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Short communication

# Astrocytic $Ca^{2+}$ responses in the spinal dorsal horn by noxious stimuli to the skin

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ABSTRACT

The role of astrocytes in the spinal dorsal horn (SDH) for sensory information processing under normal conditions is poorly understood. In this study, we investigated whether SDH astrocytes respond to noxious and innocuous stimuli to the skin of normal mice using *in vivo* two-photon $Ca^{2+}$ imaging under anesthesia. We found that noxious stimulation evoked by intraplantar formalin injection provoked an elevation in intracellular $Ca^{2+}$ levels in SDH astrocytes. By contrast, neither instantaneous noxious pinching nor innocuous stimuli (cooling or brushing) to the hindpaw elicited astrocytic $Ca^{2+}$ responses. Thus, SDH astrocytes could respond preferentially to a strong and/or sustained noxious stimulus.



The spinal dorsal horn (SDH) is a region that receives a variety of somatosensory information from the periphery through primary afferents and processes the information via neuronal circuits.[1] Emerging evidence has suggested that astrocytes, glial cells that are the abundant cell type in the central nervous system (CNS), respond to sensory stimuli. Several studies using *in vivo* two-photon $Ca^{2+}$ imaging have shown that astrocytes in the brain elevate intracellular $Ca^{2+}$ levels ($[Ca^{2+}]_i$) by odor,[2] vision[3] and whisker stimulation.[4] In the SDH, by establishing a method for *in vivo* imaging of the SDH to monitor $[Ca^{2+}]_i$ in astrocytes,[5] a recent study has reported that intense noxious mechanical stimulation to the skin produces astrocytic $Ca^{2+}$ responses.[6] However, there is currently only limited evidence, and whether SDH astrocytes commonly respond to other noxious stimuli, such as chemical irritants, and to innocuous stimuli remains unknown. Elucidating this issue would facilitate our understanding of the mechanism for sensory information processing in the SDH and also for neuron-astrocyte communications in the CNS. Thus, in the present study, we performed *in vivo* two-photon $Ca^{2+}$ imaging of SDH astrocytes and examined astrocytic activity by applying several distinct stimuli to the skin.

The materials and methods were fully described in Electronic Supplementary Materials. Briefly, male C57BL/6J mice (CLEA Japan) were used and housed at 22 ± 1 °C with a 12-h light–dark cycle with food and water ad libitum. All experimental procedures were performed under the guidelines of Kyushu University. We constructed adeno-associated virus (AAV) expression plasmid pZac2.1 (Penn Vector Core) carrying the $Ca^{2+}$ sensor protein GCaMP6m (amplified from Addgene #40754)[7] with the astrocytic promoter $gfaABC_1D$ (viral titer: approximately $8.5 \times 10^{12}$ GC/ml). AAV2/9-$gfaABC_1D$-GCaMP6m was microinjected into the left side of the SDH.[8] Three weeks later, SDH astrocyte-specific expression of GCaMP6m protein was confirmed by immunohistochemistry. For *in vivo* SDH imaging, a custom-made spinal chamber was attached at laminectomized Th13 of GCaMP6m-expressing mice anesthetized by ketamine and xylazine. Two-photon $Ca^{2+}$ imaging was

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performed under ketamine-xylazine anesthesia using an Olympus FV1000 (Olympus) and the MaiTaiHP Ti:Sapphire laser (Spectra Physics). Sensory stimulation (pinch, acetone, brush, and intraplantar formalin injection) was applied to the left hindpaw. Movies were imported into ImageJ (http://rsbweb.nih.gov/ij/), and motion artefacts were corrected using TurboReg. All data were expressed as the mean ± SEM and were analyzed by the Mann–Whitney U test. Statistical analysis was performed using Prism 7. Significance was reached at values of $P < 0.05$.

To monitor $[Ca^{2+}]_i$ in SDH astrocytes *in vivo*, we microinjected AAV2/9 containing a gene encoding GCaMP6m and the *glial fibrillary acidic protein* (*Gfap*) promoter gfaABC$_1$D (AAV2/9-gfaABC$_1$D-GCaMP6m) unilaterally into the SDH. Almost all GCaMP6m-expressing cells were positive to the astrocytic markers SRY-related high-mobility group box 9 (SOX9) (Fig. 1A) and GFAP (Fig. 1B), but not to the markers for neurons neuronal nuclei (NeuN), for microglia ionized calcium binding adapter molecule 1 (Iba1), or for oligodendrocytes adenomatous polyposis coli (APC) (Fig. 1C), indicating a selective expression of GCaMP6m in SDH astrocytes.

Using GCaMP6m-expressing mice under anesthesia, we monitored $[Ca^{2+}]_i$ in SDH astrocytes by *in vivo* imaging (Fig. 2A) after distinct sensory stimulations to the hindpaw. First, we tested mechanical pinching by forceps as an instantaneous nociceptive stimulus and found that this stimulus did not provoke $[Ca^{2+}]_i$ increases in SDH astrocytes (Fig. 2B). The failure of $Ca^{2+}$ responses was consistently observed by the second and third stimulations. Similarly, applying acetone to the planter skin (cool stimulus) also had no effect (Fig. 2C). In addition, light stroking of the plantar skin by a paintbrush (tactile stimulus) failed to induce astrocytic $[Ca^{2+}]_i$ elevations (Fig. 2D). Then, we tested intraplantar injection of the chemical irritant formalin, which is a well-known model for intense acute and persistent pain associated with tissue injury. We found that formalin injection produced robust and synchronized astrocytic $[Ca^{2+}]_i$ elevations in the SDH (Fig. 2E and F). The peak was observed within 5 min after formalin injection (Fig. 2F). Interestingly, in some astrocytes, slight $[Ca^{2+}]_i$ elevations were also observed around 20–30 min post-injection (indicated by arrowheads). Intraplantar injection of vehicle had no effect on $Ca^{2+}$ responses (Fig. 2F). The peak amplitude of individual astrocytes was significantly increased by formalin but not by other stimuli (Fig. 2G). These results suggest that SDH astrocytes respond preferentially to formalin compared with instantaneous noxious pinching or innocuous cooling or tactile stimuli.

In the present study, by combining techniques of SDH microinjection to express GCaMP6m selectively in SDH astrocytes and of two-photon microscopy to monitor $[Ca^{2+}]_i$ in SDH astrocytes *in vivo*, we demonstrated for the first time that SDH astrocytes are strongly activated by the chemical irritant formalin injected into the plantar skin. By contrast, innocuous cool (acetone) and tactile (brushing) stimuli failed to produce such activation, suggesting a somatosensory modality-selective responsiveness of SDH astrocytes. However, it should be noted that our experiments were performed under anesthesia by ketamine and xylazine, these reagents have been reported to blunt astrocytic $Ca^{2+}$ responses in the neocortex evoked by whisker stimulation.[9] In addition, a recent study using *in vivo* SDH imaging of freely behaving mice has shown that intense pinch stimulation of the tail produces $Ca^{2+}$ transients in SDH astrocytes, the frequency of which is suppressed under anesthesia.[6] In addition, glutamate released from primary afferents and/or dorsal horn neurons whose excitability may be affected by ketamine and xylazine could presumably play a role in astrocytic $Ca^{2+}$ responses. Therefore, we cannot exclude the possibility that astrocytic $Ca^{2+}$ responses in our experimental condition may be underestimated by the treatment with anesthetics. In fact, we show that the nociceptive mechanical pinch had little effect on astrocytic $Ca^{2+}$ responses, which is in contrast to the results reported previously in awake mice.[6] Although this is a technical limitation in the present study, our findings that intraplantar injection of formalin markedly provoked astrocytic $Ca^{2+}$ increases even such an anesthetized condition implies the strong ability of this stimulus to activate SDH astrocytes. Furthermore, in some SDH astrocytes, a slight increase in $[Ca^{2+}]_i$ was also observed during a later phase (around 20–30 min post-injection). These temporally biphasic responses seem to be correlated with formalin pain behaviors: the first phase started immediately after the injection and lasted for 5 min, and the second phase started from around 15 min and lasted for 60 min. The involvement of spinal astrocytes in formalin pain has been examined in several studies,[10–13] but their precise role remains unclear because of no manipulation specific to SDH astrocytic function in these studies. Nevertheless, because astrocytes have become increasingly recognized as critical elements for regulating neuronal function and plasticity in the brain[14] and SDH,[15] further investigations using a tool to manipulate $Ca^{2+}$ responses specifically in SDH astrocytes will uncover their *in vivo* role in pain processing and behaviors such as when evoked by formalin and other noxious stimuli.

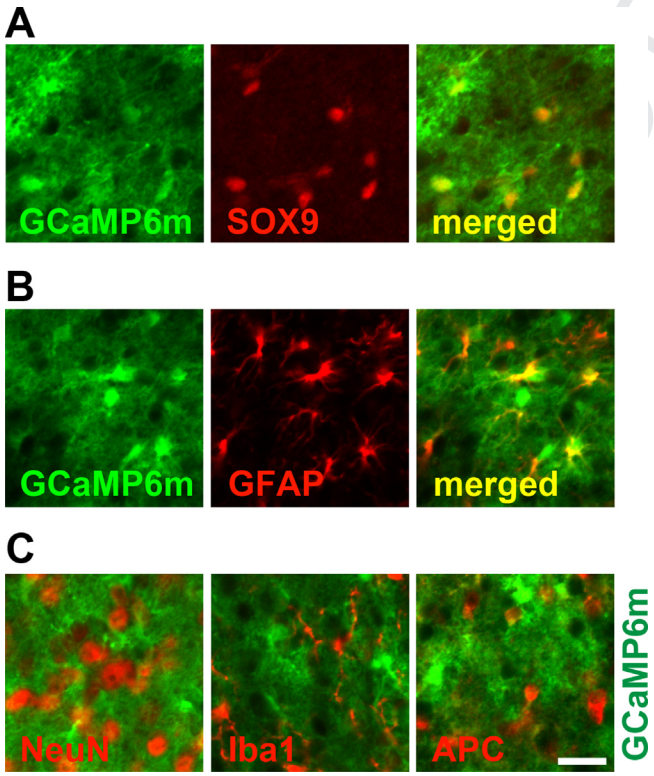


**Fig. 1.** Selective expression of GCaMP6m in SDH astrocytes. **A–C.** Immunohistochemical identification of GCaMP6m-expressing cells (green) using cell-type markers (red; **A**, SOX9; **B**, GFAP; **C**, NeuN, Iba1, and APC) in L4 SDH sections 22 days after intra-SDH injection of AAV2/9-gfaABC$_1$D-GCaMP6m (Scale bar, 20 μm).

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