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Histochemical analysis of the biphasic properties of formalin pain-induced behavior

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ABSTRACT

The formalin test has been established as a method for evaluating mouse models of pain. Although there have been numerous reports of formalin-pain-induced behavior, few reports of a detailed histochemical analysis of the central nervous system focus on behavioral biphasic properties.

To investigate the alternation of spinal neuronal activity with formalin-induced pain, we performed immunofluorescent staining with c-Fos antibodies as neuronal activity markers using acute pain model mice induced by 2% formalin stimulation. As a result, phase-specific expression patterns were observed. In the spinal dorsal horn region, there were many neural activities in the deep region (layers V−VII) in the behavioral first phase and those in the surface region (layers I−III) in the behavioral second phase. Furthermore, we conducted comparative studies using low concentrations (0.25%) of formalin and capsaicin, which did not show distinct behavioral biphasic properties. Neural activity was observed only in the spinal dorsal horn surface region for both stimuli.

Our study suggested that the histochemical biphasic nature of formalin-induced pain was attributable to the activity of the deep region of the spinal cord. In the future, treatment strategies focusing on the deep region neuron will lead to the development of effective treatments for allodynia and intractable chronic pain.

1. Introduction

The formalin test has been widely used as an evaluation method for rodent models of pain. Subcutaneous injection of formalin in the sole of the foot induces noxious stimuli in the central nervous system (CNS). Formalin is injected into the hind limbs rather than rodent forelimbs because pain-specific reactions are likely to be observed there [1]. Pain is assessed by pain-induced behavior-elapsed time, such as licking, lifting, and favoring [2,3]. Unlike other pain inducers, formalin-induced pain is known to exhibit a biphasic phase of characteristic pain-related behaviors. This behavior appears as soon as formalin is injected. The first phase of pain-induced behaviors (3−5 min after injection) presents a direct behavior induced by chemical stimulation via C fibers [4]. The rodents do not exhibit pain-induced behavior for 10−15 min, followed by the beginning of the second phase (20−40 min after injection), which presents an indirect behavior induced by an inflammatory reaction derived from formalin.

To date, there have been numerous reports on formalin pain-induced behavior. Meanwhile, histochemical analysis for formalin testing has often been used for comparative studies in drug administration of pain therapeutic drug candidates [5,6]. There have been few reports about detailed histochemical analysis of the CNS focusing on behavioral biphasic properties.

This study aimed to investigate the neural activity status of the spinal cord during the formalin test. We investigated the temporal changes in spinal activity in the mouse spinal cord that induced pain by 2% formalin stimulation.

2. Methods

2.1. Animals

Seven-or eight-week-old male C57BL/6J mice (specific pathogen free; Japan SLC, Shizuoka, Japan) were used in all experiments. The animals were housed at a controlled temperature (23−25 °C) and were supplied with standard rodent pellets and water ad libitum. All animal experiments were carried out according to the protocols approved by the Committee of Animal Experiments of Osaka University (approval

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number 02-003-002) and following the National Institute of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to reduce animal suffering. This study was conducted in compliance with the ARRIVE guidelines.

2.2. Formalin/capsaicin-induced nociceptive response

Formalin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and capsaicin (FUJIFILM Wako Pure Chemical Corporation) were used as pain-producing substances. Formalin was diluted with saline (0.85% NaCl in distilled water) to 2% or 0.25%. Capsaicin was dissolved in dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corporation) and diluted with saline to a concentration of 32 μg/μL. Following 30 min of acclimation, C57BL/6J mice were injected subcutaneously into the dorsal surface of the left hind paw with 20 μL of the above-mentioned pain-producing substance using a microsyringe with a 27-gauge needle. Control mice were injected with 20-μL saline. The number of licking responses was counted over the following period: 5, 10, 20, 40, and 90 min after injection. In addition, the perfusion fixation was performed for immunohistochemical analysis in 3 mice at each time (5, 10, 20, 40, and 90 min after injection).

2.3. Behavior test

The analysis and evaluation of pain-like behavior was performed with reference to the previous paper [7]. After injecting a pain stimulant or saline into the sole of the foot, pain-like behavior was observed for 90 min. Pain-like behavior was evaluated using the licking time as an index since licking is very important in the pain control system [8]. All mouse behavioral tests were videotaped, and movie was used to measure the licking time every 10 min.

2.4. Immunofluorescent staining

The mice were anesthetized with a triple combined anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol) [9]. Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and the fourth and fifth lumbar spine vertebrae (L4/L5) were removed. Spinal tissues were immersed in a 30% sucrose solution for cryoprotection, embedded in a compound, and then frozen on dry ice. Frozen tissue samples were scratched the pain-stimulated side with a knife to identify the right-and-left side, and sliced into 14-μm thick sections in a cryostat. Immunofluorescence staining was performed using floating sections, as previously reported [10]. The slides were immersed in a blocking solution, 0.3% Triton-X, 3% bovine serum albumin in 0.1 M phosphate-buffered saline (PBS) for 30 min and then incubated with a blocking buffer containing rabbit polyclonal antibodies against c-Fos (1:1000; catalog no. ab190289; Abcam Inc., Cambridge, MA), mouse polyclonal antibodies against Neu N (1:100; catalog no. MAB377; Merck KGaA, Darmstadt, Germany), adenomatous polyposis coli (APC; 1:500; catalog no. OP80; Merck KGaA), Glial fibrillary acidic protein (GFAP; 1:100; catalog no. g3893; Merck KGaA), and goat polyclonal antibodies against Ionized calcium-binding adapter molecule 1 (Iba1; 1:500; catalog no. 019–19741; Wako Chemical, Osaka, Japan) at 4 °C overnight. After washing thoroughly in 0.1 M PBS, the slides were reacted with the secondary antibody, that is, the donkey anti-rabbit immunoglobulin G (IgG) polyclonal antibody conjugated to Alexa Fluor 488 (1:500; catalog no. A-21206; Thermo Fisher Scientific, Waltham, MA, USA), donkey anti-mouse IgG polyclonal antibody conjugated to Alexa Fluor 568 (1:500; catalog no. A-10037; Thermo Fisher Scientific) and donkey anti-goat IgG polyclonal antibody conjugated to Alexa Fluor 568 (1:500; catalog no. A-11057; Thermo Fisher Scientific). The slides were washed with 0.1 M PBS, and sealed with an aqueous mounting medium (PermaFluor: Thermo Fisher Scientific). Observations and photographic acquisitions were performed using a BX53 microscope (Olympus Corporation, Tokyo, Japan) and a Keyence

microscope (Keyence Corporation, Osaka, Japan).

2.5. Evaluation of immunostaining signals

The c-Fos-expression sites in the mouse spinal cord were identified using the Atlas of the Spinal Cord: Mouse, Rat, Rhesus, Marmoset, and Human (Academic Press Elsevier, Amsterdam, the Netherlands). Obtained microphotographs were converted to gray scale images and inverted monochrome color tone. Immunostaining signals were evaluated based on both positive cell number and immunostaining intensity under a microscope and above-mentioned 20-fold magnification micrographics. Large red circles (high-intensity c-Fos-positive cells) and small green circles (low-intensity c-Fos-positive cells) dots corresponding to the positive signals were plotted on the mouse atlas images.

3. Results

3.1. Analysis of pain-like behavior for each pain stimulus

First, we examined the pain-like behavior (licking) of mice after 2% formalin stimulation. Licking was well observed for 10 min after stimulation, followed by a slight decrease in licking from 10 to 20 min (Fig. 1). After 20 min, licking increased rapidly, peaked at 40 min, and then decreased. On the other hand, in the saline-injected group, little licking was observed throughout (Fig. 1). These results confirmed that the 2% formalin-injected mice showed a biphasic of pain-like behavior. Subsequently, we also performed 0.25% formalin and capsaicin stimulation, for which no pain-like behavior biphasic has been reported so far. In the 0.25% formalin-injected mice, licking increased after stimulation, but decreased after 20 min (Fig. 1). At 20 min after stimulation, there was no significant difference in licking time between 2% and 0.25% formalin-injected mice (Fig. 1). On the other hand, the capsaicin-injected mice showed licking behavior for only 10 min (Fig. 1). Their licking time was about half that of the formalin-injected mice (Fig. 1). Taken together, it was clarified that 2% formalin-injected mice showed biphasic pain-like behavior, but 0.25% formalin and capsaicin-injected mice showed monophasic pain-like behavior.

3.2. The chronologic alternation of c-Fos expression in the spinal cord of 2% formalin-induced pain model mice

Neuronal activity in the spinal cord after 2% formalin stimulation was examined using fluorescent immunostaining for c-Fos, a neural activity marker. A 2% formalin stimulation of the mouse's left dorsal hind paw resulted in time-dependent alteration of c-Fos expression in the spinal cord dorsal horn. After 5 min of stimulation, a high expression of c-Fos was mostly observed in the bilateral area from lamina 5 of the spinal gray (5Sp) to lamina 7 of the spinal gray (7Sp), and a low expression of c-Fos was scattered over a wide area from lamina 2 of the spinal gray (2Sp) to 7Sp (Fig. 2A). After 10 min, c-Fos positive signals were detected more frequently than those after 5 min. A strong expression was observed in the bilateral 5Sp-7Sp and partly in the lateral region of 2Sp. Meanwhile, a low expression of c-Fos was observed bilaterally in the area of 2Sp-7Sp, as were the cases in 5 min (Fig. 2B). After 20 min, the number of c-Fos positive cells decreased by approximately half compared to that after 10 min. While a high c-Fos expression was observed bilaterally in the medial area of 5Sp and 6Sp, a low expression was scattered in 2Sp-7Sp, as in the case of 5 or 10 min (Fig. 2C). After 40 min, the number of c-Fos positive cells reached a maximum throughout the time course. A high c-Fos expression was concentrated unilaterally in the area of 2Sp-3Sp and 5Sp ipsilateral to the formalin stimulus. Most of the cells weakly expressing c-Fos were spread in the area of 2Sp-7Sp ipsilateral to the formalin stimulus, whereas a small number of neurons with a weak expression of c-Fos were dispersed in the area of 3Sp-6Sp contralateral to the formalin stimulus (Fig. 2D). After 90 min, the number of c-Fos positive cells was
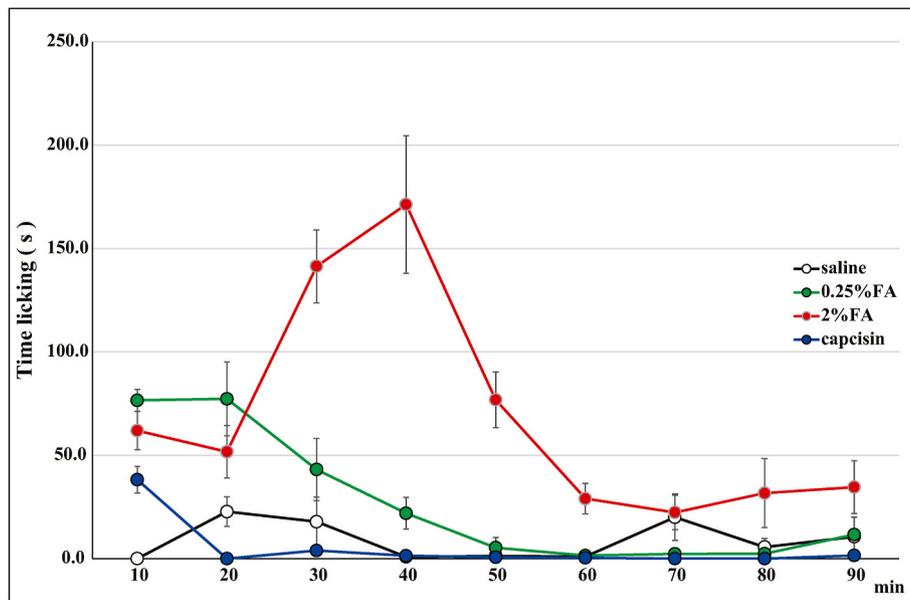
Fig. 1. The analysis of pain-like behavior after injecting a pain stimulant or saline into the sole of the foot. Line graph measured licking time every 10 min for 90min of mice injected with 2% formalin (A; red circle; n = 9), saline (B; white circle; n = 8), 0.25% formalin (C; blue circle; n = 11) and capsaicin (D; green circle; n = 8). FA: Formalin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
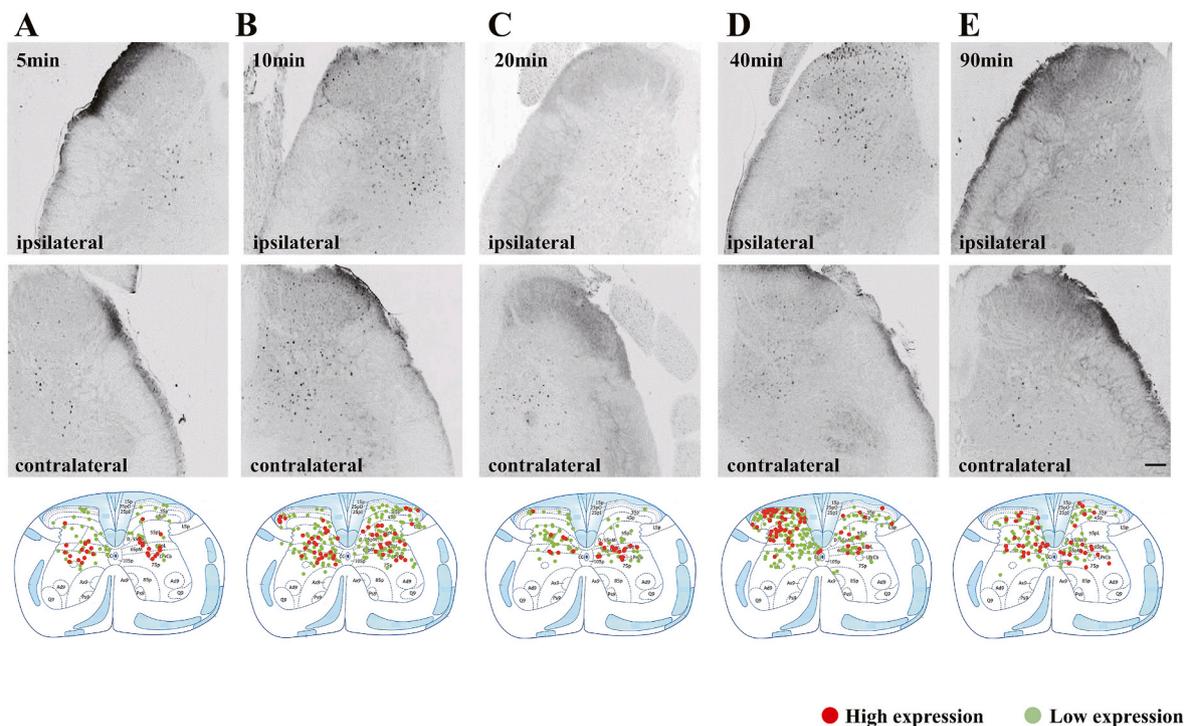
2% formalin

Fig. 2. Immunofluorescence staining of c-Fos positive neurons in the fourth and fifth lumbar spinal cord of 2% formalin-treated mice. (A–E) Upper and middle: Representative microphotographs for immunostaining with c-Fos over the respective periods: 5 (A), 10 (B), 20 (C), 40 (D), and 90 (E) min after injection in the ipsilateral (upper) and contralateral to the injection sides (middle). Bottom: Spinal illustration indicates c-Fos expression in red and green dots. Left: ipsilateral side; Right: contralateral side. Large red dot: strong expression; small green dots: weak expression. N = 3. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

approximately one-third of that after 40 min. Most c-Fos expressions were observed bilaterally in the medial area of 5Sp and partly in 2Sp and 6Sp, while a low expression was bilaterally scattered in 2Sp-7Sp (Fig. 2E).

The immunoreaction of c-Fos expression induced by 2% formalin stimulation was observed biphasically similarly to pain-induced behavior (Fig. 1). The region of high c-Fos-positive cells changed from the bilateral deep area to the spinal dorsal surface area ipsilateral to the

formalin stimulus.

3.3. The chronologic alternation of c-Fos expression in the spinal cord of 0.25% formalin-induced pain model mice

To investigate the correlation between biphasic pain behavior and neuronal activity, we analyzed the chronological alternation of c-Fos expression in the dorsal horn of the spinal cord-induced low-concentration formalin that did not exhibit biphasic pain behavior (Fig. 1). As a result, the expression of c-Fos was much weaker and less than that of 2% stimulation, and no clear biphasic pattern was observed. After 5 min of stimulation, a high expression of c-Fos was observed unilaterally in the 2Sp and 5Sp ipsilateral to the formalin stimulus. In contrast, a low expression of c-Fos was mostly scattered in the 2Sp-7Sp ipsilateral to the formalin stimulus and partly in the 4Sp contralateral to the formalin stimulus (Fig. 3A). After 10 min, there was a slight increase in the number of positive cells compared to that at 5 min (Fig. 3B). A high expression of c-Fos was mainly observed bilaterally in the medial area of 5Sp-7Sp, and a low expression of c-Fos was bilaterally scattered in 4Sp-7Sp. After 20 min, the expression of c-Fos was much higher than that at any time point (Fig. 3C). A high expression of c-Fos was mostly observed in 2Sp-3Sp and partly in 5SpL-7Sp ipsilateral to the formalin stimulus. Although the number was less than that on the ipsilateral side, a high expression of c-Fos was also observed in 2Sp contralateral to the formalin stimulus. Meanwhile, a low expression of c-Fos was mainly scattered in bilateral 2Sp-7Sp. After 40 min, c-Fos expression was lower and wider than that after 20 min (Fig. 3D). A high expression of c-Fos was mainly observed in the area of 2Sp-7Sp ipsilateral to the formalin stimulus, and a low expression of c-Fos was scattered in bilateral 2Sp-7Sp. After 90 min, c-Fos expression was limited and weak (Fig. 3E). A low c-Fos expression was observed bilaterally in 5Sp-7Sp. Reportedly, the pain-like behavior was monophasic when 0.25% formalin was

injected [11]. The immunoreaction of c-Fos expression was also observed to be monophasically similar to pain-induced behavior (Fig. 1). The number of c-Fos expressing cells peaked in the surface layer of the spinal cord and then decreased significantly.

3.4. The chronologic alternation of c-Fos expression in the spinal cord of capsaicin-induced pain model mice

Moreover, we investigated the alternation of c-Fos expression in the spinal cord of mice treated with capsaicin, which is a stimulant with a different mechanism of action that does not exhibit biphasic behavior. Our behavior test has shown that capsaicin injection into the dorsal surface of a mouse hind paw produced transient pain behavior within 10 min after capsaicin stimulation in according with previous study [12] (Fig. 1). Capsaicin stimulation showed a completely different pattern from that of formalin and tended to increase with time. After 5 min of stimulation, c-Fos was almost not expressed (Fig. 4A). After 10 min, although no strong expression was observed, a low expression of c-Fos was observed bilaterally in 5Sp-7Sp (Fig. 4B). After 20 min, a strong expression finally appeared (Fig. 4C). A high expression of c-Fos was observed bilaterally in 5Sp-7Sp, and a low expression of c-Fos was observed bilaterally in the medial area of 2Sp-7Sp. After 40 min, the number of c-Fos-positive cells increased considerably and locally compared to that at 20 min (Fig. 4D). A high expression of c-Fos was mainly observed unilaterally in the area of 2Sp ipsilateral to the capsaicin stimulus, and a low expression of c-Fos was observed in the 2Sp-7Sp of bilateral to the capsaicin stimulus. After 90 min, the number of c-Fos high expression increased from the first time-lapse (40 min after stimulus) (Fig. 4E). A high expression of c-Fos was mainly observed unilaterally in the area of 2Sp ipsilateral to the capsaicin stimulus, as was the case with 40 min; a high expression of c-Fos was also observed in 4Sp-6Sp on the ipsilateral side and 2Sp-3Sp on the contralateral side.

0.25% formalin

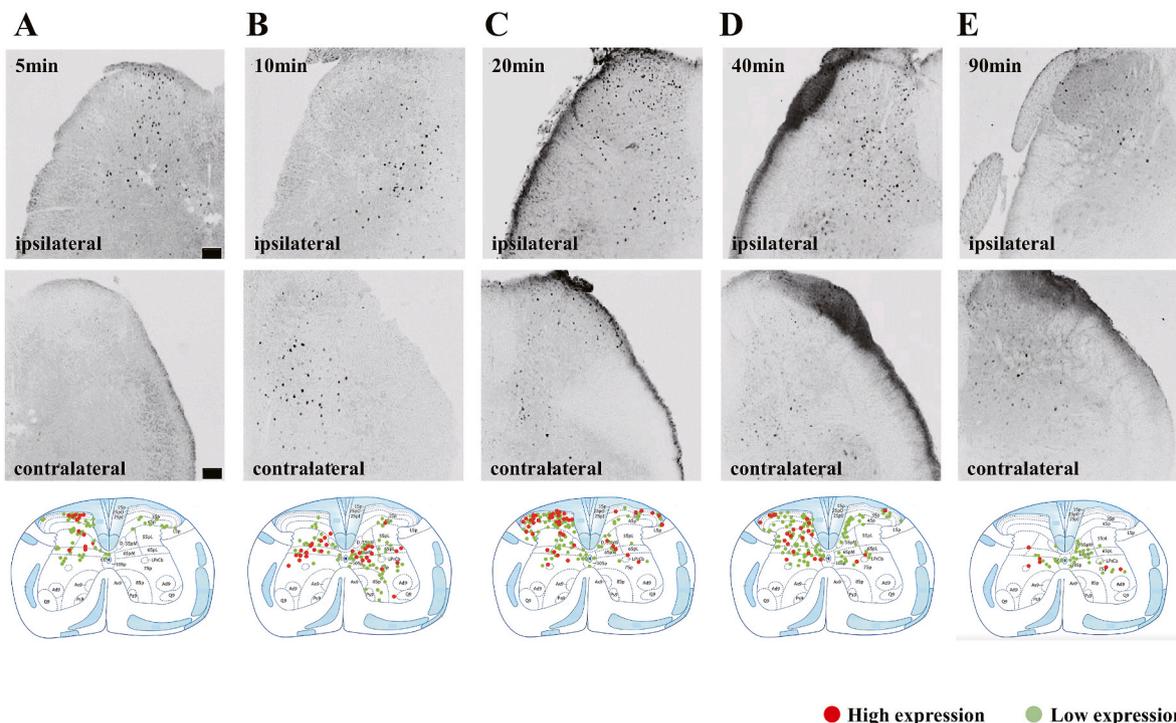● **High expression**   ● **Low expression**

Fig. 3. Immunofluorescence staining of c-Fos positive neurons in the 0.25% formalin-treated mouse fourth and fifth lumbar spinal cord. (A–E) Upper and middle: representative microphotographs for immunostaining with c-Fos over the respective periods: 5 (A), 10 (B), 20 (C), 40 (D), and 90 (E) min after injection in ipsilateral (upper) and contralateral to the injection side (middle). Bottom: Spinal illustration indicates c-Fos expression in green dots. Left: ipsilateral side; Right: contralateral side. Large red dots: strong expression, small green dot: weak expression. N = 3. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
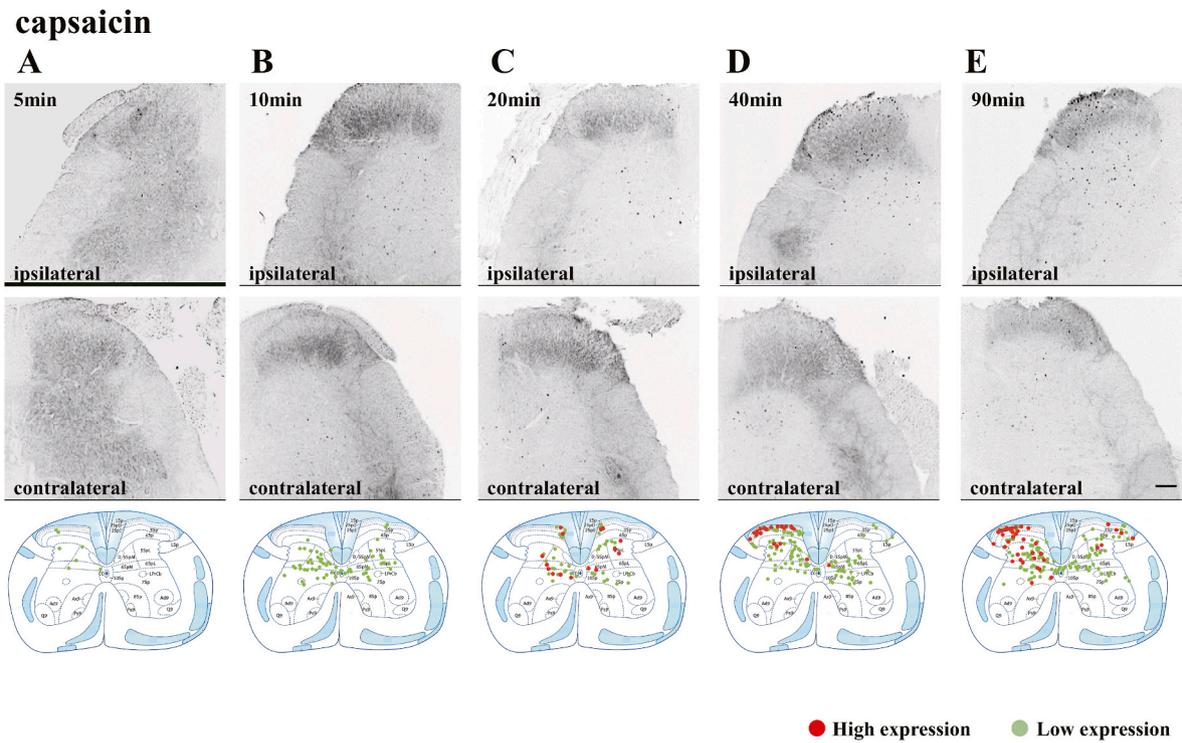
Fig. 4. Immunofluorescence staining of c-Fos positive neurons in the capsaicin-treated mouse fourth and fifth lumbar spinal cord. (A–E) Upper and middle: representative microphotographs for immunostaining with c-Fos over the respective periods: 5 (A), 10 (B), 20 (C), 40 (D), and 90 (E) min after injection in the ipsilateral (upper) and contralateral to the injection sides (middle). Bottom: Spinal illustration indicates c-Fos expression in red and green dots. Left: ipsilateral side; Right: contralateral side. Large red dot: strong expression, small green dots: weak expression. N = 3. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
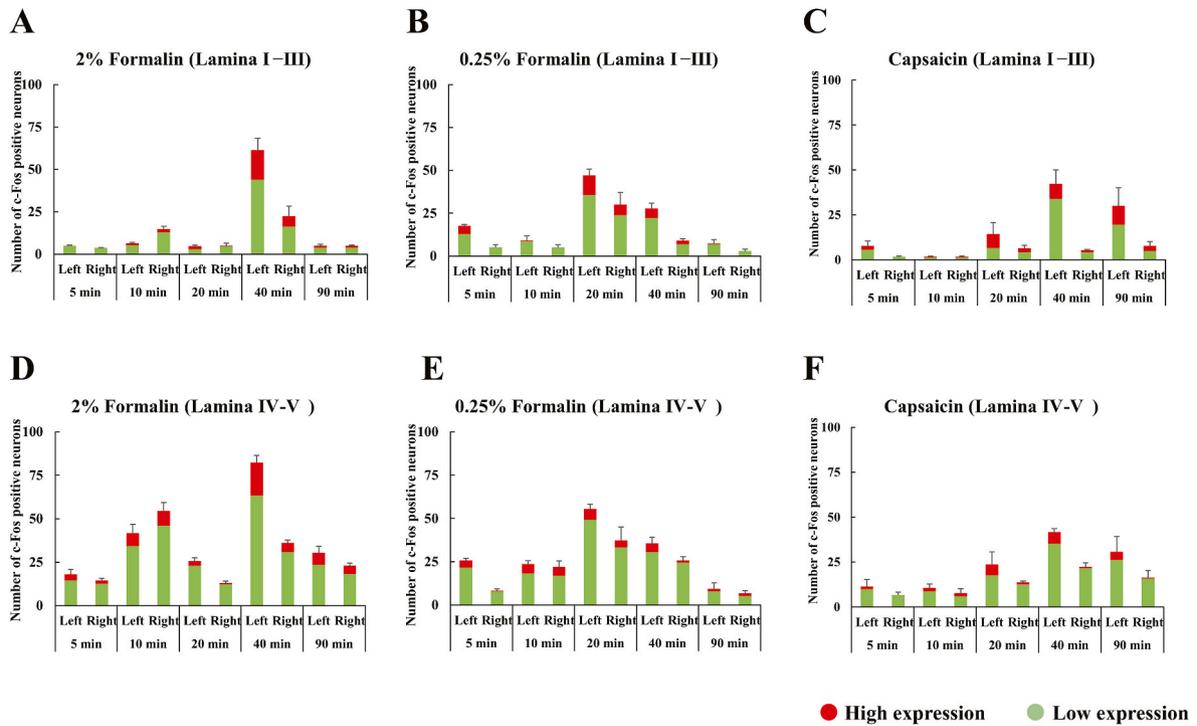

Fig. 5. Comparison of chronologic alternation of c-Fos expressing cell number between each pain stimulus. (A–F) Bar graph measured the c-Fos-expressing cell number in the layers I-III, (A–C) and IV-V (D–F) over the respective periods: 5, 10, 20, 40 and 90 min after injection in the ipsilateral (Left) and contralateral to the injection sides (Right) of mice injected with 2% formalin (A,D), 0.25% formalin (B,E) and capsaicin (C,F). Data are expressed as mean ± SEM of three mice per group.

Meanwhile, a low expression of c-Fos was observed bilaterally in 2Sp-7Sp. Unlike formalin stimulation, capsaicin stimulation did not show a rapid increase in expression in a short period of time but increased slowly and surely, showing unilaterality.

3.5. Comparison of chronologic alternation of c-Fos expressing cell number between each pain stimulus

Subsequently, the number of c-Fos positive cells was counted for each pain-inducing stimulus. In the layer 1–3, all stimuli showed monophasic (Fig. 5A–C). However, the capsaicin-stimulated group showed a gradual increase from 20 min and reached a peak at 40 min, whereas the formalin-stimulated groups showed a sudden peak and then a gradual decrease. Peak times differed between the formalin-stimulated groups (2% formalin: 40 min, 0.25% formalin: 20 min). On the other hand, in the layer 4–5, the 2% formalin stimulation group showed biphasic (peak: 10 and 40 min), whereas the other stimulation groups showed monophasic (0.25% formalin peak: 20 min, capsaicin peak: 40 min; Fig. 5D–F). The total cell number and strong expression of c-Fos-positive cells at the peak time of the 2% formalin-stimulated group were significantly higher than those of the other stimulated groups. Taken together, it was suggested that the histochemical biphasic properties in the 2% formalin-stimulated group was involved in cFos-expressing cells localized at the deep layers.

3.6. Identification of c–FOS–expressing cell types induced by 2% formalin stimulation

Finally, to examine the types of c-Fos-expressing cells induced by 25 formalin stimulation, immunostaining using each neuronal marker antibody and c-Fos antibody was performed. NeuN was used as a neuron marker, APC as an oligodendrocyte marker, GFAP as an astrocyte marker, and Iba1 as a microglial marker. As a result of immunostaining using 2% formalin-treated samples, c-Fos-expressing cells in the deep layer 10 min and in the superficial layer 40 min after stimulation were NeuN-positive (Fig. 6). All three types of glial markers were negative.

Taken together, it was clarified that the c-Fos expression-positive cells induced by 2% formalin stimulation are neurons.

4. Discussion

We demonstrated that spinal neuronal activity induced by formalin stimulation changed biphasically following pain-like behavior. For 2% formalin-injected mice, the immunoreaction of c-Fos expression was first observed in the deep area of the mouse spinal cord, and then the phase changed to the dorsal surface area of the mouse spinal cord. We revealed that mice induced by both 0.25% formalin and capsaicin did not show clear biphasic c-Fos expression similar to pain-like behavior. At 0.25% formalin concentration, the number of c-Fos expressing cells peaked in the surface layer of the spinal cord 20 min after stimulation and then decreased significantly. During capsaicin stimulation, c-Fos positive cells were frequently observed in the surface area of the spinal cord for the first time at 40 and 90 min after stimulation. During 2% formalin stimulation, a strong expression of c-Fos was observed in the deep spinal cord and the spinal cord surface layer. During other pain stimulation, a strong expression of c-Fos was only observed in the spinal cord surface layer. Moreover, c-Fos expression induced by 2% formalin stimulation was observed in neurons. We revealed that the biphasic pain induced by formalin stimulation was attributed to neuronal activity based on c-Fos expression in the deep spinal cord.

The spinal dorsal horn is an important area for nociceptive signal transduction and processing. Acute nociceptive stimuli are transmitted to neurons in the dorsal horn superficial layer via myelinated Aδ and unmyelinated C fibers. Nociceptive information is transmitted to the upper CNS by a pain transmission system, such as the lateral spinothalamic tract [13,14]. Thus, nociceptive information from the skin is transmitted to the ipsilateral spinal cord surface layer. For the spinal cords of mice injected with 2% formalin, a strong signal peak was observed in the ipsilateral surface layer in the second phase, but a signal peak in the first phase was observed in the deep layers on both sides. Since formalin is injected only into the unilateral sole and nociceptive information from the stimulation is considered transmitted unilaterally to the spinal cord, we questioned these results (Fig. 1). In most reports of biphasic pain-like behavior associated with the formalin test [1–3], such behavior suddenly reached a peak as soon as stimulation (the behavioral first phase) and then settled down and reached a second peak again. Our behavior test confirmed that the pain-like behavior after formalin
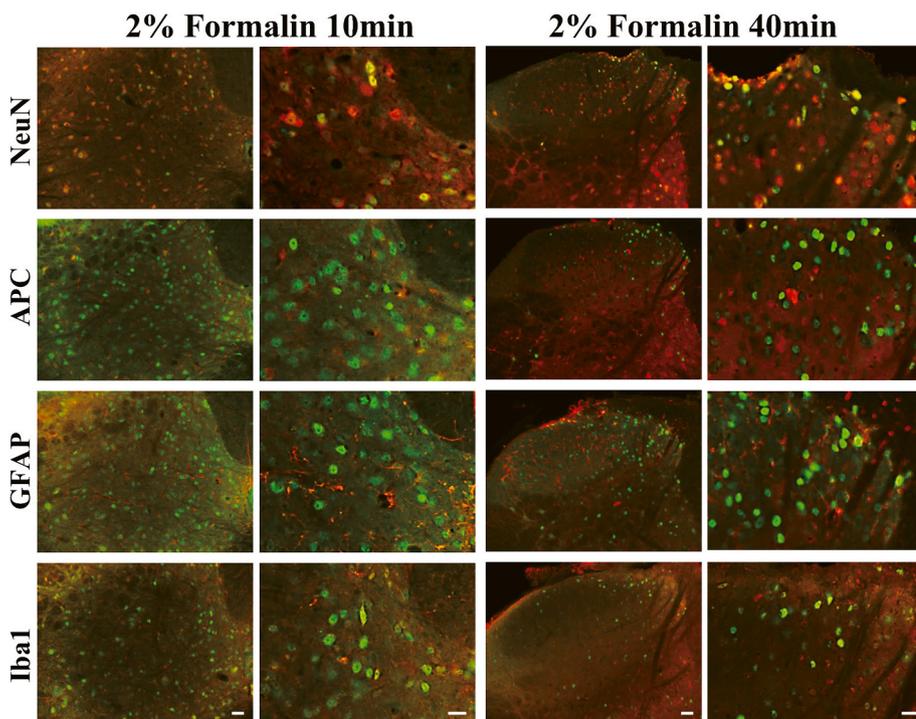

Fig. 6. Immunofluorescence staining for c-Fos and each neuronal marker in the fourth and fifth lumbar spinal cord of 2% formalin-treated mice. Low (left) and High magnification (right) at 10 min (left two panels) and 40min (right two panels) after 2% formalin treatment. Representative microphotographs for immunostaining with c-Fos (green) and neuronal marker: first (NeuN), Second (APC), Third (GFAP) and fourth (Iba1) in order from the top. N = 3. Scale bar: 40 μm (Low) and 20 μm (High magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

stimulation was observed biphasically (Fig. 1). We speculated that the neuronal activated signals were observed on the surface layer until 5 min after stimulation. In the spinal cords of mice injected with 0.25% formalin, a unilateral and superficial weak signal was observed 5 min after the stimulation, probably because the inhibitory effect of the descending pain inhibitory system on weak nociceptive stimulation is weak (Fig. 3). The superficial signal of the dorsal horn then disappeared once and was expressed 20 min after the formalin injection. Thus, although the number of signal-positive cells was small and unclear, it was considered that biphasic-like neuronal activity was observed at low concentrations of formalin. These results support our hypothesis. The first phase of formalin-induced pain-like behavior is caused by the activation of sensory nerve endings in the skin induced by formalin itself [1,15,16]. Since pain suppression works strongly against strong stimulation, the strong activity of nerve cells in the deep spinal cord in response to strong suppression action from the midbrain and pons was observed as the activity of the first phase in the spinal cord of mice injected with 2% formalin [17–19] (Fig. 2). The histochemical biphasic nature of formalin-stimulated neuronal activity is thought to be the result of a clear separation of the pain response to formalin's chemical stimulus and the pain response to inflammatory immune stimulus by the pain inhibitory response to the initial pain response.

Regarding the biphasic pain-like behavior in the formalin test, the first phase reflects the direct stimulation of the primary afferent fibers (neuropathic pain), and the second depends on peripheral inflammation (inflammatory pain) [1,15,16]. Low-concentration formalin is weak as a stimulus, and the pain-suppressing effect appears immediately so that the weak nociceptive stimulus returns to the normal state in a short time. In the case of strong nociceptive stimulation, it takes time for the pain-suppressing effect to appear, so the activity of the dorsal horn is observed for a long period of time. Since the interphase period between phase 1 and phase 2 results from formalin-induced hyperpolarization and temporary inactivation of spinal nerve cells, it became long in proportion to the amount of residual formalin at the injection site [6]. It was found that the histochemical biphasic pattern of formalin stimulation was clearly observed with high-concentration stimulation and unclearly with low-concentration stimulation.

Capsaicin, an agonist of the Transient Receptor Potential Vanilloid 1 receptor (TRPV1), is often used to prepare neuropathic pain models [12, 20]. TRPV1 is expressed not only on c-fibers but also on a few α-delta fibers [21]. Pain-like behavior is monophasically observed because capsaicin stimulation induces only a direct response to TRPV1-localized C-fibers primarily involved in inflammatory heat pain sensation [22]. Capsaicin-induced pain-like behavior peaked immediately after injection, lasted for approximately 10 min, and was rarely observed thereafter (Fig. 1). Neuronal activity on the surface of the dorsal horn was observed 40 min after injection, which was after the end of pain-like behavior. Rats treated with morphine or cocaine did not show pain-like behavior, but c-Fos expression in the dorsal horn was observed in both [23,24]. c-Fos cannot be detected at a quantifiable level unless expressed strongly and moderately for a certain period [25,26]. Capsaicin is a TRRV1-specific agonist, whereas formalin is an agonist of ankyrin-like with transmembrane domains 1 and TRPV1 [15,27]. Capsaicin has a localized site of action, and its nociceptive stimulus is not as strong as formalin, so it may take some time to integrate the stimulus to induce the expression of c-Fos. According to reports, capsaicin-induced neural activation of the dorsal horn subsided in 3 h, but formalin lasted for 6 h [28]. In the case of capsaicin stimulation, licking and biting as pain-like behaviors were hardly observed after 20 min of stimulation (Fig. 1). The capsaicin-stimulated group moved around very well in the analysis cage. Licking and biting may not have been possible because the mice moved around a lot to relieve pain. On the other hands, c-Fos was not expressed in all activated neurons [29]. No expression of c-Fos was observed in the dorsal root ganglion neurons or thalamic ventral posterolateral nucleus neurons under-stimulation [30–32]. For strong nociceptive stimuli, c-Fos expression may be

effective as an index in the histochemical analysis of behavior-related neural activity; for weak nociceptive stimuli, it is essential to consider other nerve activation markers, such as Fos B and phosphorylated extracellular signal-regulated kinase.

Our immunostaining assay was revealed that nerve cells were activated when stimulated with formalin (Fig. 6). Two types of noxious stimulus-receptive neurons in the spinal dorsal horn are essential for the transmission of pain signals. One is nociceptive-specific neurons mainly localized in superficial 1Sp and outside of 2Sp and respond only to strong noxious stimuli by Aδ and C fibers. The other is the wide dynamic range (WDR) neurons often observed in 5Sp. They were excited by a wide range of stimuli, such as heat, mechanical, and chemical stimuli via Aδ, Aβ, and C fibers, and encoded their intensity [33–35]. There are two transmission pathways to the upper CNS of WDR neurons: the lateral pathways involved in identifying pain projecting to the somatosensory area via the posterior ventral nucleus of the thalamus, and the medial pathways involved in pain emotions and cognition projecting the anterior cingulate gyrus and insular cortex through the medial nucleus of the thalamus [36]. WDR neurons are greatly involved in the intensity and discomfort in pain sensation, but a large difference in the breadth of their receptive fields is observed depending on the type of stimulus. While the receptive fields for tactile and pressure stimuli are relatively localized, WDR neurons have a wide receptive field for noxious stimuli [37]. Unlike other neurons, WDR neurons are known to increase neuronal excitability when noxious stimuli persist [38]. These characteristics of WDR neurons are significantly involved in allodynia and chronic pain formation [34,35]. Although formalin-induced pain is initially severe pain due to neuropathic pain for a short period of time, the second formalin-induced pain is longer than the first, and the pain area feels wider. This formalin-induced pain is remarkably similar to pain associated with trauma. The wind-up of WDR neurons was induced by stimulation with C fibers [39]. Although C fiber activity against formalin stimulation is consistent with pain-like behavior in the first phase, that in the second phase exhibits only weak excitement enough to not generate the pain-like behavior [4,40]. The activity pattern of dorsal horn neurons is consistent with the pain-like behavior [41]. Since the activity of C fibers is not so high in the second phase and the activity of the posterior horn cells is increased, it is considered that wind-up in WDR neurons contributes to the enhancement of the pain in the second phase. The neural activity of the first phase is directly stimulated by formalin, whereas that of the second phase is due to central sensitization. Unlike capsaicin and low-concentration formalin, a large number of strong signals are observed in layers 2–3 and 5 during all periods after stimulation. In fact, Fig. 5 revealed that only the number of expressing cells in layer 4–5 under 2% formalin stimulation shows chronological biphasic properties, and these layers are very important in the histochemical biphasic properties under formalin stimulation. The 5th layer is occupied by many WDR neurons. Persistent pain induces constant activation of layer 5 WDR neurons, resulting in allodynia and chronic pain. In the future, treatment strategies focusing on WDR neurons will lead to the development of effective treatments for allodynia and chronic pain.

In the present study, we analyzed the chronological alternation of neuronal activity based on the c-Fos expression in the spinal cord stimulated by three pain-producing substances. Our study suggested that the histochemical biphasic nature of formalin-induced pain was attributable to the activity of the deep region of the spinal cord. Our results are of great importance as they suggest new implications for the role of c-Fos on neuronal activity. On the other hands, biphasic pain-like behavior induced by formalin stimulation has been evaluated by follow-up study until now. With this method, there is a risk of prejudice because the analysis is done by humans. However, mechanical analysis of weight-bearing mice was an objective evaluation method and demonstrated a biphasic nature of pain-like behaviors with formalin stimulation [42]. In the future, the immunostaining using the c-Fos antibody in this study will become objectively method to evaluate biphasic nature of pain-like

behavior induced by formalin stimulation.

Data availability

No data was used for the research described in the article.

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