechanical acupuncture-evoked region-specific pain relief. We have not covered other parts of the central nervous system.

In summary, we report that mechanical acupuncture evoked region-specific calcium transients of cortical neurons and astrocytes during acupuncture stimulation, providing the first in vivo neuronal and astrocytic activity induced by acupuncture. Mechanisms of acupuncture, we confined our research in relation to pain relief. We have not covered other parts of the central nervous system. It is not uncommon to observe long-term depression of synaptic transmission after repeated excitation. We thus speculate that the acute activation of neurons and astrocytes may produce chronically inhibitory effects, probably via neuromodulators that are produced in an activity-dependent manner, such as endorphin or endocannabinoid. These phenomena indicatedider than the discrepancy of acute and chronic effects. Induced both neuronal and astrocytic activity. These seemingly paradoxical phenomena can be explained by the discrepancy of acute and chronic effects. It is not uncommon to observe long-term depression of synaptic transmission after repeated excitation. We thus speculate that the acute activation of neurons and astrocytes may produce chronically inhibitory effects, probably via neuromodulators that are produced in an activity-dependent manner, such as endorphin or endocannabinoid. These phenomena indicatedider than the discrepancy of acute and chronic effects. Induced both neuronal and astrocytic activity. These seemingly paradoxical phenomena can be explained by the discrepancy of acute and chronic effects. It is not uncommon to observe long-term depression of synaptic transmission after repeated excitation. We thus speculate that the acute activation of neurons and astrocytes may produce chronically inhibitory effects, probably via neuromodulators that are produced in an activity-dependent manner, such as endorphin or endocannabinoid. These phenomena indicatedider than the discrepancy of acute and chronic effects. Induced both neuronal and astrocytic activity. These seemingly paradoxical phenomena can be explained by the discrepancy of acute and chronic effects. It is not uncommon to observe long-term depression of synaptic transmission after repeated excitation. We thus speculate that the acute activation of neurons and astrocytes may produce chronically inhibitory effects, probably via neuromodulators that are produced in an activity-dependent manner, such as endorphin or endocannabinoid. These phenomena indicatedider than the discrepancy of acute and chronic effects. Induced both neuronal and astrocytic activity. These seemingly paradoxical phenomena can be explained by the discrepancy of acute and chronic effects. It is not uncommon to observe long-term depression of synaptic transmission after repeated excitation. We thus speculate that the acute activation of neurons and astrocytes may produce chronically inhibitory effects, probably via neuromodulators that are produced in an activity-dependent manner, such as endorphin or endocannabinoid. These phenomena indicatedider than the discrepancy of acute and chronic effects. Induced both neuronal and astrocytic activity. These seemingly paradoxical phenomena can be explained by the discrepancy of acute and chronic effects. It is not uncommon to observe long-term depression of synaptic transmission after repeated excitation. We thus speculate that the acute activation of neurons and astrocytes may produce chronically inhibitory effects, probably via neuromodulators that are produced in an activity-dependent manner, such as endorphin or endocannabinoid. These phenomena indicatedider than the discrepancy of acute and chronic effects. Induced both neuronal and astrocytic activity. These seemingly paradoxical phenomena can be explained by the discrepancy of acute and chronic effects.

In addition, astrocytic calcium currents can be initiated independently from neurons. A previous study suggested the activation of adenosine receptors for local anti-nociception during acupuncture stimulation (Goldman et al., 2010). Because of the prominent distribution of adenosine receptors on the astrocyte membrane (Orr et al., 2015; Martín-Fernández et al., 2017), it is possible that adenosine receptors mediate the acupuncture-induced calcium currents in astrocytes. This mechanism may be related to the pain-relieving function of acupuncture treatment, as one mouse study demonstrated the dependence of acupuncture-mediated analgesia on the activation of the adenosine pathway (Liao et al., 2017). It is also interesting to note that in the S1 cortex, there has been a history of acupuncture for chronic neuropathic pain disorders (Chicon et al., 2017). However, our mechanical acupuncture induced both neuronal and astrocytal activity. 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Additional Figure 1 Co-localization of GCaMP6s in cortical excitatory neurons.

(A) Immunofluorescent staining of co-localization (arrows) of GCaMP6s (green) and NeuN (red, stained by Alexa Fluor 594), Neurogranin (red, stained by Alexa Fluor 594) or parvalbumin (red, stained by Alexa Fluor 594). A higher co-localization ratio of GCaMP6s in excitatory (Neurogranin, NG) neurons but not in GABAergic parvalbumin (PV) cells. Scale bar: 50 μm. (B) Percentage of co-localization of GCaMP6s and NeuN, Neurogranin or parvalbumin. Data are expressed as mean ± SEM. The experiments were repeated twice. GCaMP6s: green fluorescent-calcium proteins.
Additional Figure 2 Specific expression of GCaMP6f in astrocytes.

(A) Immunofluorescent staining of co-localization of GCaMP6s (green) and S100β (red, stained by Alexa Fluor 594), Iba-1 (red, stained by Alexa Fluor 594) or Olig2 (red, stained by Alexa Fluor 594). GCaMP6f under the driven of gfaABC1D promoter was mainly expressed in astrocytes (S100β) but not microglia (Iba-1) or oligodendrocytes (Olig2). Scale bar: 50 μm. (B) Percentage of co-localization of GCaMP6s and S100β, Iba-1 or Olig2. Data are expressed as mean ± SEM. The experiments were repeated twice. GCaMP6s: green fluorescent-calmodulin proteins; Iba-1: ionized calcium binding adaptor molecule 1; ND: non-detectable level.