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Involvement of A5/a7 Noradrenergic Neurons and B2 Serotonergic Neurons in Nociceptive Processing: A Fiber Photometry Study

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Short report

Keywords: nociception, A5 NA neurons, A7 NA neurons, B25-HT neurons, fiber photometry, G-CaMP6, mCherry, DBH-tTA mice, TPH-tTA mice

Posted Date: June 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-31869/v1

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Version of Record: A version of this preprint was published at Neural Regeneration Research on January 1st, 2022. See the published version at https://doi.org/10.4103/1673-5374.322465.

Abstract

In nociceptive processing, the descending antinociceptive system (DAS) is well known to include a number of descending pathways.As the main pathway of DAS, the locus coeruleus (A6) – spinal horn circuit including the noradrenergic (NAergic) system andthe periaqueductal gray-rostral ventromedial medulla (B3) – spinal horn circuit including the serotonergic (5-HTergic) system are well known.OtherNAergic or 5-HTegic systems, however,are less well known. Some studies have reported that A5/A7 noradrenaline (NA) neurons or B2 serotonin (5-HT, 5-hydroxytryptamine) neurons partially participate in DAS.In this study, we recorded G-CaMP6 green fluorescence signal intensities in theA5/A7/B2 sites of awake mice in response to acute tail pinch stimuli, acute heat stimuli, and in the context of a noninvasive control test, using fiber photometry with acalcium imaging system.We first introduced G-CaMP6 in the A5/A7 NA neuronal soma or B2 5-HT neuronal soma, using transgenic mice carrying tetracycline-controlled transactivator transgene (tTA) under the control of either a dopamine β-hydroxylase promoter or a tryptophan hydroxylase-2 and by the site-specific injection of adeno-associated virus (AAV-TetO(3G)-G-CaMP6).The specific expression patterns of G-CaMP6 were clearly confirmed in the A5/A7 areasand B2 area.After confirming the specific expression patterns of G-CaMP6, we recorded G-CaMP6 green fluorescence signals in these sites fromawake mice in response to acute nociceptive stimuli.G-CaMP6 fluorescence intensity in the A5, A7, and B2 groups wasrapidly increased by acute nociceptive stimuli,soon returning to baseline fluorescence intensity.This was not observed in the context of the noninvasive control test.Weused mCherry red fluorescent protein as a control indicator. mCherry fluorescence intensity was not significantly changed by acute nociceptive stimuli or in the noninvasive control test in any of the groups. These results clearly indicate that acute nociceptive stimuli rapidly increase the activities of A5/A7 NA neurons or B2 5-HT neurons, suggesting that A5/A7 NA neurons or B2 5-HT neurons play important roles in nociceptive processing in the central nervous system.

Introduction

In modern clinical practice, pain symptomsareone of the important symptoms thatclinicians treat. Pain symptomsarefrequently present themselves with various mental disorders as well as physical disorders and are sometimes difficult to treat [1]. Especially in the case of mental disorders, the pathogenesic mechanismsunderlying theironsetaresometimes unclear. In the field of psychiatry, pain symptomspresent themselves in the form of neuropathic pain [2], somatoform pain disorder [3], major depressive disorder [4], and sleep-related disorder [5]. The monoaminergic system in the central nervous system (CNS) is thought to be involved in the etiology of these diseases [6, 7]. The role of monoaminergic signaling in nociceptive processing in the CNS iswell known. In the pain management system, there are some descending pathways termedthe descending antinociceptive system (DAS) [8, 9]. As the mainpathways of the DAS, the locus coeruleus (LC, A6) – spinal horn circuit including the noradrenergic (NAergic) system andthe periaqueductal gray (PAG)-rostral ventromedial medulla (RVM, B3) – spinal horn circuit includingthe serotonergic (5-HTergic) system are well known [10, 11]. Descending monoaminergic terminals are distributed in the spinal dorsal horn and regulate nociceptive signals. The NAergic system

has extensive innervation in the CNS, and subsets of noradrenaline (NE) cells are designed as A1-A7 groups in a caudal to rostral direction [12, 13]. The A6NE cell group is a well-recognized player in the DAS [10], while other NE cell groups are less well known. However, a few papers have reported that A5 and A7 NE cell groups are implicated in the regulation of antinociception [14, 15]. The A5 group is located in the ventrolateral part of the pons and the A7 group is located in the lateral part of the pons [16]. A5/A7 NE cells as well as A6 cellsmainly project to thesuperficial spinal horn [16]. The5-HTergic andNEergic systemshaveextensive innervations in the CNS, and subsets of serotonin (5-HT, 5-hydroxytryptamine) cells are identified as forming part of the B1–B9 group in a caudal to rostral direction [12]. The B3 5-HT group is a well-recognizedplayer in the DAS, while other 5-HT cell groups are less well known [17]. The B2 (nucleus raphe obscurus) 5-HT cell group is located in the ventral part of medullary reticulum [18]. Unlike the B3 group,the B2 groupmainly projects to the spinal intermediolateral cell column (IML) [19, 20] andspinal ventral horn [21]. The B2 group contributes to the regulation of autonomic function [19, 20] and motor function [20, 21]. To the best of our knowledge, few studies have assessed thecontributing functions of the B2 group in the regulation of nociceptive processing. Recently, we reported that the activities of A6 NA neurons and B3 5-HT neurons in awake mice are rapidly increased in responseto acute nociceptive stimuli using a fiber photometry system [22]. Asaforementioned, a few studies have reported that A5/A7 NA neurons are implicated in the regulation of pain management, but to the best of our knowledge, only few studies have assessed how the activities of A5/A7 NA neurons are affected by nociceptive stimuli. A fiber photometry system adopting acalcium (Ca^{2+}) imaging system is an advanced tool for evaluating nociceptive signaling, as demonstrated in a recent paper focused onB9 5-HT neurons [23], A10 (ventral tegmental area, VTA)dopamine (DA) neurons [24], and A13 DA neurons [25]. Therefore, we deemed it meaningfulto assessthe activities of A5/A7 NA neurons and B2 5-HT neurons in response to acute nociceptive stimuli. We focused on these sites because these sites mainly form the descending pathways to the spinal cord.

We first introduced G-CaMP6 into A5/A7 NA neurons or B2 5-HT neurons using transgenic mice carrying tetracycline-controlled transactivator transgene (tTA) under the control of either a dopamine β-hydroxylase (DBH) promoter or a tryptophan hydroxylase-2 (TPH2), with a site-specific injection of an adeno-associated virus (AAV-TetO(3G)-G-CaMP6). After confirming the specific expression patterns ofG-CaMP6 in the A5/A7 or B2 sites, we recorded the G-CaMP6 green fluorescence signal in these sites inawake mice in response to acute nociceptive stimuli.

Materials And Methods

Animals

We usedtransgenic mice carryingtTA under the control of a DBHorTPH2 promoter (DBH-tTA mice or TPH2-tTA mice, respectively) [22, 23, 24] (Fig. 1a);10–14-week-old male mice were usedin this experiment. Rearing was carried outin standard conditions,with lights on at 7:00 AM and off at 7:00 PM, atemperature of 24 ± 1°C, and food and water *adlibitum*. We made efforts to minimize animal affliction and reduce the

number of animals used.All experimentalprocedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of laboratory Animals and approved by the Institutional Animal Use Committee of Kagoshima University (MD17105).

Stereotaxic AAV injection

Adeno-associated virus (AAV) vectors were produced using the AAV Helper-Free system (Agilent Technologies, Inc., Santa Clara, CA, USA) and purified as described previously[25]. AAV-TetO(3G)-G-CaMP6 (Serotype:DJ; 0.3µl/injection, 4×10^{13}copies/ml) and AAV-Tet(3G)-mCherry (Serotype:DJ; 0.3µl/injection, 6×10^{12}copies/ml)were produced using the Tet system (Fig. 1b). We slowly sucked up AAV into the glass micropipette (1B150F-3, World Precision Instruments, Inc., Sarasota, FL, USA) connectedto an injection manipulator (I-200J, Narishige, Tokyo, Japan) linked toa nitrogen pressure source through polyethylene tubing. Under 2-3% isoflurane anesthetic conditions, mice were fixed witha stereotaxic instrument (ST-7, Narishige) with the help of supportive ear bar (EB-6, Narishige). To minimize suffering, the surfaces of their ears were covered with local anesthetic jelly (lidocaine, 2%xylocaine),and both eyes were preserved with vaseline. Head hair was shaved using an electric shaver and the cranial dura mater was cut open withsmall scissors. AAV was unilaterally injected into thetarget sites(A5: bregma -5.52mm, lateral +1.4mm, and ventral -5.30mm from the cranium; A7: bregma -4.96mm, lateral +1.88 mm, and ventral -3.10 mm from the surface of the brain; B2: bregma -7.56mm, and ventral -3.93mm from the cranium) (Fig. 1c)and the glass microtube was let sitin the sites for 10minutes before being withdrawn.Postoperative antibiotic administration was carried out by asubcutaneous injection (penicillin G, 40,000 U kg^{-1}) to prevent postoperative infections. After the operation, mice were maintained in normal rearing conditions (see adobe) for 14 days (twoweeks) to recover from thedamage and to set aside a period allowing for the G-CaMP6/mCherry fluorescent protein to be fully expressed prior to the experimental sessions.

Immunohistochemistry

After the experiment, we performed immunohistochemistry to confirm the AAV-induced site-specific expression of G-CaMP6 and mCherry in mice. We transcardially perfused mice with 20ml of phosphate-buffered saline (PBS) and 20 ml of a 4% paraformaldehyde (PFA) solution under anesthesia with urethane (1.6 g/kg, ip). The brain was removed and post-fixed with 4% PFA and soaked in 30% sucrose in PBS for two days.We created a series of 30 µm slices including the target sites with the cryostat (Cryotome FSE, Thermo Scientific, Yokohama, Japan) and immersed the sections in PBS for 24 hours at 4°C.Every third section was usedfor analysis. We washed the sections with PBS three times and incubated the sections with primary antibodies overnight. We diluted primary antibodies in blocking buffer and set the concentration as follows: A5/A7; anti-Tyrosine Hydroxylase raised in rabbit antibody (AB152, EMD Millipore Corp., Temecula, CA, USA) at 1:500, B2; anti-TPH antibody (AB1541, EMD Millipore Corp.) at 1:1000. The next day, we washed the sections with PBS three times and incubated the sections

with secondary antibodies at room temperature for two hours. We diluted secondary antibodies in PBS and set the concentration as follows;A5/A7: CF647 donkey anti-rabbit (20047, Biotium Inc., Fremont, CA, USA) in PBS at 1:200;B2: CF647 donkey anti-sheep (20284, Biotium Inc.) in PBS at 1:200.Next, we washed the sections with PBS once and mounted them onmicroscope slides (PRO-02, Matsunami, Osaka, Japan) and covered them with a micro cover glass (C024601, Matsunami). We observed the sections undera fluorescence microscope (BZ-X700, Keyence, Osaka, Japan), and created imagesusingAdobe Photoshop CC software (Adobe Systems Inc., San Jose, CA, USA).

In vivo fiber photometry system

In this study, we usedthe same fiber photometry system with two channels that we usedin previous papers [22, 23, 24, 25]. We portray the scheme of the fiber photometry system (Fig. 2a). In brief, a high-power LED driver (LEDD1B/M470F3, Thorlabs Inc., Newton, NJ, USA) generates anexcitation blue light (470nm, 0.5mW at the tip of the silica fiber) or yellow light (590nm) and the blue or yellow lights pass the excitation bandpass filter (blue light: pass 475±12.5nm; yellow light; 590 ± 12.5nm) and are reflected by a dichroic mirror-1 and joined into the single silica fiber (diameter: 400μm, numerical aperture = 0.6). Blue/yellow lights emitted from the tip of the silica fiber reflect G-CaMP6/mCherry fluorescent proteins andgreen/red fluorescence signalsare detected and transmitted to the same tube. The signals pass the dichroic mirror-1 and are reflected by a dichroic mirror-2 and pass the bandpass emission filter (green: pass 510±12.5nm; red: 607±12.5nm). At the endof the line, the signalsare guided to a photomultiplier tube (PMT) (PMTH-S1-1P28, Zolix Instruments, Beijing, China). The signals were digitized by an A/D converter (PowerLab8/35, ADInstruments Inc., Dunedin, New Zealand) and recorded by Labchart version-7 software (ADInstruments Inc.).

Experimental protocol

In this study, we divided experimental mice into three groups: the A5, A7, and B2 groups. Fourteen days prior torecording, AAV was unilaterally injected into A5/A7 regions in DBH-tTA mice and B2 regions in TPH2-tTA mice(Fig. 1c). Each mouse was individually kept in normal breeding conditions for 14 days after the operation. In this study, we recorded the G-CaMP6/mCherry green/red fluorescence intensityof A5/A7 NA neuronal cell bodies and B2 5-HT neuronal cell bodiesin response to acute nociceptive stimuli. Each of the four stimuli was presentedonce to each mouse(Fig. 2b). We applied an acute tail pinch stimulus at aforce of 400 gusing a pinch meter (PM-201, Soshin-Medic, Chiba, Japan) and an acute heat stimulus at a temperature of 55°Cusing a heating probe (5R7-570, Oven Industries Inc., Mechanicsburg, PA, USA) as per previous reports [23, 25]. As noninvasive control stimuli, we applied a gentle touch using a cotton stick and a low temperature stimulus corresponding to a temperature of 25℃ using the same heating probe (5R7-570, Oven Industries Inc.).

We carried out the recording according to the following procedure. Each mouse was anesthetized with 2–3% isoflurane using a vaporizer and fixed with a stereotaxic instrument (ST-7, Narishige)using a

supportive ear bar (EB-6, Narishige). Surfaces of the ears werecovered with a local anesthetic jelly (lidocaine, 2% xylocaine) to minimize suffering. We then carried out the silica fiber implantation operation. The silica fiber was placed just above the A5 site (bregma -5.52mm, lateral +1.4mm, and ventral -5.30mm from the cranium), A7 site (bregma -4.96mm, lateral +1.88 mm, and ventral -3.10 mm from the surface of the brain) and B2 site (bregma -7.56mm, and ventral -3.93mm from the cranium). We implanted the silica fiber slowly while monitoring the fluorescence signal intensity and confirmed that the fluorescence intensity increased rapidly when the optical position approached the target sites(Fig. 2c). After implantation, we ceased the administration of anesthesia. We waited for two hours after ceasinganesthesia until the first stimulus to reduce any possible effects of anesthesia. To reduce any possible effects of a previous stimulus, we set the inter-stimulus interval to30 minutes and the order of stimuli as follows: the first was thelow heat stimulus (25℃), the second was thegentle touch, the thirdwas the heat stimulus(55℃) and the last was thepinch stimulus(Fig. 2b).We usedthe pinch meter (PM-201, Soshin-Medic) for the tail pinch stimulus and attached the apparatus to the root of the tail for three seconds with a force of 400g.We alsoused aheating probe (5R7-570, Oven Industries, Inc.) for the heat stimulus set to55°C and attached the probe to the root of the tail for three seconds.For noninvasive control stimuli, we used alow heat stimulus using the same heat probe by touching the root of the tail for three seconds and by gentlytouching the mouse using a cotton stick at the root of the tail for three seconds.

We defined the neuronal activity characteristic index as follows: F: averaged fluorescence signal intensity value for three seconds immediately prior toeach stimulus and defined as 100%; ΔF: maximum fluorescence signal intensity value during each stimulus – F; onset latency: time from the start of the stimulus to the time when the fluorescence signal intensity exceeded the maximum value during the baseline period; peak latency: time from the start of the stimulus to the maximum peak signal intensity.

Statistical analysis

Statisticalanalyses were conductedusing atwo-way analysis of variance (ANOVA) with theSidak's test for post hoc analyses. The twofactors ofΔF/F were the stimulus style (mechanical vs thermal) and stimulus intensity (nociceptive vs control). The twofactors ofonset/peak latency were the stimulus style (pinch vs heat) and target sites (A5 vs A7 vs B2).Data values wereexpressed as the mean ± standard error of the mean (S.E.M). Probability values of $p < 0.05$ were considered statically significant. Analyses were performed using GraphPad Prism version 7 (GraphPad software, San Diego, CA, USA).

Results

Expressionpatterns of AAV-induced G-CaMP6/mCherry

The specific expression patterns of G-CaMP6/mCherry were confirmed in A5/A7 NA neuronal soma(Fig. 3a, b)and B2 5-HT neuronal soma(Fig. 3c). G-CaMP6/mCherry-positive neurons were hardly observed

outside the A5, A7, and B2 areas. TH-positive cells were found in the A5 and A7 areas.Alarge proportion of them expressed G-CaMP6 (A5 area: 90.6%, A7 area: 90.9%)(n = 6, each) (Fig. 3d, e). All G-CaMP6 positive cells expressed mCherry, and a large proportion of them expressed TH (A5 area: 97.0%, A7 area: 85.7%) (Fig. 3d, e). TPH-positive cells were found in the B2 area.Alarge proportion of them expressed G-CaMP6 (91.8%) (n = 6, each)(Fig. 3f),and allG-CaMP6-positive cells expressed mCherry,while94.3% expressed TPH(Fig. 3f). Therefore, green/red fluorescence was confirmed to bederived from specific A5/A7/B2 sites.

Effects of acute nociceptive stimuli on G-CaMP6/mCherry fluorescence intensity

We histologically confirmed that the fiber was properly positioned (see Additional file 1: Figure S1). G-CaMP6/mCherry fluorescence intensity were abruptly increased when the optical position was just above thetarget sites (Fig. 2c).Figure 4showsthe averaged traces of G-CaMP6/mCherry fluorescence intensity in response toacute nociceptive stimuli or a noninvasive control test (n = 6, each)(Fig. 4a-d). A two-way factorial ANOVA revealed that G-CaMP6 fluorescence intensity was significantly different acrossstimulus intensities (A5 group: pinch vs touch: $F(1, 5) = 36.84$, $p = 0.0018$, heat vs low heat: $F(1, 5) = 24.28$, $p = 0.0044$; A7 group: pinch vs touch: $F(1, 5) = 12.36$, $p = 0.0170$, heat vs low heat: $F(1, 5) = 11.48$, $p = 0.0195$; B2 group: pinch vs touch: $F(1, 5) = 50.10$, $p = 0.0009$, heat vs low heat: $F(1, 5) = 23.81$, $p = 0.0046$).An ANOVA revealed no significant difference inthe onset/peak latency valuesacross stimulus intensities(onset latency: pinch vs touch: $F(2, 10) = 0.3465$,$p = 0.7153$; heat vs low heat: $F(2, 10) = 0.1266$,$p = 0.8825$; peak latency:pinch vs touch: $F(2, 10) = 0.4141$,$p = 0.6718$; heat vs low heat: $F(2, 10) = 0.1148$,$p = 0.8927$). Atwo-way factorial ANOVA revealed that mCherry fluorescence intensity was not significantly different acrossstimulus intensities (A5 group: pinch vs touch: $F(1, 5) = 0.3742$, $p = 0.5675$, heat vs low heat: $F(1, 5) = 1.0350$, $p = 0.3557$; A7 group: pinch vs touch: $F(1, 5) = 0.3585$, $p = 0.5755$, heat vs low heat: $F(1, 5) = 0.0225$, $p = 0.8867$; B2 group: pinch vs touch: $F(1, 5) = 0.0014$, $p = 0.9719$, heat vs low heat: $F(1, 5) = 0.3598$, $p = 0.5747$).

The fluctuation influorescence intensity during the baseline period wasas follows; G-CaMP6:A5group (0.67 ± 0.14%), A7 group (0.68 ± 0.15%), B2 group (0.62 ± 0.14%), mCherry: A5 group (0.74 ± 0.11%), A7 group (0.53 ± 0.12%),B2 group (0.51 ± 0.11%). G-CaMP6 fluorescence intensity in all groups wasrapidly increased by acute nociceptive stimuli but not by a noninvasivecontrol test(ΔF/F values; A5 group: pinch/touch = 7.98 ± 1.07/0.91 ± 0.12, $p< 0.001$, 55°C/25°C = 7.22 ± 1.41/0.98 ± 0.22, $p< 0.001$; A7 group: pinch/touch = 8.20 ± 2.16/ 0.68 ± 0.22, $p= 0.0018$, 55°C/25°C = 6.63 ± 1.84/0.71 ± 0.19, $p= 0.014$; B2 group: pinch/touch = 7.97 ± 0.89/0.70 ± 0.19, $p< 0.001$, 55°C/25°C = 9.00 ± 1.56/0.74 ± 0.27, $p< 0.001$, n=6, by aSidak'stest.mCherry fluorescence intensity in all groupswasnot significantly modifiedby any stimuli (ΔF/F values; A5 group: pinch/gentle = 0.59 ± 0.13/0.70 ±0.11, 55°C/25°C = 0.70 ± 0.13/0.89 ± 0.10; A7 group: pinch/gentle = 0.79 ± 0.26/0.61 ± 0.19, 55°C/25°C = 0.52 ± 0.11/0.51 ± 0.16; B2 group: pinch/gentle = 0.62 ± 0.13/0.61 ± 0.16, 55°C/25°C = 0.76 ± 0.12/0.63 ± 0.18, n=6, by Sidak's test. The response patterns to a tail pinch and heat stimuli in all groupswerenot significantly different, as shownbythe statisticalresults ofthe onset and peak latency=ies ($p> 0.05$, bySidak's test) (Fig. 5b, c).

Discussion

Our study clearly showed that the activities of A5/A7 NA neuronsorB2 5-HT neurons were rapidly increased by acute nociceptive stimuli but not by a noninvasive control test. As mentioned earlier, A5 NA neurons are located in the ventrolateral pons and A7 NA neurons are located in the lateral part of the pons, withA5/A7 NA neurons projecting to the spinal cord and forming part ofthe pontospinal NAergic system [16]. The neuronal population of theNAergic DAS comprised about 80% A6 NA neurons, 12% A5 NA neurons, and 8% A7 neurons [26].A few studies have demonstrated that the activation of A5 NA neurons appears to be chemically inducedin conditions of pain[27, 28].Martins et al. reported that pCREB expression was significantly increased in the A5 site of spinal nerve injury animals [29].Another study reported that the A5-spinal pathway increased noxious-evoked c-fos expression [30].These findings are consistent with our data.

A few studies have also reported that the chemical [31, 32, 33] or electrical [34] stimulation of A7 NA neurons regulates nociceptive processing.Few studies have assessed the physiological state of A7 NA cells. To the best of our knowledge, our study usinga fiber photometry system is the first study to assess the activities of A5/A7 NA neuronal soma in response to nociceptive stimuli usingelectrophysiological methods.Our data clearly show a momentary reaction of A5/A7 and lay forth a new perspective with regard to the DAS.

With regardto the onset latency/peak latency, A5/A7 NA neurons did not show anysignificant differences, meaning that theresponse patterns of A5/A7 NA neurons did not depend on the typesof stimuli.In a previous study, we showed that acute nociceptive stimuli rapidly increased the activity of A6 NA neurons in awake mice inreal time conditionsusing a fiber photometry system [22].In this study, the onset latency/peak latency values in theA6 site wereas follows: pinch group, 0.4 ± 0.2/1.9 ± 0.3, heat group, 0.7 ± 0.1/3.5 ± 0.7.Given our data, it may be that A5/A6/A7 NA neurons nearlysimultaneously react to acute nociceptive stimuli. In the future, studies comparing these characteristics in the same experimental system will be of interest. In addition,it may be that the LC-spinal NAergic system (A6 related) and pontospinal system (A5/A7 related)work nearly simultaneously in response to acute nociceptive processing.Wei et al. reported that A7 NA neurons affected A6 NA neurons by projectingtheir neuronal axons to the A6 area and regulating pain sensitivity [33].Taking these findings into consideration, our data suggest that the A7-spinal NAergic pathway, A6-spinal NAergic pathway and A7-A6 NAergic pathway might influence each other. Studies investigating these possibilities will be needed in the future.

B2 5-HT neurons are little understood in the context of nociceptive processing. As mentioned in the Introduction, B2 5-HT neurons hardly project to the spinal dorsal horn, mainly targeting the IML [19, 20] or spinal ventral horn [21], and regulate autonomic function [19, 20] or motor function [20, 21]. To the best of our knowledge, our study is first study to assess the activities of B2 5-HT neurons by an electrophysiological method. Our data clearly showed that B2 5-HT neurons were implicated in

nociceptive processing. TheIML or spinal ventral horn,like B2,are not well understood in the context of nociceptive processing. In addition to the DAS, the B2-IML/spinal 5-HTergic pathway may be implicated in nociceptive processing. Studies assessing this will be of interest in the future.

In a previous study, we confirmedthat the activities of B3 5-HT neurons were rapidly increased by acute nociceptive stimuli [22].Some electrophysiological studies have pointed to theactivation of B3 5-HT neurons in response to nociceptive stimuli [35, 36]. As with A5/A7 parts, B2 parts did not showsignificant differences in theonset latency/peak latency, meaning that theresponse patterns of B2 5-HT neurons did not depend on the types of stimuli.In our previous paper, onset latency/peak latency values in B3 wereas follows: pinch group, 0.5 ± 0.1/2.4 ± 0.5, heat group, 1.8 ± 1.0/5.2 ± 1.8) [22]. It may be that B2/B35-HT neurons nearlysimultaneously react to acute nociceptive stimuli. Future studies assessing this will be of interest. Our present results provide new evidenceofdescending 5-HTergic nociceptive processing.

In this study, we useda fiber photometry system using G-CaMP6/mCherry proteins as intracellular calcium ionindicators. This calcium imaging system enables ahigh temporal resolution and is notaffected by time lag and metabolism, differing as such from other electrophysiological and chemical methods. Therefore, our resultsare robust in that they are the product ofmomentary and non-medicated data.

In psychiatric clinical medicine, antidepressants are frequentlyprescribed for pain symptoms. Among antidepressants, serotonin noradrenaline reuptake inhibitors (SNRIs) are themost frequently prescribed. SNRIs mainly act on the synapses of NA neurons in the CNS and take at least a few weeks to improve pain symptoms[37]. Some studies have suggested that SNRIs inhibit the transmission of nociceptive information [38, 39]. In theory, a few weeks after starting to take SNRIs, the amountof NA in A5-A7 synapses is increased. This may affect theactivities of A5/A7 NA neurons.Alongside SNRIs, selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants are also frequentlyprescribed. SSRIsmainly act on the synapses of 5-HT neurons in the CNS and affect the amountof 5-HT. This may affect the activities of B1/B2 5-HT neurons. We suggest that A5/A7/B2 neurons may represent new therapeutic targets.

There are some limitationstothis study. We did notuseSNRI/SSRI drugs and therefore did notassesshow these drugs affect the activities of A5/A7 NA neurons or B2 5-HT neurons. We focused on nuclei origins located in the brain stem but not on the areas to which they project. In the future, the simultaneous measurement of neuronal activities in nuclei origins and spinal cord will be of acute interest. In addition, we usedacute nociceptive stimuli rather than chronic nociceptive stimuli.In the future, studies usingchronic stimuli will be needed.

In conclusion, acute nociceptive stimuli rapidly increased the activities of A5/A7 NA neurons or B2 5-HT neurons. This suggests that A5/A7 NA neurons or B2 5-HT neurons play important roles in nociceptive processing in the CNS.

Abbreviations

adeno-associated virus (AAV); central nervous system (CNS); descending antinociceptive system (DAS); dopamine (DA); dopamine β-hydroxylase (DBH); intermediolateral cell column (IML); locus coeruleus (LC); noradrenaline (NA); noradrenergic (NAergic); paraformaldehyde (PFA); periaqueductal gray (PAG); phosphate-buffered saline (PBS); photomultiplier tube (PMT); rostral ventromedial medulla (RVM); selective serotonin reuptake inhibitor (SSRI); serotonergic (5-HTergic); serotonin (5-HT, 5-hydroxytryptamine); serotonin noradrenaline reuptake inhibitor (SNRI); standard error of the mean (S.E.M); tetracycline-controlled transactivator transgene (tTA); tryptophan hydroxylase-2 (TPH2); ventral tegmental area (VTA)

Declarations

Acknowledgments

We thank all staff members of the Institute of Laboratory Animal Sciences at Kagoshima University for keeping the animals in good condition. We acknowledge the Joint Research Laboratory, Kagoshima University Graduate School of Medical and Dental Sciences, for the use of their facilities.

Authors’ contributions

SM and TK designed the study. SM, A.Yamashita, JS, HS, and YI conducted the study with the supports of A.Yamanaka and TK. SM and DM analyzed the data and created the figure. SM and TK wrote the manuscript.

Funding

This work was supported by JSPS KAKENHI grants (19K17093 to SM; 20K06858 to A. Yamashita; 16H05130 to TK) and CREST JST (JPMJCR1656 to A. Yamanaka).

The authors have no conflicts of interest to declare.

Availability of data and material

The datasets analyzed in this study are available from the corresponding author uponrequest.

Ethics approval

All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of laboratory animals and approved by the institutional Animal Use Committee of

Kagoshima University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

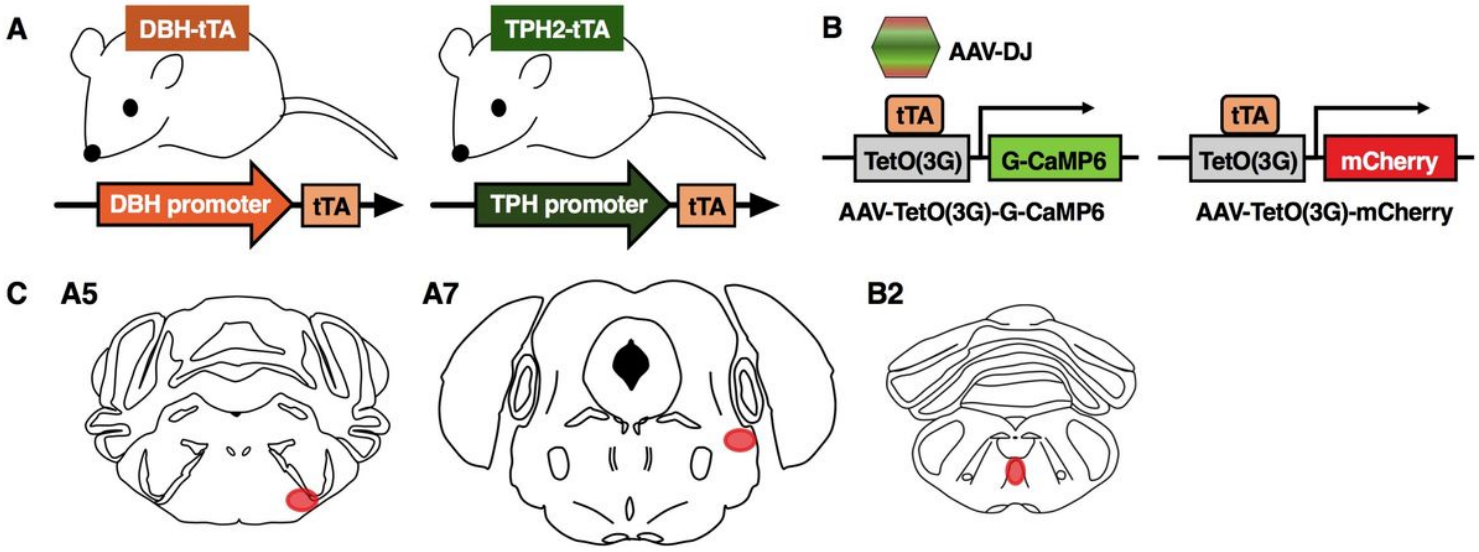


Figure 1

5-HT or NA neuron-specific expression patterns of G-CaMP6 or mCherryfluorescent proteins adopting Tet system. (a)genetic scheme of DBH-tTA mice or TPH-tTA mice, carrying tTA under the control of DBH-tTA or TPH2 promoter. (b) G-CaMP6 and mCherry fluorescent proteins expressed using the tTA/TetO system. (c) AAV injection into the target sites (A5,A7 and B2).

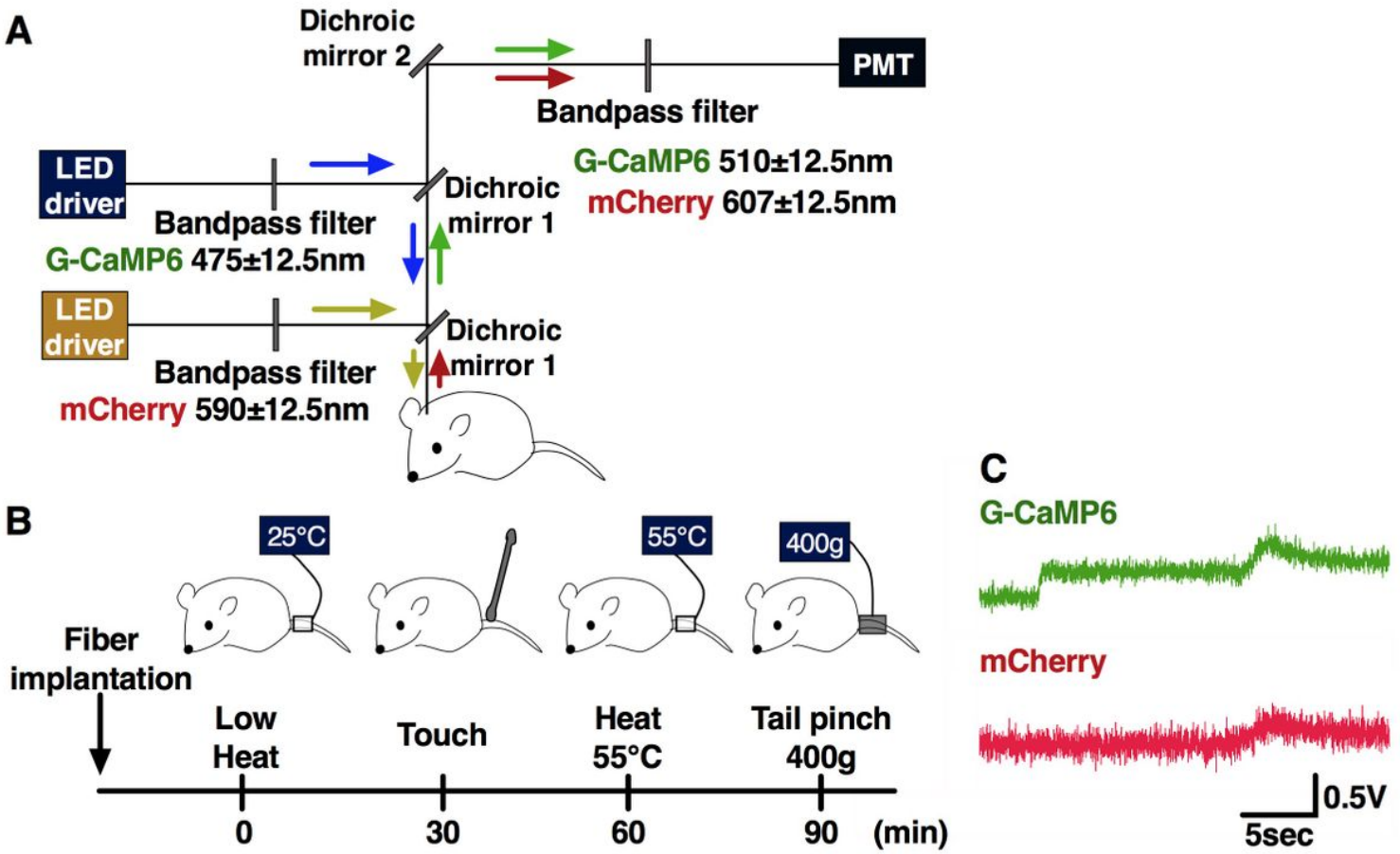
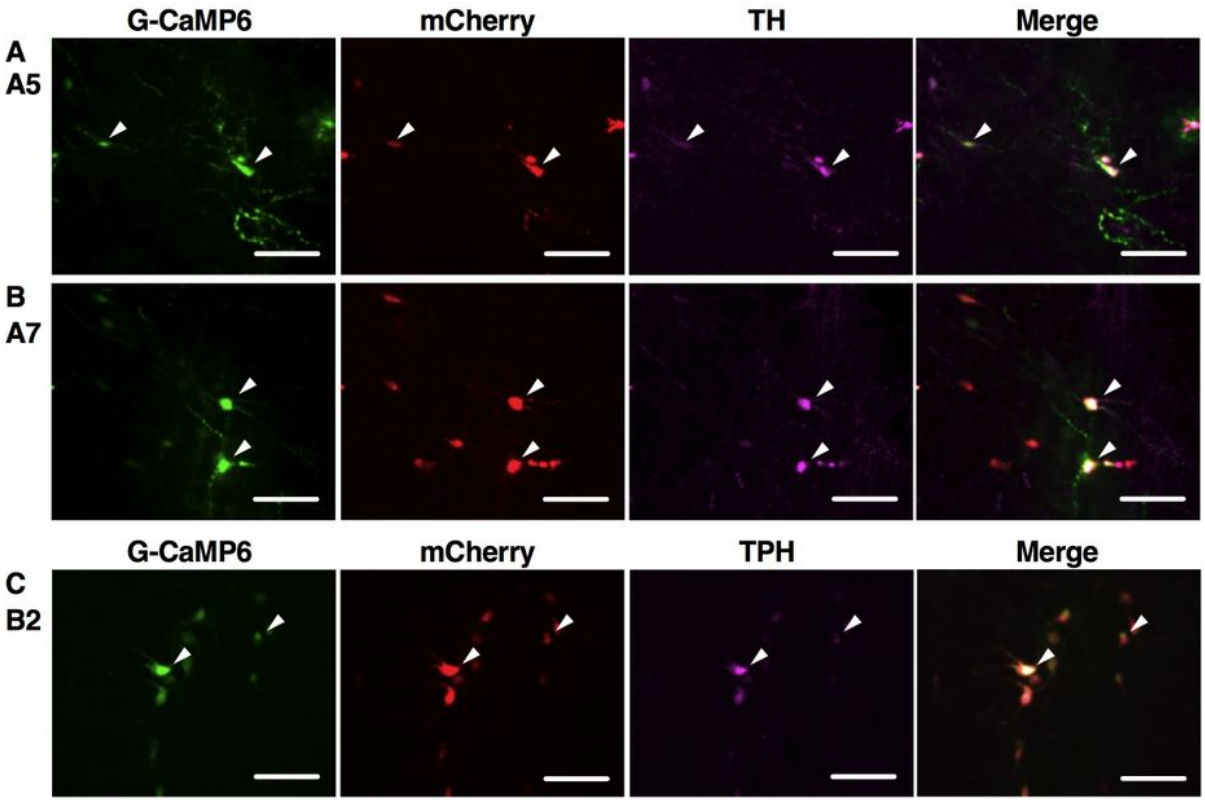


Figure 2

Experimental procedures. (a) Schematic representation of the fiber photometry system with two channels. (b) Recording procedure. Each of the four stimuli deliveredonce to each mouse. We set the inter-stimulus interval to be 30 minutes to reduce any possible effects of previous stimuli. (c) Monitoring of the fluorescence signal intensity. It is confirmed that the fluorescence intensity increased rapidly when the optical position approached the target sites.



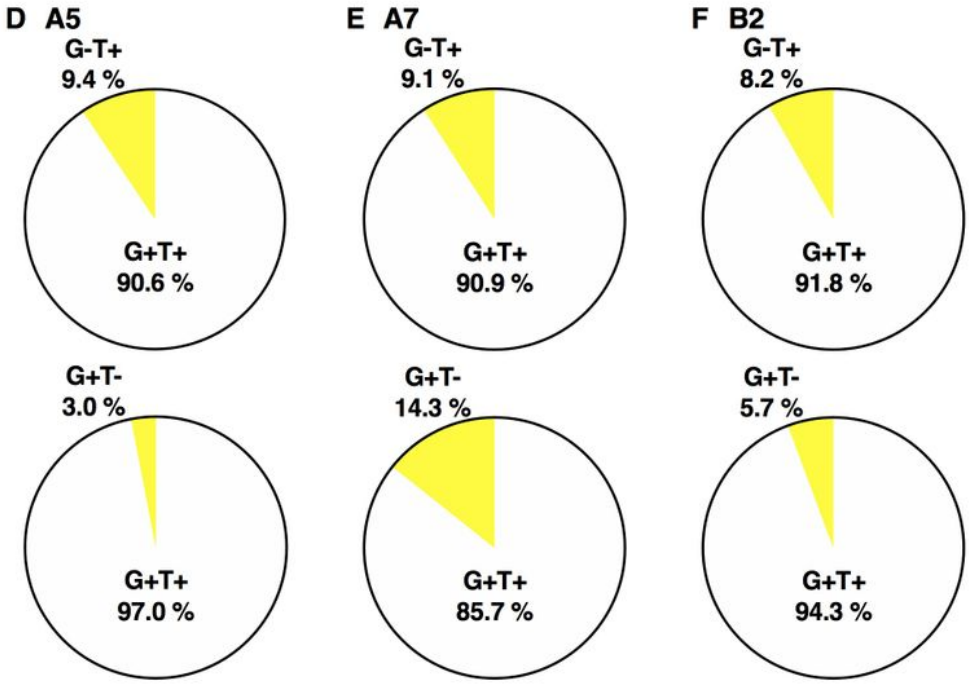


Figure 3

Expression of AAV-induced G-CaMP6/mCherry.(a – c)Specific expression patterns of G-CaMP6/mCherry confirmed in A5/A7 NA neuronal soma (white rectangles) (a, b) and B2 5-HT neuronal soma(white rectangles) (c).TH-positive cells found in the A5 and A7 areas (a, b).TPH-positive cells found in the B2 area (c). Alarge proportion of TH-positive cells expressed G-CaMP6 (A5 area: 90.6%, A7 area: 90.9%)(n = 6, each) (d, e).All G-CaMP6-positive cells expressed mCherry and a large proportion of them expressed TH

(A5 area: 97.0%, A7 area: 85.7%) (d, e).Alarge proportion of TPH-positive cells expressed G-CaMP6 (91.8%) (n = 6, each) (f).All G-CaMP6 positive cells expressed mCherry and 94.3% expressed TPH(f). Scale bar: 100μm.

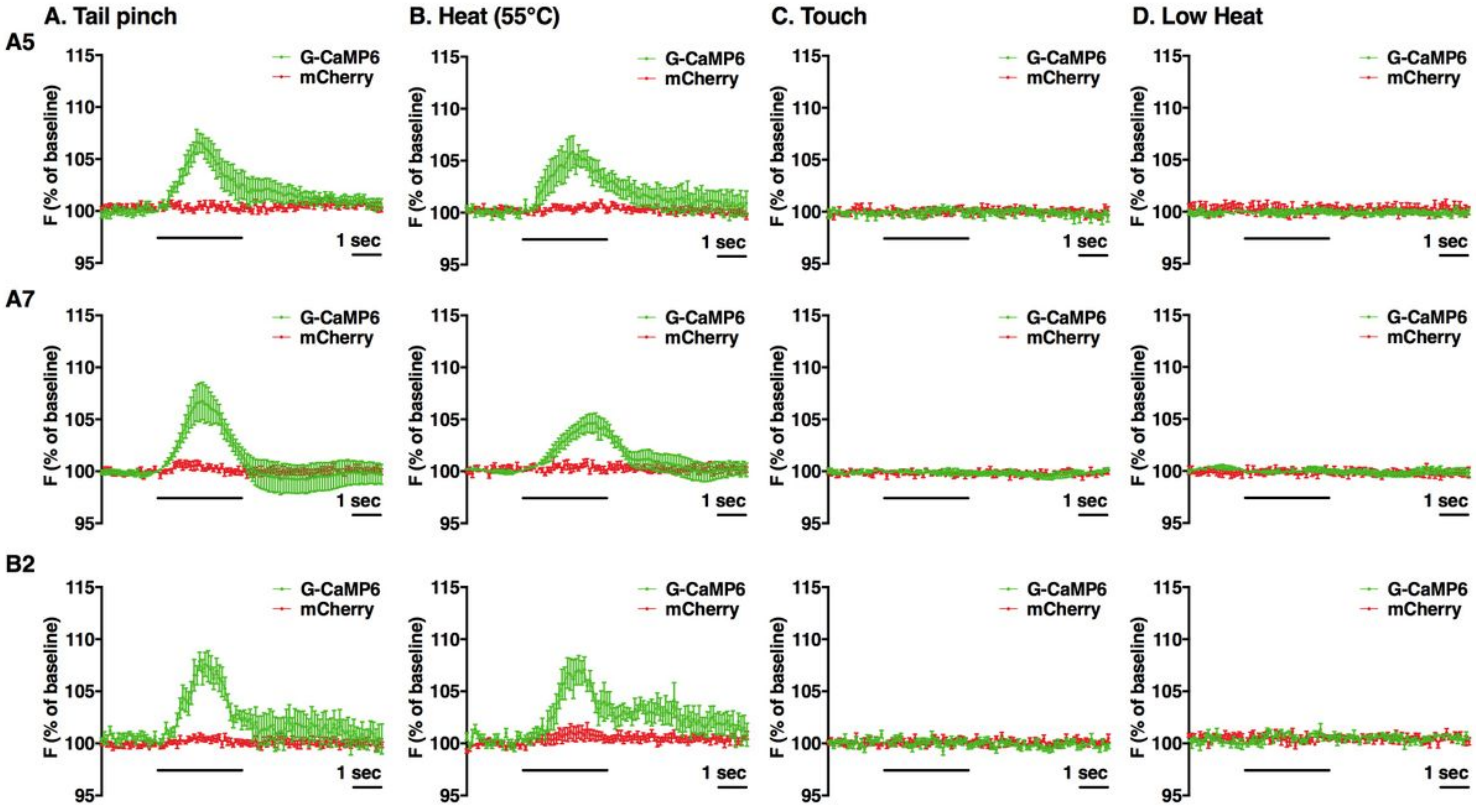


Figure 4

Averaged traces ofG-CaMP6/mCherry (green/red)fluorescence intensity in response to stimuli. (a, b) acute nociceptive stimuli or (c, d)a noninvasive control test (n = 6, each).Brief scale bar: 1sec; long scale bar: 3sec.

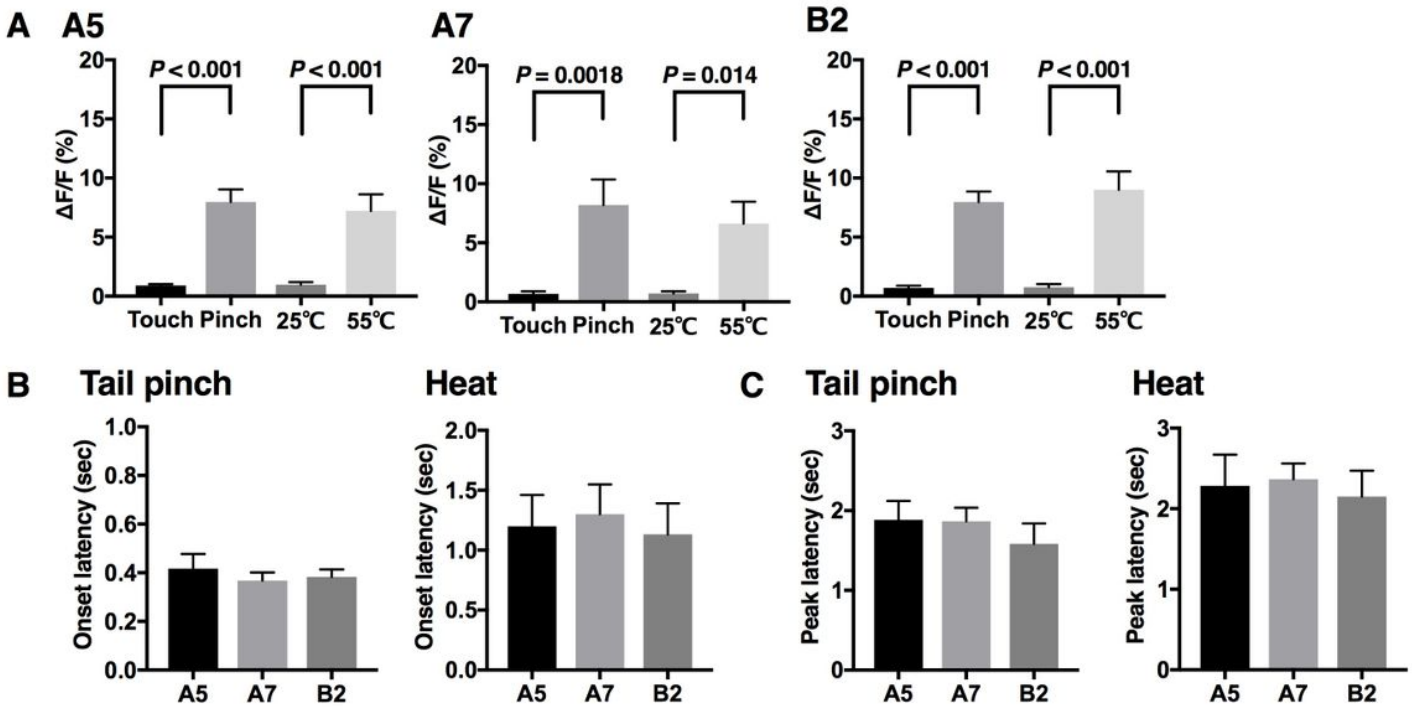


Figure 5

Effects of acute nociceptive stimuli or a noninvasive control test on G-CaMP6/mCherry fluorescence signals. (a) G-CaMP6 fluorescence intensity in all groups rapidly increased by acute nociceptive stimuli but not by a noninvasive control test. (b) The response pattern to a tail pinch and heat stimuli in all groups not significantly different, as indicated bythe statisticalresults of the onset and peak latency values. Data are expressed as values of the mean ± S.E.M and analyzed using a two-way ANOVA withtheSidak'stest (n = 6, each). P values represent the statistical results ofthe Sidak'stest.

Supplementary Files

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