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Sorting nexin 25 regulates C5a-induced nerve growth factor expression in macrophage-like J774.1 cells

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Although the complement cascade is the basis of innate immunity, little is known about its mechanism. NGF acts as an important mediator of the cascade acting downstream of the C5a receptor 1 (C5aR1). Although NGF is known to be involved in inflammatory pain, the mechanism by which NGF causes pain remains unclear. It is known that an injection of Complement 5a (C5a) into the hind paw skin of mice elicits painful behavior and enhances NGF expression in dermal macrophages. Sorting Nexin25 (SNX25) is part of a family of proteins that play important roles in membrane trafficking, intracellular signaling, membrane remodeling, and organelle motility, some of which also contribute to the immune system. Using the macrophage cell-line, J774.1, we examined whether SNX25 is involved in C5a-induced NGF expression at the cellular level. Treatment of J774.1 with C5a enhanced NGF and SNX25 expression. Western blotting revealed that C5a-induced NGF expression was reduced by *Snx25* siRNA. *Snx25*+/− mice had lower NGF levels and showed a pain-insensitive phenotype in response to C5a. We propose that the expression level of C5a-induced NGF is controlled by macrophages through SNX25 signaling. (J Osaka Dent Univ 2023; 57: 55-62)

Key words: Macrophage; SNX25; NGF

INTRODUCTION

Tissue damage in the skin causes the release of inflammatory mediators by nonneural cells that reside within or infiltrate into the injured area, including macrophages, mast cells and neutrophils. These inflammatory mediators such as nerve growth factor (NGF), serotonin, histamine, glutamate and ATP act directly on the nociceptors. Although peripheral sensitization after tissue injury is well described in the skin,[1] the role of inflammatory cells under acute pain sensation is poorly understood.

The complement system is a critical part of innate immunity, serving as the first line of defense against infection and conditions associated with tissue damage. Recent studies have highlighted the importance of the complement cascade in the regulation of nociceptor function and pain processing.[2] Among the many components of the complement system, C5a is a highly potent proinflammatory and pronociceptive product that is rapidly generated in response to injury or infection.[2] C5a acts primarily through a canonical G-protein-coupled receptor, C5aR1. Shutov *et al.* showed that C5a-induced macrophage activation via C5aR1 results in mobilization of NGF and NGF/TrkA-dependent sensitization of TRPV1 in nociceptive fibers.[2] NGF is expressed in immune cells including macrophages and facilitates pain transmission by sensory neurons through a variety of mechanisms. Although the level of NGF content should be maintained within an optimal range for evading noxious pain sensa-

tion, the mechanisms underlying its regulation remain to be determined.

SNX25 is one of the SNX family involved in membrane trafficking, cell signaling, membrane remodeling and organelle motility.[3] SNX plays an important role in membrane transport, cell signaling, membrane remodeling, and organelle motility, some of which are involved in the immune system.[4-6] However, little is known about the relationship between SNX25 and the immune system. Recently, we found that SNX25 modulates NGF levels and sets pain sensitivity under normal condition in macrophages.[14] However, the role of SNX25 in NGF expression and pain regulation in inflammatory conditions remains unclear. In this study, we investigated whether NGF was regulated by SNX25 in response to C5a stimulation in J774.1 cells. Treatment of J774.1 with C5a increased NGF and SNX25 expression at 30 min. Western blotting showed that C5a-induced NGF expression was attenuated by *Snx25* siRNA. Additionally, *Snx25*＋/− mice had lower NGF levels and showed a pain-insensitive phenotype in response to C5a. These findings demonstrate that SNX25 is an important factor in C5a-induced NGF expression.

MATERIALS AND METHODS

Animals

C57 BL/6 mice were purchased from Japan SLC, Shizuoka, Japan. SNX25 constitutive KO (*Snx25*＋/−) mice (B6/N-*Snx25* tm 1a/Nju, strain name: B6/N-*Snx25* tm 1 a/Nju, strain number: T 001400) were obtained from Nanjing BioMedical Research Institute (NBRI) of Nanjing University. All the protocols for the animal experiments were approved by the Animal Care Committee of Nara Medical University in accordance with the policies established in the NIH Guide for the Care and Use of Laboratory Animals.

Behavioral test

The 50% withdrawal threshold to mechanical stimuli in the paws was assessed using von Frey's filaments based on the up-down method developed by Chaplan.[7] Animals were acclimatized for at least 15 min in individual clear acrylic cubicles (10×10×10 cm) placed on top of an elevated wire mesh. Quick withdrawal or licking of the paw within the 3 sec stimulus was considered a positive response. Threshold values were derived according to the method described by Chaplan.[7]

Cell culture

The macrophage cell-line, J774.1 was cultivated in Roswell Park Memorial Institute (RPMI) 1640, supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown at 37°C in a humidified chamber containing an atmosphere of 95% air/5% CO_2.

Western blotting

To prepare whole cell lysates, cells were lysed with RIPA buffer (10 mM Tris, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, and 0.1% sodium dodecyl sulfate (SDS)) supplemented with sodium vanadate and protease inhibitor cocktail (L9A7903; Nacalai Tesque, Japan). They were then sonicated. The homogenates were centrifuged at 15,000 rpm for 5 minutes and the protein concentration in the supernatant was measured using a Micro BCA protein assay kit (23225; Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein per lane were electrophoresed on an SDS-polyacrylamide gel and transferred to the polyvinylidene difluoride membrane. After blocking 5% skimmed milk in phosphate buffered saline containing 20% Tween 5 for 1.5 hours, the target protein was incubated at 4°C overnight and bound with anti-SNX25 (13294-1-AP; Proteintech, Rosemont, IL, USA), anti-NGF (sc-548; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-GAPDH (ABS 16, Burlington, MA, USA) antibodies. An anti-rabbit horseradish peroxidase-binding antibody was used as the secondary antibody, followed by an enhanced chemiluminescent Western blotting detection reagent (297-72403; Wako, Osaka, Japan).

Real-time PCR

J774.1 was cultured on a 12-well plate at a density

of 9×10^6 cells per well. Total RNA was extracted using the NucleoSpin RNA kit (740955; Takara Bio, Shiga, Japan) and reverse transcribed with a random primer using the Quantitect reverse transcription kit (205311; Qiagen, Hilden, Germany). Real-time quantitative PCR was performed using a Thermal Cycler Dice Real Time System (Takara Bio), with THUNDERBIRD SYBR qPCR Mix (QPS-201; Toyobo, Osaka, Japan). PCR primers used in this study were as follows: *β-actin* sense primer, 5'-AG CCATGTACGTAGCCATCC-3'; *β-actin* antisense primer, 5'-CTCTCAGCTGTGGTGGTGAA-3'; *C5ar* sense primer, 5'- TACTCGGTGGTGTTCCTGGT-3'; *C5ar* antisense primer, 5'- ACTATACAGGCGGTG GCATC-3', *Ngf* sense primer, 5'-TCAGCATTCCCT TGACACAG-3'; *Ngf* antisense primer, 5'-GTCTGA AGAGGTGGGTGGAG-3'; *Snx25* sense primer, 5'-CATGGATCGTGTTCTGAGAG-3'; *Snx25* antisense primer, 5'-GAAGTCATCTAAGAGCAGGATGG-3'.

Snx25 siRNA transfection

Snx25 siRNA or scramble siRNA, were transfected to J774.1 using Lipofectamine RNAiMAX transfection reagent (13778030, Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

Quantification and statistical analysis

Quantifications were performed from at least three independent experimental groups. Data are presented as mean ± SEM. Statistical analyses were performed using Student's t-test. Statistical significance was set at $*p < 0.05$, $**p < 0.01$. The number of samples (n) are indicated in the Figures.

RESULTS

NGF and SNX25 are expressed in the macrophages

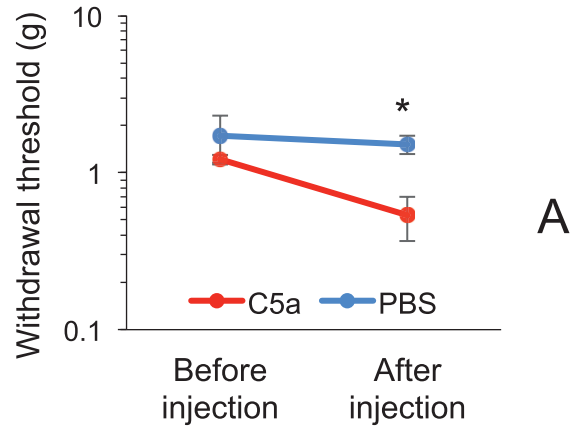
Previous reports have described a C5a-induced mechanical hypersensitivity in rodents.[2] First, C5a and PBS were injected into hind paw skin, and 2 hr later, the von Frey test was performed. The pain sensation was more sensitive when C5a was injected into hind paw skin (50% threshold, 0.54 ± 0.17 g) compared to PBS (50% threshold, 1.51 ± 0.20 g, $p = 0.02$) (Fig. 1 A). It has been reported that NGF derived from macrophages is important factor in C5a-induced hyperalgesia.[2] We confirmed that NGF was expressed in macrophages in hind paw dermis (Fig. 1 B). To investigate the roles of SNX25 in NGF production and pain sensation, we examined the expression of SNX25 in macrophages. Immunohistochemistry revealed that SNX 25 was expressed in the macrophages of the hind paw skin (Fig. 1 C).

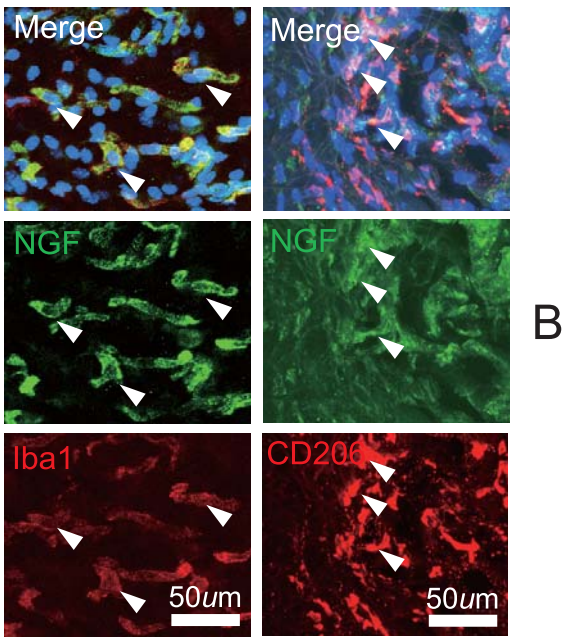
C5a induces NGF and SNX25 in macrophage cell-line J774.1 cells

To investigate the relationship between SNX25 and NGF, we used C5a treated macrophage-like cell-line, J774.1 cells. Western blot analysis revealed that the expression levels of both NGF and SNX25 were increased at 0.5 h after C5a treatment (Figs. 2 A-C). We hypothesized that SNX25 regulates C5a-induced NGF expression. To test this hypothesis, we investigated changes in C5a-induced NGF expression in J774.1 cells following exposure to *Snx25* siRNA (Fig. 2 D). We confirmed that the expression of SNX25 was suppressed by siRNA (0.35 ± 0.14 fold of scramble siRNA, $p = 0.055$; Figs. 2 E and F). As expected, NGF expression was reduced by *Snx25* siRNA (0.38 ± 0.15 fold of scramble siRNA, $p = 0.019$; Figs. 2 E and G). These results suggest that C5a-induced NGF expression is regulated by SNX25.

C5ar1 expression was not changed in SNX25-knocked down cells

C5a-induced NGF expression may have decreased due to decreased expression of C5aR1 as a result of SNX25 being knocked down. Therefore, we investigated whether the expression level of *C5ar1* mRNA was changed in SNX25-knocked down J 774.1 cells. qPCR analysis revealed that *C5ar1* mRNA expression was not changed in SNX25-knocked down cells (*Snx25,* 0.40 ± 0.08 fold of scramble siRNA, $p = 0.00053$; C5ar 1, 1.42 ± 1.08 fold of scramble siRNA, $p = 0.55$; Fig. 3 A-B). These data suggest that C5a-induced NGF expression was modulated by SNX25, but not C5aR1.





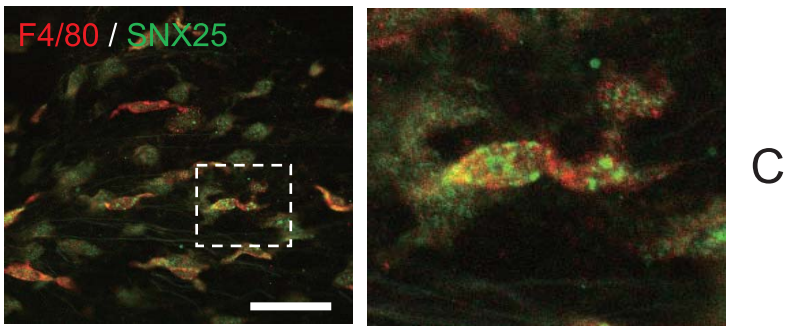


Fig. 1 NGF and SNX25 are expressed in macrophages. (A) Paw withdrawal thresholds to mechanical stimulation with von Frey filaments after C5a (10 μg/mL, 50 μL) injection (2 h) compared to thresholds before injection in WT mice (n＝3). The pain sensation was more sensitive when C5a (red) was injected into the hind paw skin than when PBS (blue) was injected (*p＜0.05). (B) Confocal images of the hind paw skin (naive) of WT mice, immunolabeled for Iba 1 (red), CD206 (red) and NGF (green). Arrowheads denote double-labeled cells (Bar: 50 μm). (C) Confocal images of the hind paw skin (naive) of WT mice, immunolabeled for F 4/80 (red) and SNX25 (green) (Bar: 50 μm).

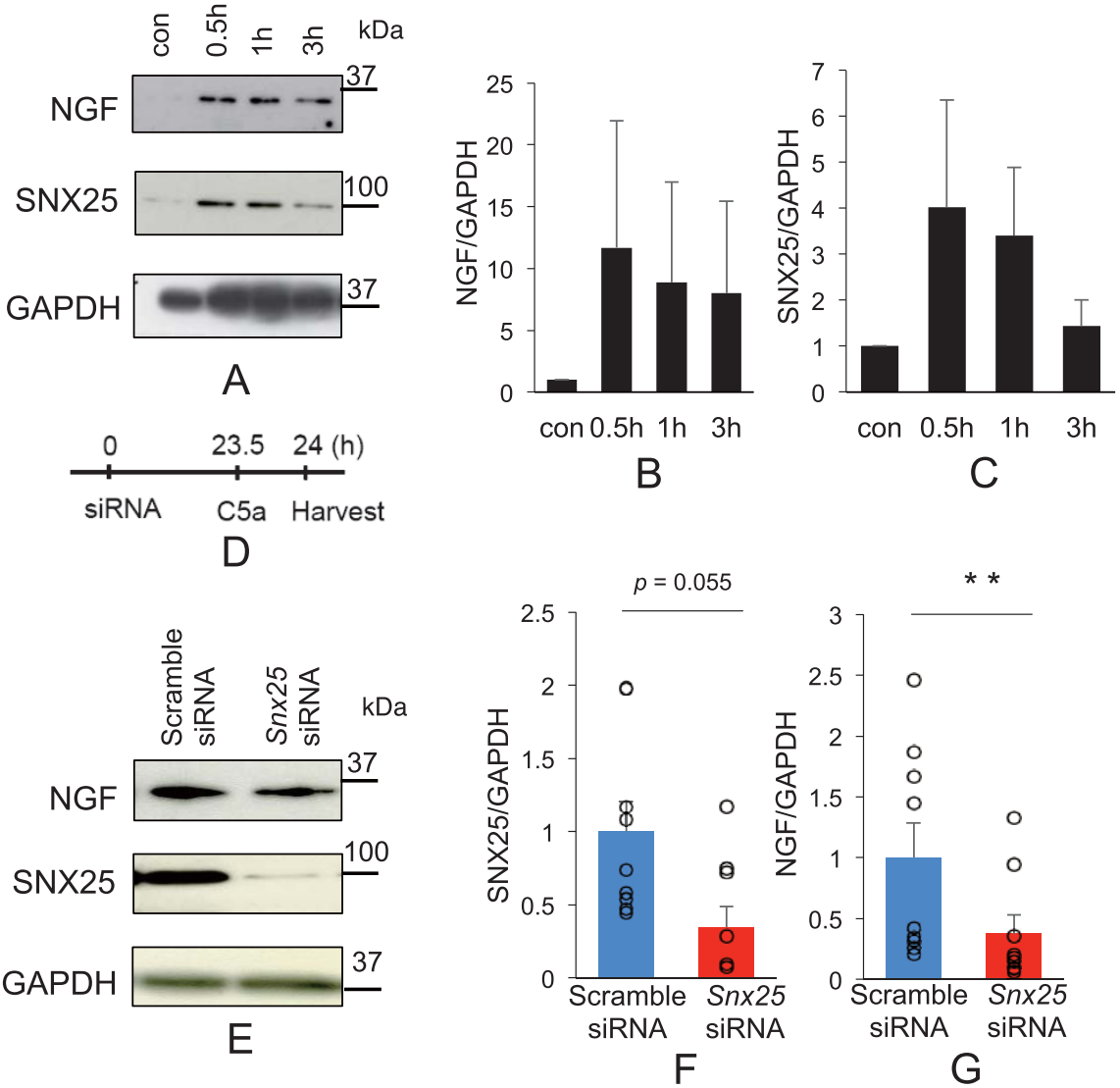


Fig. 2 NGF expression in SNX25 knockdown J774.1 cells after C5a stimulation. (A) Expression level of NGF and SNX25 in J774.1 cells after C5a stimulation. J774.1 cells were stimulated with C5a (50 ng/mL) for 0.5, 1 and 3 hours. The expression of SNX25 and NGF was analyzed by Western blotting. GAPDH was used as a loading control. (B-C) Semi-quantitative analyses of NGF and SNX25 levels in J774.1 cells after C5a injection. The data is expressed as mean±SEM (D) Diagram of the scheme of siRNA (scramble siRNA or *Snx25* siRNA) and C5a treatment (50 ng/mL). (E-G) Expression level of NGF and SNX25 after C5a stimulation in SNX25 knocked down J774.1 cells. J774.1 cells were stimulated with C5a for 0.5 hours. The expression of SNX25 and NGF was analyzed by Western blotting. GAPDH was used as a loading control. Data are presented as the mean values±SEM. Circles indicate individual experimental values (*$p < 0.05$).

***Snx25*+/− mice yields a pain-insensitive phenotype in response to C5a**

We used *Snx25*+/−mice to investigate the involvement of SNX25 to NGF expression and pain phenotype *in vitro.* First, we confirmed that C5aR1 was expressed in macrophages and the expression level of C5aR1 in hind paw skin of *Snx25*+/− mice was similar to that of WT mice (0.74±0.26 fold of *Snx25*+/− mice, $p = 0.31$; Fig. 4 A-B). Interestingly, Western blot analysis revealed that the NGF expression in the hind skin was reduced in *Snx25*+/− mice compared to WT mice (Fig. 4 C). It has been reported that NGF plays a critical role in hyperalgesia and its mutation causes painless phenotypes.[9, 10]

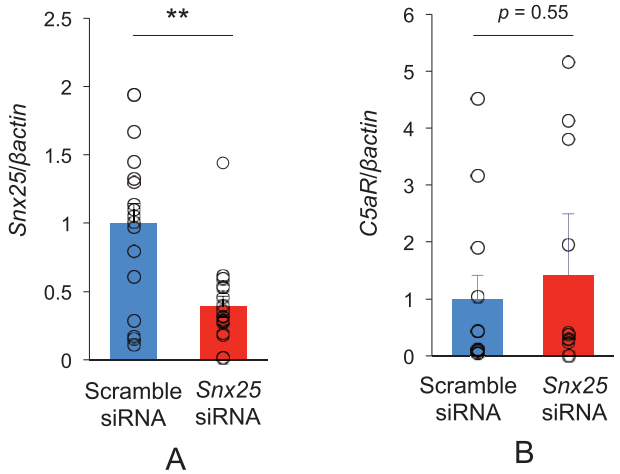


Fig. 3 Expression of C5aR1 in SNX25 knockdown J774.1 cells. (A) Expression level of *Snx25* mRNA after C5a stimulation (0.5 h) in SNX25 knocked down J774.1 cells. (B) Expression level of *C5ar1* mRNA after C5a stimulation (0.5 h) in SNX 25 knocked down J774.1 cells. *β-actin* was used as an endogenous control. Data are presented as the mean values ± SEM. Circles indicate individual experimental values (n = 12, $^{**}p < 0.01$).

We next evaluated the pain behavior to C5a-induced mechanical stimulation. In *Snx25* +/− mice, the von Frey test after C5a injection at 2 h had a higher threshold (50% threshold, 0.90 ± 0.14 g) than in WT mice (50% threshold, 0.52 ± 0.07 g, $p = 0.03$) (Fig. 4 D). These data suggest that SNX25 in macrophages regulates pain behavior by controlling the expression of C5a-induced NGF.

DISCUSSION

In this study, we investigated whether NGF was regulated by SNX25 in response to C5a stimulation. Treatment of J774.1 with C5a increased NGF and SNX25 expression. We also showed that C5a-induced NGF expression was attenuated by *Snx25* siRNA. In addition, *Snx25* +/− mice had lower NGF levels and showed a pain-insensitive phenotype in response to C5a.

SNXs play important roles in membrane trafficking, cell signaling and membrane remodeling, and some SNXs are known to participate in the functioning of the immune system.[4-6] It has been reported that SNX25 is widely expressed in different tissues.[11] However, little is known about the relationship between SNX25 and the immune system. Recently, we found that SNX25 knockdown in the macrophage cell line RAW 264.7 promoted ubiquitination of IκBα after lipopolysaccharide stimulation, indicating that SNX25 is an important factor in immune cells.[8] Neurotrophic factors such as NGF may modulate gene expression after nerve injury or long-term inflammation, leading to the development of persistent pain. However, the mechanisms underlying the induction of persistent pain by NGF are not fully understood.[4, 12] We observed that SNX25 was expressed in macrophages. We showed that SNX 25 in macrophages modulates C5a-induced NGF production. Based on these data, we conclude that SNX25 in macrophages modulates acute pain sensing via NGF signalling under C5a-injected painful conditions. The increase of tissue NGF levels is well characterized in several inflammatory conditions and in several models of pain.[13] We propose that the tissue content of C5a-induced NGF is controlled at least in part by macrophages through SNX25 signaling.

The complement system is a critical part of innate immunity, serving as the first line of defense against infection and tissue damage—associated conditions. It has been reported that C5a-induced macrophage activation via C5aR1 results in mobilization of NGF and NGF/TrkA-dependent sensitization of TRPV1 in nociceptive fibers.[2] In this study, we showed that C5a-indduced NGF and SNX25 expression levels reached their peaks at 30 min and that NGF expression was reduced by *Snx25* siRNA (Fig. 2). The decreased NGF expression in SNX25 knocked down cells may have decreased due to decreased expression of C5aR1. Therefore, we investigated whether the expression level of C5aR1 was changed in SNX25-knocked down J774.1 cells and *Snx25* +/− mice. C5aR1 expression was not changed in SNX25-knocked down cells (Fig. 3) or in *Snx25* +/− mice (Fig. 4). These data suggest that C5a-induced NGF expression was modulated by SNX25.

In conclusion, we found that the expression level of C5a-induced NGF is controlled by macrophages through SNX25 signaling. This work may further our understanding of SNX25 and may prove to be

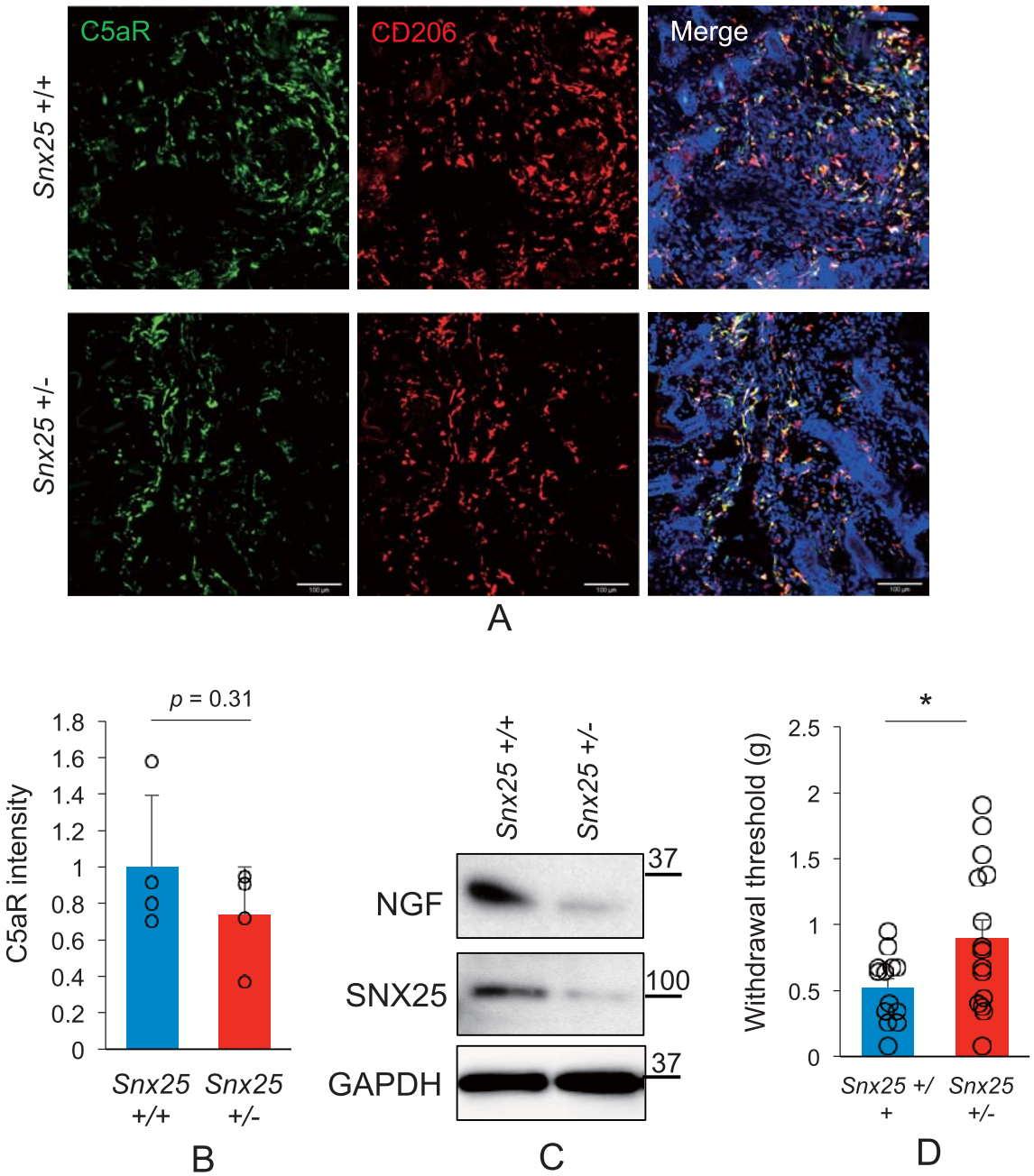


Fig. 4 *Snx25*+/− mice had lower NGF levels and showed a pain-insensitive phenotype in response to C5a. (A) Confocal images of hind paw skin immunolabeled for C5aR1 and CD206 in WT and *Snx25*+/− mice. (B) Semi-quantitative analysis of the C5aR1 intensity in hind paw skin (Mean±SEM, n=4 sections from each of two mice, Circles indicate individual experimental values). (C) Expression level of NGF and SNX25 in the hind paw skin of WT and *Snx25*+/− mice. (D) Comparison of paw withdrawal thresholds to mechanical stimulation with von Frey filaments between WT (n=13) and *Snx25*+/− mice (n=16) measured 2 h after C5a injection. Circles indicate individual experimental values. (*$p < 0.05$).

a useful strategy for treating various pain conditions, including NGF.

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