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Background and Aim: Repeated water avoidance stress (WAS) induces visceral hypersensitivity. Additionally, it is also known to activate corticotropin-releasing factor (CRF), mast cells and pro-inflammatory cytokines systems, but their precise roles on visceral sensation have not been determined definitely. The aim of the study was to explore this issue.

Methods: Abdominal muscle contractions induced by colonic balloon distention, i.e. visceromotor response (VMR) was detected electrophysiologically in conscious rats. WAS or sham stress as control for 1 hr daily was loaded and the threshold of VMR was determined before and at 24 hr after the stress.

Results: Repeated WAS for 3 consecutive days reduced the threshold of VMR, but sham stress did not induce any change. Astressin, a CRF receptor antagonist (50 μg/kg) intraperitoneally (ip) at 10 min prior to each WAS session prevented the visceral allodynia, but the antagonist (200 μg/kg) ip at 30 min and 15 hr before measurement of the threshold after completing 3-day stress session did not modify the response. Ketotifen, a mast cell stabilizer (3 mg/kg), anakinra, an interleukin (IL)-1 receptor antagonist (20 mg/kg) or IL-6 antibody (16.6 μg/kg) ip for 2 times before the measurement abolished the response.

Conclusions: Repeated WAS for 3 consecutive days induced visceral allodynia which was mediated through mast cells, IL-1 and IL-6 pathways. Inhibition of peripheral CRF signaling prevented but did not reverse this response, suggesting that peripheral CRF may be an essential trigger but may not contribute to the maintenance of repeated WAS-induced visceral allodynia.

Key words

Repeated water avoidance stress; Visceral allodynia; Corticotropin-releasing factor; Cytokine; Mast cell

Introduction

Irritable bowel syndrome (IBS) is one of the functional gastrointestinal (GI) disorders characterized by the presence of recurrent abdominal pain with altered bowel habits without any organic cause.[1] Stress induces onset and/or exaggeration of GI symptoms in the majority of IBS patients.[2] The pathophysiology has not been completely defined but disturbed gut motility and altered visceral sensory function play an important role at least in part.[3] Since stress alters GI motility and visceral sensation through central and peripheral corticotropin-releasing factor (CRF), CRF-mediated GI responses are thought to be involved in IBS pathophysiology.[3, 4]

The actions of CRF are mediated through the activation of two receptors, CRF receptor type 1 (CRF_1) and type 2 (CRF_2),[5] and activation of each CRF receptor induces distinct responses in GI tract, i.e. stimulation of colonic motility and inducing visceral hypersensitivity to colorectal distension (CRD) by CRF_1, and delayed gastric emptying by CRF_2 exclusively.[3, 4, 6]

Water avoidance stress (WAS) is a conventional psychological stress protocol, and several studies reported that repeated WAS induced visceral hyperalgesia in rodents.[7-10] Since accumulation of continuous life stress (chronic stress) often causes the exacerbation of symptoms in IBS,[11] chronic repeated WAS is thought to be an appropriate animal stress model of IBS.[12] Although WAS is well known to activate CRF signaling [13] and increase the expression of pro-inflammatory cytokines in GI tract,[7, 8] but their roles on visceral hypersensitivity have not been determined definitely and directly.

We have very recently shown that lipopolysaccharide (LPS)-induced visceral allodynia was mediated through peripheral CRF, interleukin (IL)-1 and IL-6 in rats.[14] Incidentally, several investigators reported that circulatory LPS and pro-inflammatory cytokines were increased in IBS [15, 16] and suggested that LPS-cytokines system may be involved in the visceral hypersensitivity observed in IBS.[17] In this context, LPS-induced visceral hypersensitivity is thought to simulate the pathophysiology of IBS. Then, we hypothesized that repeated WAS-induced visceral hypersensitivity is mediated through common pathways to LPS, i.e. peripheral CRF, IL-1 and IL-6.

In the present study, we established the rat model of visceral hypersensitivity induced by repeated WAS, and the roles of peripheral CRF signaling, IL-1 and IL-6 were determined in this model. Additionally, since LPS or WAS was also reported to activate mast cells,[18] the role was also explored.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (Charles River Laboratory, Atsugi, Japan) weighing about 300 g were housed grouply (3−4 rats/cage) under controlled conditions of illumination (12 hr light/dark cycle starting at 7 a.m.) and temperature at 23−25 ºC with food (Solid rat chow, Oriental Yeast, Tokyo, Japan) and water available ad libitum.

Chemicals. Astressin (Sigma-Aldrich, St. Louis, MO, USA), a non-selective CRF_1 and CRF_2 receptor antagonist was dissolved in double-distilled water. Anakinra (Swedish Orphan Biovitrum, Stockholm, Sweden), an IL-1 receptor antagonist and ketotifen (Sigma-Aldrich), a connective tissue mast cell stabilizer were dissolved in normal

saline. Goat anti-rat IL-6 neutralizing antibody and normal goat IgG (R&D Systems, Minneapolis, MN, USA) were dissolved in sterile phosphate buffered saline. The doses of the chemicals were determined according to the previous reports.[14, 19] The volume of injection was 0.2 mL/rat.

Measurement of visceral sensation. Visceral sensation was assessed by abdominal muscle contractions induced by colonic distention (visceromotor response; VMR) using electromyogram (EMG) in conscious rats, which was validated as quantitative measure of visceral nociception.[20]

Implantation of electrodes and placement of colonic distention balloon. Under brief ether anesthesia, a small skin incision about 5 mm in length was made in non-fasted rats, and electrodes (Teflon coated stainless steel, 0.05 mm diameter, MT Giken, Tokyo, Japan) for EMG were inserted approximately 2 mm into left side external oblique musculature through the incision. They were fixed to musculature and the incised skin was closed by cyanoacrylate instant adhesive (Aron Alpha, TOAGOSEI, Tokyo, Japan). The electrode leads were externalized directly through this closed incision without subcutaneous tunnel and threaded through a urethane tube. Distension balloon (6-Fr disposable silicon balloon-urethral catheter, JU-SB0601, Terumo Corporation, Tokyo, Japan) was inserted intra-anally with the distal end positioned 2 cm proximal to the anus. The volume and length of maximally inflated balloon was 1.5 mL and 1.2 cm.

Colonic distention and measuring abdominal muscle contractions. After completing the surgery for the electrodes implantation and balloon placement, the rats were placed in Bollmann cages and allowed to recover from the anesthesia, and acclimated to the experimental condition for 30 min before testing. (The animals were trained to the experimental conditions by placing them singly in Bollmann cages for 1 hr before the day of experiment.) Later electrode leads were connected to an EMG amplifier, and EMG signals were amplified, filtered (3000 Hz), digitized by a PowerLab system (AD Instruments, Colorado Springs, CO, USA) and recorded using computer software (LabChart 7, AD Instruments). Colonic distension was performed at 30 min after the surgery according to a previous publication,[14] namely, ascending method of limits phasic distension was applied in increments of 0.1 mL for 5 sec by inflating the balloon by water using a syringe manually until significant abdominal muscle contractions, i.e. VMR, were detected. The threshold of VMR was defined as the distended balloon volume (mL) inducing VMR (Fig.1A). Tang et al.[21] previously demonstrated that the pain threshold induced by CRD assessed by the observation of abdominal withdrawal reflex using the balloon quite similar to ours could be determined as distended balloon volume in rats and also showed that intracolonic pressure was linearly associated with intraballoon volume. The threshold was assessed two times (2 min interval) and the mean of the threshold was calculated as the data of the animals.

Stress protocol. Exposure to WAS was performed as described previously.[22] Rats were placed individually on a plastic platform (height, 8 cm; length, 6 cm; width, 6 cm) positioned in the middle of a plastic cage filled with water up to 7 cm of the height of the platform. To avoid contact with water, rats stood on a platform during the entire

stress period. Control animals were also put on the same plastic platform in a plastic cage but not filled with water (sham stress).

Experimental procedures. The non-fasted rats were submitted to colonic balloon distention and the basal threshold of VMR was measured. Then the electrodes and distention balloon were removed, and either WAS or sham stress (controls) for 1 hr was applied. First, in order to establish the rat model of visceral hypersensitivity, we determined the time course effect of repeated WAS from single session (Fig.1B). Each group of animals (n = 6−9) was loaded by different times of daily 1-hr stress session, and the experiment continued until detecting significant visceral allodynia after repeated WAS. After completing each stress session, the animals were returned to their group housing cages. Stress sessions were performed at 8−12 a.m. After 24 hr from the last stress session, the animals underwent surgery for the electrodes implantation and balloon placement, and put in the Bollmann cages again. After 30 min, the threshold was determined.

Next, the mechanisms of repeated WAS-induced visceral hypersensitivity were explored using this established model. The effect of astressin, ketotifen, anakinra and IL-6 antibody were tested. These drugs were administered at 30 min and 15 hr before colonic distention performed after completing repeated WAS or sham stress session through intraperitoneal (ip) route (Fig.1C). In another series of experiment, astressin was also administered at 10 min before each stress session, repeatedly (Fig.1D).

Statistical analysis. Data were expressed as means ± SE. Multiple comparison was performed by two-way ANOVA followed by Fisher's Least-Significant-Difference Test. Comparison between two groups was performed using the Student's t or paired t test. SYSTAT 13 software (Systat Software, Chicago, IL, USA) was used throughout the study.

Ethical considerations. Approval by the Research and Development and Animal Care Committees at the Asahikawa Medical University (#15132, approved on April 1, 2015) was obtained for all studies.

Results

Repeated daily WAS for 3 consecutive days induced visceral allodynia. Single and 2-day WAS session did not change the threshold of VMR at 24 hr after the last WAS (Fig.2A). On the other hand, WAS for 3 consecutive days significantly reduced the threshold (mL) from 0.53 ± 0.020 to 0.36 ± 0.031 ($p < 0.05$). Sham stress did not alter it. Moreover, the % change threshold, i.e. the value of the threshold after stress divided by the one of the basal threshold, multiplied by 100 was significantly reduced in 3-day WAS group as compared with sham stress one (67.2 ± 5.8 for WAS vs. 97.8 ± 5.4 for sham stress, $p < 0.05$), but it was not changed in single or 2-day WAS group (Fig.2B). Since 3-day WAS was definitely demonstrated to induce visceral allodynia as described above, the % change threshold was presented in the following experiments in order to assess the effect of drugs on this response.

CRF receptor antagonist, astressin prevented but did not reverse repeated WAS-induced visceral allodynia. Ip repeated injections of astressin at 50 μg/kg before the each stress session prevented the allodynia without altering the threshold in sham stress group (effect of WAS: $F = 6.0$, $p < 0.05$, effect of astressin: $F = 11.8$, $p < 0.05$, interaction of WAS and astressin: $F = 11.1$, $p < 0.05$, 96.2 ± 7.0 for vehicle + sham vs. 64.8 ± 4.9 for vehicle + WAS, $p < 0.05$, 101.7 ± 3.7 for astressin + WAS vs. vehicle + WAS, $p < 0.05$, Fig.3A).

On the other hand, ip astressin at 200 μg/kg twice before measuring the threshold after completing 3-day stress session neither modified the response induced by repeated WAS nor the threshold in sham-stressed rats (effect of WAS: $F = 40.7$, $p < 0.05$, effect of astressin: $F = 0.11$, $p > 0.05$, interaction of WAS and astressin: $F = 0.22$, $p > 0.05$, Fig.3B). This dose of antagonist was known to block CRD- or LPS-induced visceral hypersensitivity in rats.[14, 23]

Mast cell stabilizer, ketotifen attenuated repeated WAS-induced visceral allodynia. Ketotifen at 3 mg/kg twice before measuring the threshold did not alter the threshold in sham-stressed rats but reversed the WAS-induced response (effect of WAS: $F = 7.0$, $p < 0.05$, effect of ketotifen: $F = 11.2$, $p < 0.05$, interaction of WAS and ketotifen: $F = 4.5$, $p < 0.05$, 94.1 ± 4.0 for sham + vehicle vs. 68.1 ± 5.0 for WAS + vehicle, $p < 0.05$, 92.3 ± 7.0 for WAS + ketotifen vs. WAS + vehicle, $p < 0.05$, Fig.4) .

IL-1 receptor antagonist, anakinra reversed repeated WAS-induced visceral allodynia. The administration of anakinra at 20 mg/kg twice before measuring the

threshold blocked the allodynia, but the antagonist did not change the threshold in sham stress group (effect of WAS: $F = 14.4$, $p < 0.05$, effect of anakinra: $F = 11.2$, $p < 0.05$, interaction of WAS and anakinra: $F = 6.0$, $p < 0.05$, 95.7 ± 2.9 for sham + vehicle vs. 66.3 ± 4.5 for WAS + vehicle, $p < 0.05$, 93.6 ± 5.5 for WAS + anakinra vs. WAS + vehicle, $p < 0.05$, Fig.5).

IL-6 antibody blocked repeated WAS-induced visceral allodynia. IL-6 antibody at 16.6 μg/kg injected twice before measuring the threshold also abolished the response by repeated WAS without affecting the threshold in sham stress group (effect of WAS: $F = 5.3$, $p < 0.05$, effect of IL-6 antibody: $F = 5.8$, $p < 0.05$, interaction of WAS and IL-6 antibody: $F = 17.0$, $p < 0.05$, 107.1 ± 7.4 for sham + vehicle vs. 70.0 ± 3.8 for WAS + vehicle, $p < 0.05$, 107.8 ± 7.2 for WAS + IL-6 antibody vs. WAS + vehicle, $p < 0.05$, Fig.6).

Discussion

There are several studies investigating the effects of repeated WAS on visceral sensation in rodents, and the majority of them demonstrated that repeated WAS induced visceral hypersensitivity.[7-10] However, a few studies reported that it induced hyposensitivity or no change.[24, 25] The discrepancy of the results may be explained by the difference of animals, sex or method measuring abdominal contractions induced by colonic distention, such as EMG and noninvasive manometry.[24, 26]

Our method detected the threshold of VMR by EMG with acute preparation, and we showed previously that acute single WAS induced visceral hyposensitivity using this

235 method.[27] In the current study, we showed that 3-day WAS induced visceral allodynia,
236 and tried to determine the roles of peripheral CRF, mast cells, IL-1 and IL-6, which
237 were previously proved to mediate LPS-induced visceral allodynia.[14]

238 Several studies showed that acute WAS activates both central and peripheral
239 CRF signaling, which mediates altered GI motility and visceral sensation induced by
240 WAS.[13, 27, 28] However, there were only few studies reporting the role of CRF signaling
241 on chronic WAS-induced visceral hypersensitivity. Larauche et al.[10] showed that
242 repeated WAS-induced visceral hyperalgesia was prevented by repeated peripheral
243 injections of astressin before daily WAS session in rats. In the present study, the same
244 result was obtained, which further suggests that the development of visceral
245 hypersensitivity by chronic WAS may be mediated through peripheral CRF receptors,
246 because astressin is peripherally restricted CRF receptor antagonist.

247 Incidentally, the current study also demonstrated the interesting result that the
248 administration of astressin after 3-day WAS did not reverse the response by WAS. It
249 implies that activating peripheral CRF signaling may be an essential trigger for the
250 development of allodynia, but once established the hypersensitivity, it no longer may
251 contribute to the response.

252 Activating peripheral CRF/CRF_1 induces visceral hyperalgesia [29] and acute
253 stress such as CRD-induced visceral hyperalgesia was abolished by the peripheral
254 administration of astressin.[23] On the other hand, we previously demonstrated that
255 peripheral activation of CRF_1 did not induce visceral allodynia in the similar
256 experimental settings to the current study.[14] Together with the results that astressin
257 prevented but did not reverse the allodynia, these lines of evidence suggest that

peripheral CRF/CRF_1 may directly contribute to the visceral hypersensitivity induced by acute stress as opposed to chronic repeated WAS. Therefore we hypothesized that peripheral CRF signaling activated by repeated WAS triggers to activate other systems, thereby inducing visceral allodynia, but does not directly contribute to the response.

Mast cells have CRF receptors at their surface [30] and acute stress evokes mast cells degranulation to release a large variety of mediators such as serotonin, prostaglandins, etc., which is triggered by peripheral CRF in GI tract.[31] These mediators were demonstrated to activate visceral afferents,[32] leading to induction of visceral sensitization.[28] Additionally, chronic WAS is also known to increase and activate mast cells,[7, 18] and mast cells degranulation directly contributes to WAS-induced visceral hypersensitivity in maternally separated rats.[33] The result of that ketotifen abolished the allodynia by WAS in the present study is consistent with the evidence above. In addition, we also confirmed that ketotifen abolished LPS-induced allodynia (data were not shown), indicating that activation of mast cells may be one of the common pathways mediating visceral hypersensitivity by chronic WAS and LPS.

Chronic WAS is well demonstrated to increase pro-inflammatory cytokines in both gene and protein level in GI tract.[7, 8, 34] However, it has not been shown that these cytokines are directly involved in WAS-induced visceral hypersensitivity so far. We believed that this is the first report showing the direct link between the cytokines and chronic WAS-induced visceral hypersensitivity, since anakinra or IL-6 antibody abolished the response.

IL-1 receptors are proved to be located in the neurons in rat dorsal root ganglia (DRG).[35] IL-6-receptor complex binds to the transmembrane signal-transducing subunit,

281 glycoprotein 130, which is also expressed in DRG neurons.[36] Thus these cytokines
282 possibly evoke visceral hypersensitivity through directly activating sensory afferents
283 through these receptors.

284 The link between peripheral CRF and the pro-inflammatory cytokines on
285 modulating visceral sensation has been also suggested. Repeated WAS increases gut
286 permeability through peripheral CRF receptors and exogenous CRF administration
287 mimics this response.[37] Increased permeability leads to an increase in bacterial
288 translocation resulting in activating local immune system and inflammation, thereby
289 increasing the release of LPS and the cytokines.[38] CRF potentiates the response of
290 macrophages to LPS, i.e. enhancing the synthesis of the pro-inflammatory cytokines
291 through CRF_1.[39] Additionally, we have very recently shown that IL-1β-induced
292 allodynia was not blocked by ip astressin,[14] suggesting that IL-1 system may be more
293 downstream signaling to modulate the visceral sensation as compared to CRF on WAS-
294 induced allodynia. In this context, in addition to mast cells, these cytokines pathways
295 might also be one of the systems which CRF signaling modulates to develop visceral
296 allodynia. These scenarios may also explain the similarity of the pathways mediating the
297 visceral sensory response by chronic WAS and LPS.

298 We did not demonstrate the direct link between CRF and the cytokines. Besides,
299 various cells other than macrophage or monocyte, such as fibroblasts, endothelial,
300 neuronal, smooth muscle cells, etc.,[40, 41] secrete our tested cytokines, but the sources
301 responsible for the allodynia were not determined. These are the limitations of our
302 study. Since other cytokines such as TNF-α, IL-8, etc., have been suggested to be
303 associated with the pathogenesis of IBS,[42] the role of these cytokines should be also
304 evaluated in the future.

Growing evidence supports that peripheral immune mechanisms could contribute to the IBS pathophysiology.[42, 43] Some IBS patients exhibit low-grade gut mucosal inflammation with activated mast cells and enhanced expression of pro-inflammatory cytokines with increased serum LPS, resulting in abnormal neural responses, leading to altered colonic functions.[3, 15, 44] In this context, we previously proposed that LPS-induced visceral allodynia is rat model of IBS.[14] Present study definitely showed that WAS activated mast cells, pro-inflammatory cytokines and peripheral CRF systems, which contributed to chronic psychological stress-induced visceral hypersensitivity. These systems are common pathways inducing visceral allodynia by LPS and WAS, speculating that the link between brain and immune system via peripheral CRF may play a crucial role in the pathophysiology of IBS.

In summary, we demonstrated that repeated WAS for 3 consecutive days induced visceral allodynia which was mediated through mast cells, IL-1 and IL-6 pathways. Inhibition of peripheral CRF signaling prevented but did not reverse this response, suggesting that peripheral CRF may be an essential trigger but may not contribute to the maintenance of repeated WAS-induced visceral allodynia. These results further suggest the crucial contribution of CRF, mast cells and cytokines systems to the pathophysiology of IBS.

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Figure legends

Figure 1

A The threshold of visceromotor response (VMR) was determined by the distended balloon volume (mL) inducing apparent sustained abdominal muscle contractions. Demonstrable EMG recording was depicted. The threshold of VMR was 0.4 mL in this rat. **B** Schematic representation of experimental protocol. The surgery for EMG electrodes and balloon placement were performed and 30 min later, basal VMR threshold was measured. Then the animals were submitted to1-hr stress session such as water avoidance or sham stress. First, in order to establish the rat model of visceral hypersensitivity, different times of daily stress session were loaded to each group of animals until detecting significant visceral allodynia after repeated WAS. After 24 hr from the last stress session, the animals underwent surgery again and the threshold was determined. **C** Next, the mechanisms of repeated WAS-induced visceral hypersensitivity was explored using this established model. Pharmacological intervention was performed at 30 min and 15 hr before measurement of the threshold. **D** In another series of experiment, astressin was administered at 10 min before each stress session, repeatedly.

Figure 2

A Effect of repeated water avoidance stress (WAS) on the threshold of visceromotor response (VMR) induced by colonic distention. 1- or 2-day WAS did not alter the threshold, but 3-day WAS significantly reduced it. Sham stress did not modify

488 the threshold. * $p < 0.05$ vs. basal threshold by paired t test. **B** % change threshold was
489 significantly reduced in 3-day WAS group as compared with sham stress group. There
490 was no significant difference between 1 or 2-day WAS and respective control group. * p
491 < 0.05 vs. Sham by Student's t test. Each column represents the mean ± SE. Number of
492 rats examined is shown in the parenthesis. Sham, sham stress.

493

494 Figure 3

495 The effect of astressin on repeated water avoidance stress (WAS)-induced
496 visceral allodynia. **A** Astressin (50 μg/kg) injected 10 min before each stress session
497 prevented WAS-induced allodynia without altering the threshold in sham-stressed rats.
498 * $p < 0.05$ vs. Vehicle + Sham, # $p < 0.05$ vs. Vehicle + WAS by two-way ANOVA
499 followed by Fisher's Least-Significant-Difference Test. **B** Astressin (200 μg/kg) injected
500 twice before the measurement of threshold after completing the 3-day stress session
501 neither modify the response induced by WAS nor the threshold in sham-stressed rats. *
502 $p < 0.05$ vs. Sham + Vehicle by two-way ANOVA followed by Fisher's Least-
503 Significant-Difference Test. Each column represents the mean ± SE. Number of rats
504 examined is shown in the parenthesis. Sham, sham stress.

505

506 Figure 4

507 The effect of ketotifen on repeated water avoidance stress (WAS)-induced
508 visceral allodynia. Ketotifen at 3 mg/kg twice after completing the 3-day stress session
509 abolished the response induced by WAS without altering the threshold in sham-stressed
510 rats. * $p < 0.05$ vs. Sham + Vehicle, # $p < 0.05$ vs. WAS + Vehicle by two-way

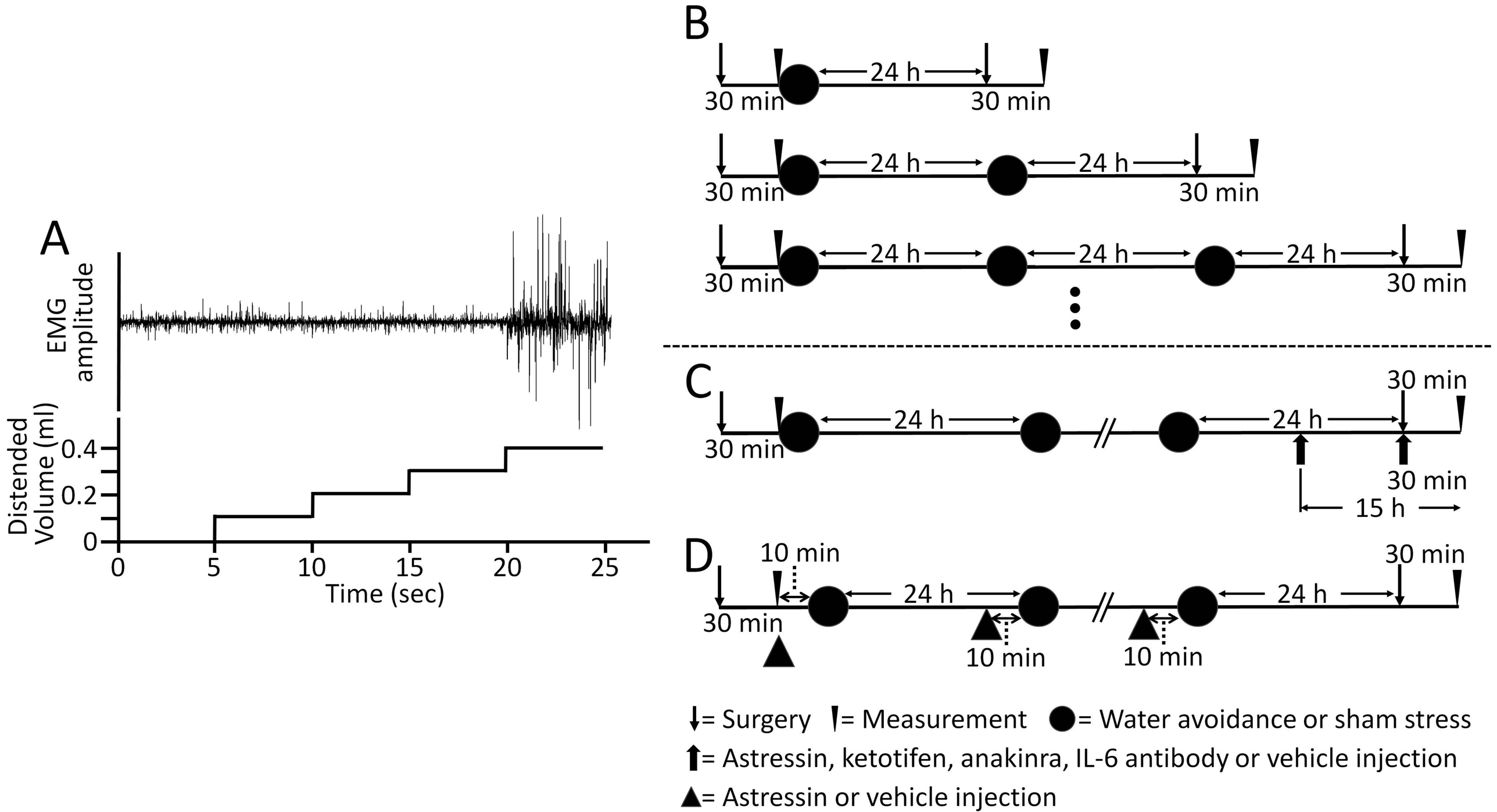
ANOVA followed by Fisher's Least-Significant-Difference Test. Each column represents the mean ± SE. Number of rats examined is shown in the parenthesis. Sham, sham stress.

Figure 5

The effect of anakinra on repeated water avoidance stress (WAS)-induced visceral allodynia. Anakinra at 20 mg/kg twice before measuring the threshold after stress sessions reversed WAS-induced allodynia without modifying the threshold in sham-stressed rats. * $p < 0.05$ vs. Sham + Vehicle, # $p < 0.05$ vs. WAS + Vehicle by two-way ANOVA followed by Fisher's Least-Significant-Difference Test. Each column represents the mean ± SE. Number of rats examined is shown in the parenthesis. Sham, sham stress.

Figure 6

IL-6 antibody (ab) at 16.6 µg/kg injected twice before measuring the threshold blocked repeated water avoidance stress (WAS)-induced allodynia. However, IL-6 ab per se did not alter the threshold. * $p < 0.05$ vs. Sham + Vehicle, # $p < 0.05$ vs. WAS + Vehicle by two-way ANOVA followed by Fisher's Least-Significant-Difference Test. Each column represents the mean ± SE. Number of rats examined is shown in the parenthesis. Sham, sham stress.


A
EMG amplitude
Distended Volume (ml)
0.4
0.2
0
0
5
10
15
20
25
Time (sec)
B
24 h
30 min
30 min
24 h
24 h
30 min
30 min
24 h
24 h
24 h
30 min
30 min
C
30 min
24 h
24 h
30 min
30 min
15 h
D
10 min
30 min
24 h
24 h
30 min
30 min
10 min
10 min
= Surgery
= Measurement
= Water avoidance or sham stress
= Astressin, ketotifen, anakinra, IL-6 antibody or vehicle injection
= Astressin or vehicle injection

A

Basal

After stress

VMR threshold (ml)

0.6

0.5

0.4

0.3

0.2

0.1

0

(6) (6) (8) (8) (8) (9)

*

Sham WAS

1-day

Sham WAS

2-day

Sham WAS

3-day

B

% change threshold

120

100

80

60

40

20

0

(6) (6) (8) (8) (8) (9)

*

Sham WAS

1-day

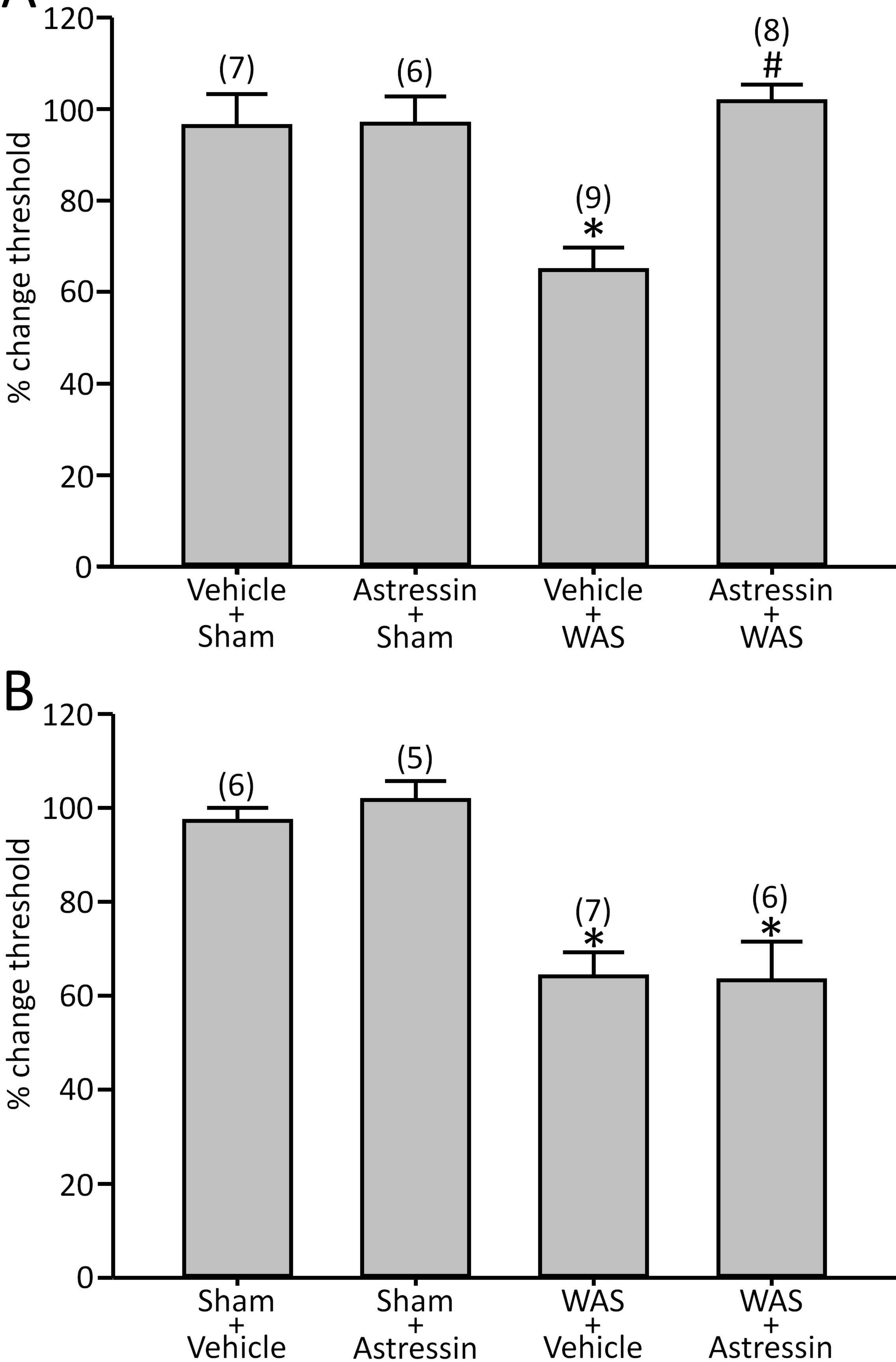
Sham WAS

2-day

Sham WAS

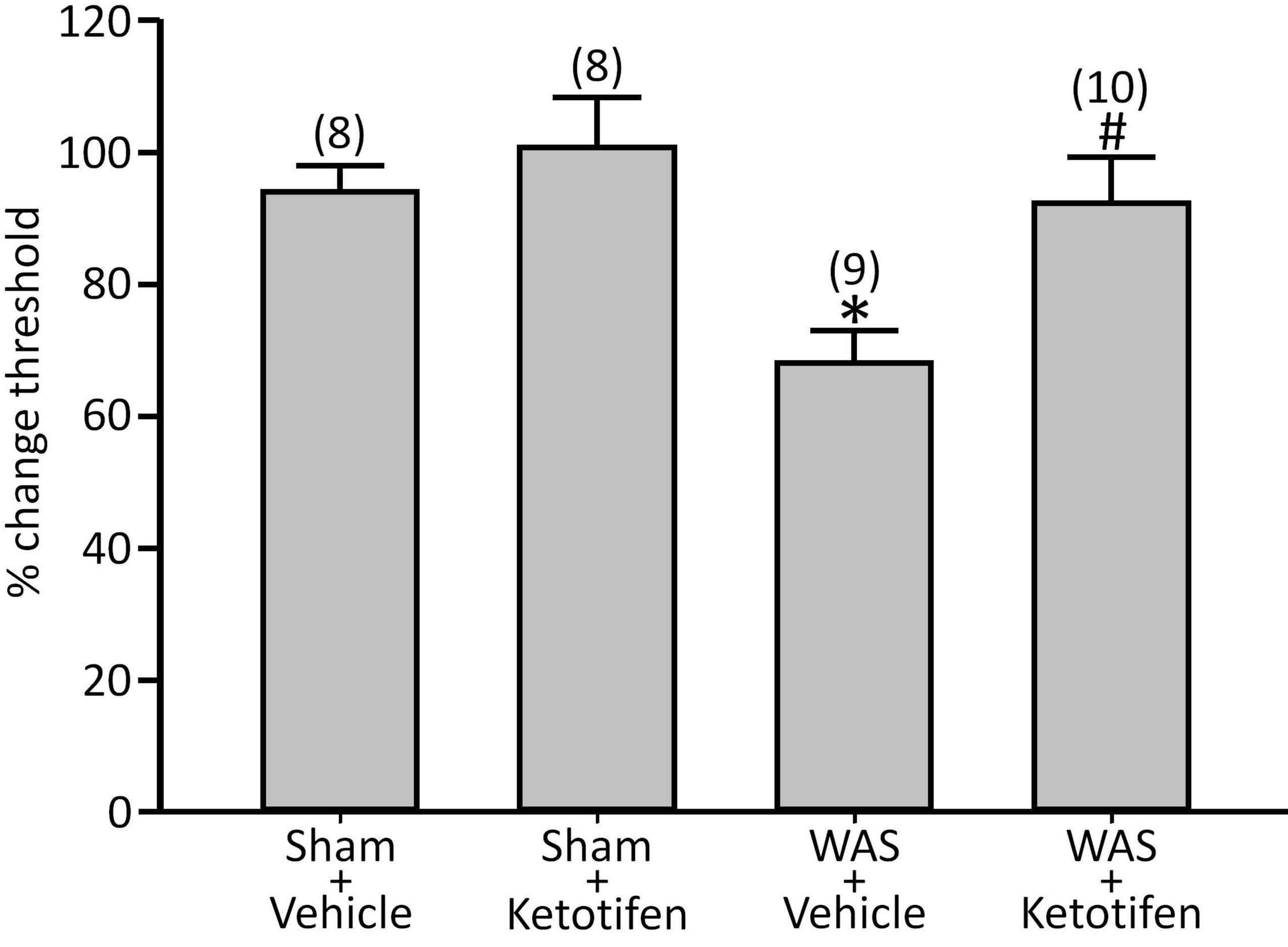
3-day

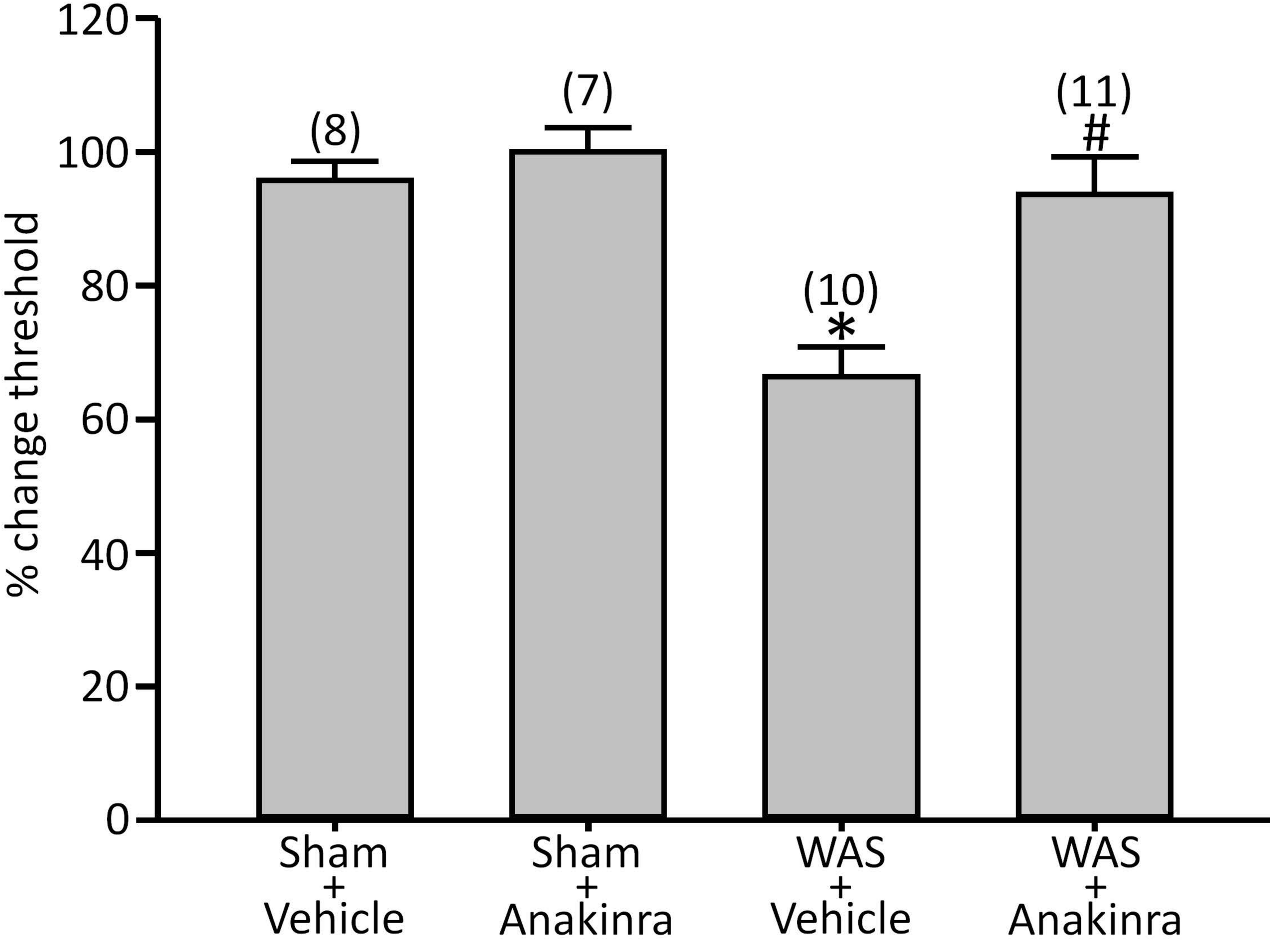
A

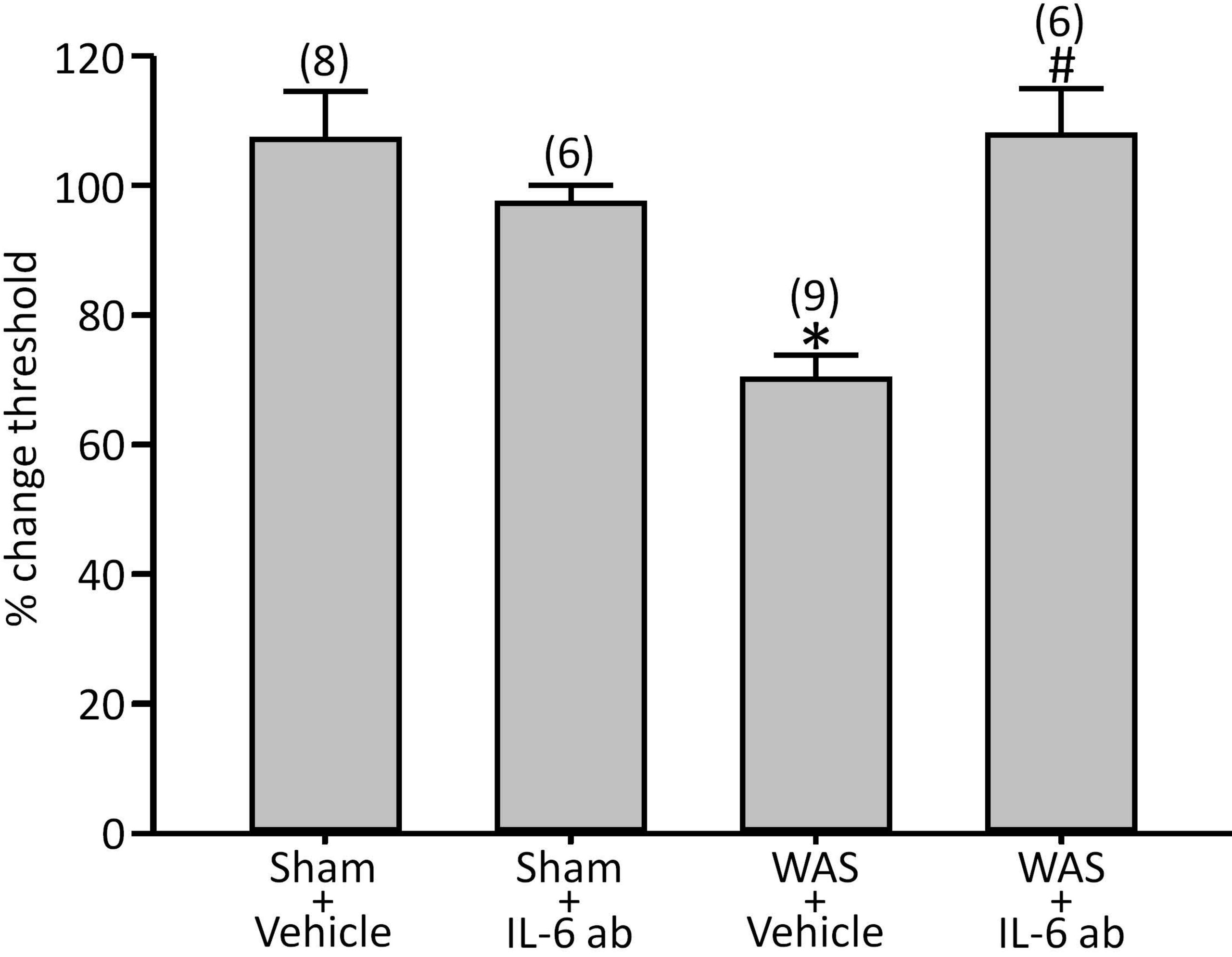

120
100
80
60
40
20
0
% change threshold
(7)
(6)
(9)
*
(8)
#
Vehicle + Sham
Astressin + Sham
Vehicle + WAS
Astressin + WAS

B

120
100
80
60
40
20
0
% change threshold
(6)
(5)
(7)
*
(6)
*
Sham + Vehicle
Sham + Astressin
WAS + Vehicle
WAS + Astressin


120
100
80
60
40
20
0
% change threshold
(8)
(8)
(9)
*
(10)
#
Sham + Vehicle
Sham + Ketotifen
WAS + Vehicle
WAS + Ketotifen


120
100
80
60
40
20
0
% change threshold
(8)
(7)
(10)
*
(11)
#
Sham + Vehicle
Sham + Anakinra
WAS + Vehicle
WAS + Anakinra


120
100
80
60
40
20
0
% change threshold
(8)
(6)
(9)
*
(6)
#
Sham + Vehicle
Sham + IL-6 ab
WAS + Vehicle
WAS + IL-6 ab