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Abstract

To clarify the mechanisms by which compound 48/80 (C48/80) induces scratching behavior, the involvement of dopamine D_1 receptors was investigated. The intracisternal (i.t.) administration of SCH23390 (1.0 μg), a selective dopamine D_1 receptor antagonist, significantly decreased C48/80-induced scratching behavior in mice. These results suggest that dopamine D_1 receptors contribute to scratching behavior or the itch sensation induced by subcutaneous injection of C48/80 in mice. Co-administration of SCH23390 and C48/80 enhanced c-fos immunoreactivities in the peduncular part of the lateral hypothalamus (PLH), whereas the immunoreactivities in the other groups were unchanged. The dopaminergic system may be playing an important role in the suppression of C48/80-induced scratching behavior by SCH23390.

Key words: compound 48/80, scratching behavior, SCH23390, c-fos

1. Introduction

In 1660, Haffenreffer defined itching as an unpleasant cutaneous sensation that provokes the desire to scratch (Rothman, 1941). This definition remains valid today, and this feeling is easily understandable. We can attenuate the itch sensation by scratching, but overscratching adversely affects the skin (e.g., damage to skin barriers, release of inflammatory factors, and exacerbation of the itch sensation) (Paus et al., 2006). This 'itch-scratch cycle' worsens serious dermatitis, and it is important to break out of the cycle to improve the skin condition. However, detailed mechanisms and the key signals of the itch sensation remain unclear until today.

Recently, Sun and Chen (2007) suggested that the receptor for gastrin-releasing peptide (GRP), a mammalian bombesin-like peptide, probably mediates the itch sensation in the dorsal horn of the spinal cord. Therefore, it can be assumed that GRP and its receptor are very important factors for mediating or feeling the itch sensation, whereas some reports have suggested that dopamine D_1 receptors are involved in mediating the itch sensation. Central administration of bombesin or GRP elicited excessive grooming/scratching behavior, and these behaviors were reduced by a dopamine D_1 receptor antagonist (Merali and Piggins, 1990; Van Wimersma Greidanus and Maigret, 1991). Accordingly, dopamine and dopamine D_1 receptors are predicted to mediate the itch sensation in the central nervous system (CNS).

Compound 48/80 (C48/80), a condensation product of N-methyl-p-methoxy- phenethylamine with formaldehyde, is well known as a peripheral pruritogen; it produces the itch sensation and vigorous scratching

behavior by subcutaneous (s.c.) injection, and it causes degranulation of mast cells and results in the release of pruritogens such as histamine and leukotriene B_4 (Andoh and Kuraishi, 1998). However, C48/80 was shown to induce the itch sensation in mast cell-deficient mice (Inagaki et al., 2002). Thus, it appears that C48/80 induces scratching behavior via a mast cell-independent pathway. Furthermore, C48/80-induced scratching behavior was inhibited by intrathecal injection of a GRP receptor antagonist (Sun and Chen, 2007).

Thus, we investigated the effect of a dopamine D_1 receptor antagonist on the scratching behavior induced by s.c. injection of C48/80 in mice, since we predicted that dopamine D_1 receptors may be involved in the vigorous scratching behavior induced by the peripheral pruritogen. Then, we investigated whether SCH23390 and/or C48/80 influence c-fos immunoreactivity in the brain, and surveyed co-localized immunoreactivities of c-fos and dopamine D_1 or D_2 receptors.

2. Materials and methods

2.1. Animals

Six-week-old, male ICR mice (Japan SLC, Shizuoka, Japan) were used. All animals were housed individually at 25°C with a 12-h light/dark cycle (8:00 light on), and they were fed a commercial diet (MF; Oriental Yeast, Tokyo, Japan) with water available *ad libitum*. After 7 days of acclimatization, behavioral tests were conducted. The experimental procedures followed the guidelines for animal experiments of the Faculty of Agriculture and the Graduate Course of Kyushu University, as well as Law No.

105 and Notification No. 6 of the Japanese Government.

2.2. Dose determination of C48/80

To determine an administration dose of C48/80, a pilot study was performed. After 7 days acclimatization, mice were divided into four groups, i.e., sham, saline and C48/80 (1 mg and 2 mg/ml) treatments. Experimental conditions were the same as behavioral test described as below. After the experimental acclimatization, 50 µl of C48/80 solution or saline was administered by s.c. injection into the rostral part of the back. The sham treatment group was only entered the tip of the needle and administered no reagent. Then, their scratching behavior was recorded using a digital video camera under unmanned conditions for 30 min.

2.3. Behavioral test

Drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SCH23390 hydrochloride (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5,-tetrahydro-1H-3-benzazepine hydrochloride), a selective dopamine D_1 receptor antagonist, was dissolved in 0.85% saline containing 0.1% Evans Blue solution and administered by intracisternal (i.c.) injection (0.5 and 1.0 µg per 5 µl). All i.c. injections were performed with a 50-µl Luer tip micro syringe (Hamilton Co., Reno, NV, USA), a disposable extension tube (Atom Medical Corp., Tokyo, Japan), and disposable 27-gauge needles (Terumo Corp., Tokyo, Japan). These needles were curved 40° at 3.5 mm from the tip. According to the technique for i.c. injection, the drugs were given to mice that were anaesthetized with isoflurane (Escain, Merck Ltd., Tokyo, Japan). To

confirm the injection area, the presence of Evans Blue dye was checked after sacrificing the animals. Following successful i.c. injection, the distribution of Evans Blue dye was limited mainly to the cisterna magna and the ventral surface of the brain stem (Ueda et al., 1979). A fixed s.c. dose of C48/80 (2 mg/ml, 50 µl per site) was used as a pruritogen, based on a pilot study.

SCH23390 or vehicle was administered by i.c. injection, and immediately after this procedure, C48/80 solution or saline was administered by s.c. injection into the rostral part of the back. According to the previous study (Kuraishi et al., 1995), mice scratched pruritogenic agent-injected sites with the hind paws when they probably felt the itch sensation. A series of scratching behaviors was counted as one bout of scratching. In this experiment, the numbers of body (C48/80 injected area) and head (adjacent to the i.c. injected site) scratching behaviors with a hind paw were counted for 30 min after injections. The reason for counting the number of head scratching behaviors was to rule out the possibility that the method of i.c. injection elicited the itch sensation.

2.4. Immunohistochemistry

To clarify the effects of SCH23390 and C48/80 on c-fos immunoreactivities in the brain, immunohistochemical analysis was performed. Mice were administered 1.0 µg SCH23390 or vehicle by i.c. injection, followed by saline or C48/80 (2 mg/ml, 50 µl per site) by s.c. injection into the rostral part of the back, and then the mice were returned to their home cages. Two hours after treatment, the mice were sacrificed by cervical dislocation and decapitated. Whole brains were quickly removed

and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) overnight at 4°C and cryoprotected by 20% sucrose solution. Coronal frozen sections (20 μm) were incubated in a rabbit polyclonal anti-c-fos antibody (F7799, diluted 1:5000; Sigma Chemical Co.) overnight at room temperature. Subsequently, the sections were washed and incubated with biotinylated goat anti-rabbit IgG (Sigma Chemical Co. 1:100, 1 h) and ExtrAvidin (Sigma Chemical Co., 1:100, 1 h) at room temperature. Visualization of immunocomplexes was performed using 0.05% 3,3-diaminobenzidine tetrahydrochloride hydrate and 0.015% H_2O_2. The sections were observed with an optical microscope (CX41LF, Olympus Co., Tokyo, Japan) connected to a digital camera, and the signal intensity was densitometrically analyzed by image software (ImageJ 1.43, National Institute of Health, Bethesda, MD, USA). Areas of c-fos immunoreactions were identified according to the stereotaxic brain atlas (Franklin and Paxinos, 2007). For comparison, serial sections were stained with hematoxylin and eosin (HE), and the number of cells in respective areas was counted. .

2.5. Double immunofluorescence

To compare the localization of c-fos- and dopamine D_1 or D_2 receptors-immunopositive cells, the sections were prepared as described for immunohistochemistry for double immunofluorescence method. In this method, a Mouse on Mouse immunodetection kit (Vector M.O.M Basic Kit, Vector Lab. Inc., CA, U.S.A) was used for the detection of mouse primary monoclonal antibodies in mouse tissue. Coronal frozen sections were washed and incubated for 30 min in 5% normal donkey serum which was

diluted with M.O.M. Diluent. Sections were then incubated overnight at room temperature in the mixture of a rabbit polyclonal anti-c-fos antibody (F7799, diluted 1:5000; Sigma Chemical Co.) and either a mouse monoclonal anti-dopamine receptor D_1 antibody (ab78021, diluted 1:100; Abcam Co. Ltd, Tokyo, Japan) or a mouse monoclonal anti-dopamine receptor D_2 antibody (H00001813-M01A, diluted 1:100; Abnova Co. Ltd, Taipei, Taiwan) which was diluted with M.O.M. Diluent. Immunosignals for c-fos were detected by Alexa-Fluor-488 donkey anti-rabbit IgG (Invitrogen Lab., OR, U.S.A., 1:400), whereas those for dopamine D_1 and D_2 receptors were detected by Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Lab., PA, U.S.A., 1:400), respectively for 1 h. The sections were observed with confocal laser scanning microscope (A1R MP, Nikon Co., Tokyo, Japan).

2.6. Statistical analysis

All analyses were conducted using two-way analysis of variance. When significant ($P<0.05$) effects were detected, comparisons between means were made using the Tukey-Kramer test. These analyses were performed with StatView (ver. 5, SAS Institute, Cary, NC, USA). Results are shown as means±S.E.M.

3. Results

3.1. Pilot study

Fig. 1 shows the total number of scratching behavior for 30 min in the pilot study. C48/80 significantly ($F(3,9)=103.708$, $P<0.0001$) increased scratching behavior in a dose-dependent manner. On the other hand, sham

and saline treatment groups remained nearly unaffected. Since higher dose of C48/80 (2 mg/ml) produced marked and significant ($P<0.01$) increase of scratching behavior compared with other groups, 2 mg/ml of C48/80 was fixed throughout the present study.

3.2. Scratching behavior test

Fig. 2 shows the effect of SCH23390 administration on C48/80-induced scratching behavior. In the event of body scratching behavior (Fig. 2a), the effects of SCH23390 administration ($F(2,39)=5.958$, $P<0.01$) and C48/80 injection ($F(1,39)=59.077$, $P<0.0001$) were significant. Furthermore, a significant ($F(2,39)=4.282$, $P<0.05$) interaction between SCH23390- and C48/80-treatments was detected. In the absence of SCH23390, the C48/80 group had significantly ($P<0.01$) more vigorous body scratching behavior than the saline control group. However, SCH23390 administration at a dose of 1.0 μg significantly ($P<0.01$) decreased the C48/80-induced scratching behavior.

In the event of head scratching behavior (Fig. 2b), the effect of SCH23390 administration was significant ($F(2,39)=5.740$, $P<0.01$), whereas the effect of C48/80 injection was not significant ($F(1,39)=0.373$, $P=0.5449$). No significant ($F(2,39)=1.132$, $P=0.3329$) interaction between SCH23390 and C48/80 was detected.

3.3. Immunoreactivity

As shown in Fig. 3, c-fos immunoreactive cells were detected in the region near the peduncular part of the lateral hypothalamus (PLH) in all

groups. In particular, c-fos immunoreactivities were enhanced in mice given both SCH23390 and C48/80 compared with other groups. To clarify the differences of immunoreactive levels, the ratio of c-fos-immunopositive cells to HE stained cells was calculated. The effects of SCH23390 administration ($F(1,8)=50.817$, $P<0.0001$) and C48/80 injection ($F(1,8)=79.246$, $P<0.0001$) were significant. Furthermore, a significant ($F(1,8)=34.460$, $P<0.0005$) interaction between SCH23390 and C48/80 was detected. Co-administration of SCH23390 and C48/80 significantly ($P<0.01$) increased c-fos immunoreactivities as compared with that in the other groups (Fig. 3E). To confirm the specificity of c-fos immunoreactivities, serial sections (SCH23390 and C48/80 co-administration group) were stained in absence of c-fos primary antibody. The ratio of staining cells per HE staining cells in these sections was significantly ($P<0.05$) decreased as compared with the ratio of those detected in the co-administration group with c-fos primary antibody (data not shown).

3.4. Double immunofluorescence

By means of double immunofluorescence, c-fos-immunopositive cells were detected in the region near the PLH and striatum (Figs. 4, 5 and 6). C-fos-immunopositive cells were co-localized with dopamine D_1 and D_2 receptors in the striatum (Fig. 6). Although c-fos was also co-localized with dopamine D_1 and D_2 receptors in the PLH area of C48/80 treated (without SCH23390) mice (Fig. 4), c-fos and dopamine D_1 receptors-immunoreactivities did not overlap in the PLH area of co-administrated (SCH23390 and C48/80) mice (Fig. 5).

4. Discussion

In the present study, C48/80-induced body scratching behavior was suppressed by i.c. injection of SCH23390. It has been reported that scratching behavior may be due to the itch sensation and an impulse to scratch, but not due to pain (Kuraishi et al., 1995); therefore, scratching is an itch-specific behavior. Thus, dopamine D_1 receptors were expected to mediate the itch sensation. However, consideration of the effect of SCH23390 on locomotor activity was required, since SCH23390 decreased locomotor activity following intraperitoneal injection (Merali and Piggins, 1990; Benturquia et al., 2008). On the other hand, central administration of SCH23390 had no significant effects on locomotor activity at doses of 0.5 or 1.0 μg (Hall et al., 2009; Rezayof et al., 2009), which were the same doses used in the present study. Furthermore, spontaneous and normal activities were maintained in the cases of administration of physiological saline or distilled water by i.c. injection (Ueda et al., 1979). Therefore, it seems unlikely that the technique of i.c. injection itself affected behavior, and the reduction of body scratching behavior by i.c. injection of SCH23390 was not attributed to the inhibition of locomotor activity in the present study. For these reasons, it appears that dopamine D_1 receptors contributed to locally stimulated scratching behavior or the itch sensation.

In the periphery, pruritogens provoke the itch sensation, and this information is transmitted via the dorsal root ganglia to the CNS. This neural pathway exists in the spinothalamic tract and requires the activity of primary afferent C-fibers (Ständer et al., 2003; Twycross et al., 2003; Ikoma et

al., 2006). However, little is known about the details of the mechanisms of the itch sensation. This is the first report of the suppression of C48/80-induced scratching behavior by central administration of SCH23390. Further study is required, but it is predicted that dopamine D_1 receptor activation or release of dopamine in the CNS is important for mediating the itch sensation and causing scratching behavior induced by a peripheral pruritogen. Co-administration of SCH23390 and C48/80 enhanced c-fos immunoreactivities in the PLH, whereas the immunoreactivities in the other groups were unchanged. In this region, non-specific staining was not different among groups. Thus, these effects of SCH23390 and C48/80 are considered to be specific. These results suggest that the suppression of C48/80-induced scratching behavior by SCH23390 was related to the strong activation of this region. No significant change of c-fos immunoreactivities were noted with SCH23390 administration (Hunt and McGregor, 2002), and SCH23390 effectively counteracted the massive c-fos increase by SKF38393, a dopamine D_2 receptor agonist (Blandini et al., 2003). These reports suggest that SCH23390 contributed little to increasing c-fos immunoreactivities when it was administered alone. In line with this hypothesis, the enhanced c-fos immunoreactivities were detected in the presence of both SCH23390 and C48/80 in the present study. The PLH constitutes the part of the corticospinal tract (CST) that is able to initiate precise movement of the entire musculature of the axial and limb muscle groups (Canty and Murphy, 2008). Hence, SCH23390 may suppress C48/80-induced scratching behavior via the activation of CST.

Co-localization of immunoreactivities was found between c-fos and

dopamine D_2 receptors, but not between c-fos and dopamine D_1 receptors in the region near the PLH of co-administration group (Fig. 5). A dopamine D_2 receptor is involved in an inhibitory action. SCH23390 increases dopamine and its metabolites, but it was not mediated by dopamine D_1 receptors (Bourne, 2001). The specific dopamine D_2 receptor agonist PPHT (2-(Nphenethyl-N-propyl)amino-5-hydroxytetralin hydrochloride) suppressed bombesin-induced scratching behavior (Merali and Piggins, 1990). Thus, the dopamine D_2 receptors seem to be playing a positive role in the treatment suppression of scratching behavior.

5. Conclusions

Control of central dopamine D_1 receptor activation is important to relieve itch sensation and scratching behavior caused by peripheral pruritogen. The dopamine D_2 receptors in the PLH seem to be involved in alleviation of C48/80-induced scratching behavior by SCH23390 administration. In conclusion, the dopaminergic system may be playing an important role in the suppression of C48/80-induced scratching behavior.

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Figure captions

Fig. 1.

Scratching behavior induced by s.c. injection of compound 48/80 (C48/80) and sham treatment. Results are expressed as mean±S.E.M. (n=3-4). *P<0.05 and **P<0.01 compared with sham or C48/80 (0 mg/ml)-treated mice.

Fig. 2.

Effect of SCH23390 on compound 48/80 (C48/80) associated scratching behavior. Effect of intracisternal administration of several doses of SCH23390 on C48/80-induced (A) body scratching behavior and (B) head scratching behavior during 30 min period following C48/80 injection. Results are expressed as mean±S.E.M. (n=7-9). **P<0.01 compared with the control group in the same dose of SCH23390.

Fig. 3.

The c-fos immunoreactivities in the brain sites of mice administered with SCH23390, compound 48/80 (C48/80) or both. Immunoreactive patterns of c-fos in (A) vehicle and saline-, (B) vehicle and C48/80-, (C) SCH23390 and saline-, (D) SCH23390 and C48/80-treated mice brains (f: fornix; ic: internal capsule; opt: optic tract, modified from Franklin & Paxinos, 2007, Fig. 39). (E): Coadministration of SCH23390 and C48/80 enhanced c-fos expression in the peduncular part of the lateral hypothalamus. Data are shown as the number of c-fos-positive cells per hematoxylin and eosin (HE) stained cells. Results are expressed as mean±S.E.M. (n=3). **P<0.01 compared with other groups. Scale bars =100 μm.

Fig.4.

Double-staining of c-fos (A, D:green) and dopamine D_1 or D_2 receptors (B, E: red), and co-localization of c-fos/dopamine D_1 receptors (C), c-fos/dopamine D_2 receptors (F) (yellow) in adjacent area of peduncular part of the lateral hypothalamus of compound 48/80 treated mice (without SCH23390). Almost all of immunoreactive cells were overlapped (C, F). D_1R: dopamine D_1 receptors, D_2R: dopamine D_2 receptors, C48/80: compound 48/80. Scale bars =50 µm.

Fig.5.

Double-staining of c-fos (A, D:green) and dopamine D_1 or D_2 receptors (B, E: red), and co-localization of c-fos/dopamine D_1 receptors (C), c-fos/dopamine D_2 receptors (F) (yellow) in adjacent area of peduncular part of the lateral hypothalamus of co-administrated (SCH23390 and compound 48/80) mice. Almost all of c-fos/dopamine D_2 receptors immunoreactive cells were overlapped (F). Scale bars =50 µm. D_1R: dopamine D_1 receptors, D_2R: dopamine D_2 receptors, C48/80: compound 48/80.

Fig.6.

Double-staining of c-fos (A, d:green) and dopamine D_1 or D_2 receptors (B, E: red), and co-localization of c-fos/dopamine D_1 receptors (C), c-fos/dopamine D_2 receptors (F) (yellow) in striatum of co-administrated (SCH23390 and compound 48/80) mice. Almost all of immunoreactive cells were overlapped (C, F). Scale bars =50 µm. D_1R: dopamine D_1 receptors, D_2R: dopamine D_2 receptors, C48/80: compound 48/80.

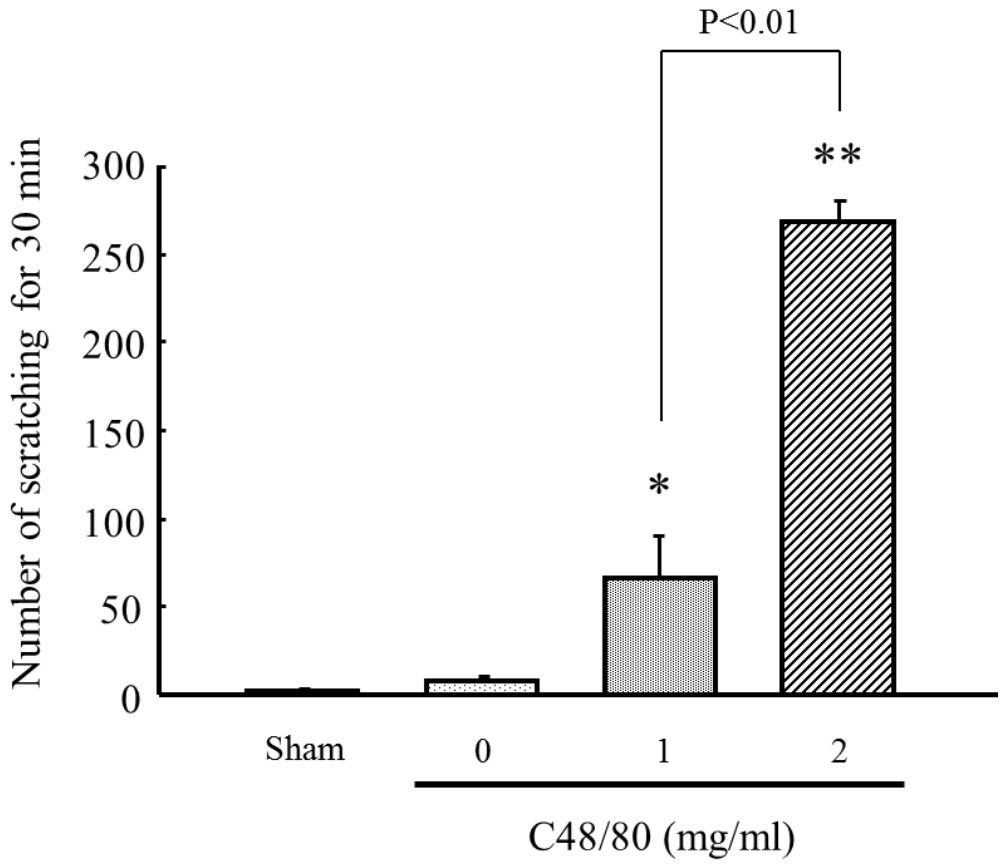


Fig. 1 Akimoto and Furuse

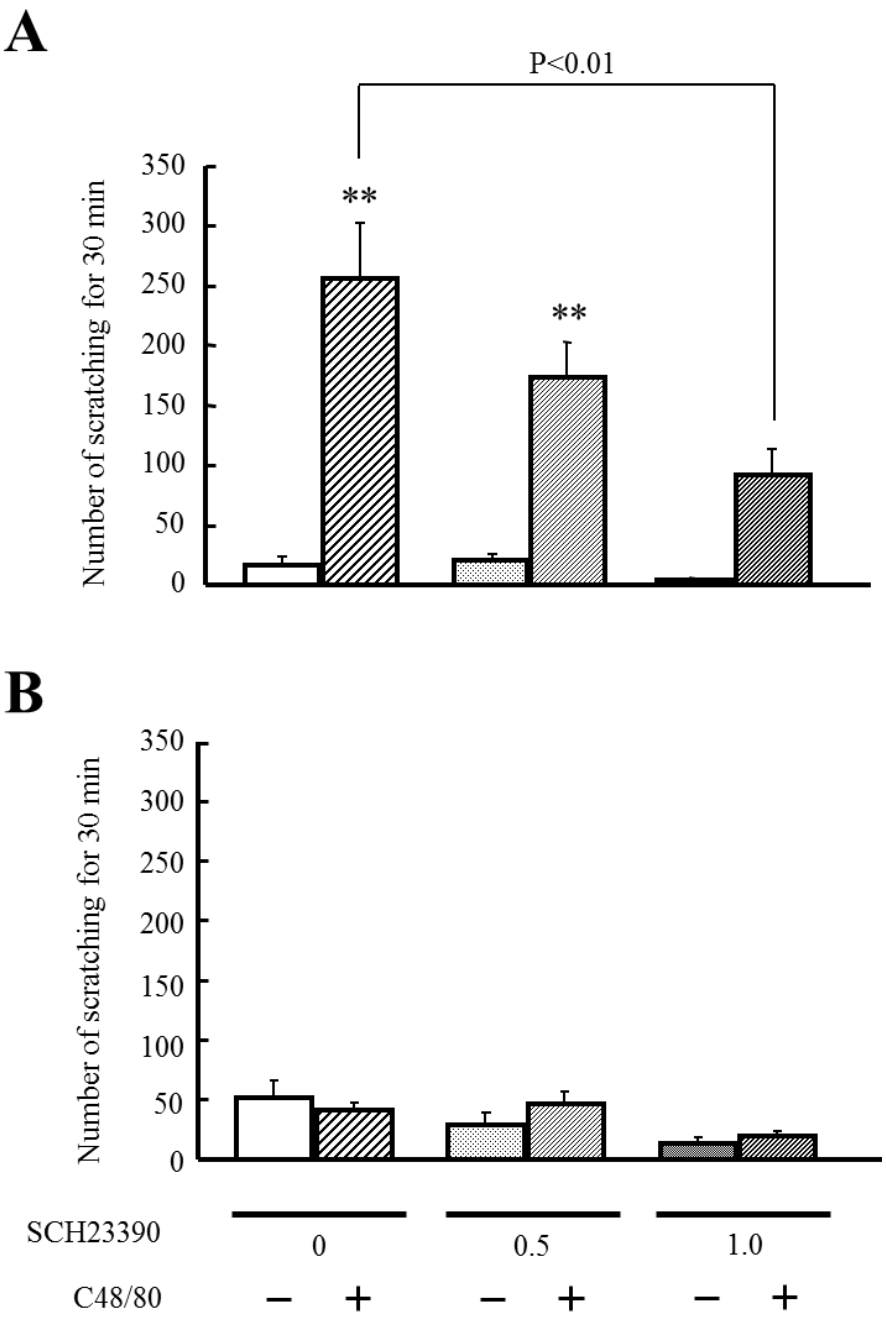


Fig. 2 Akimoto and Furuse

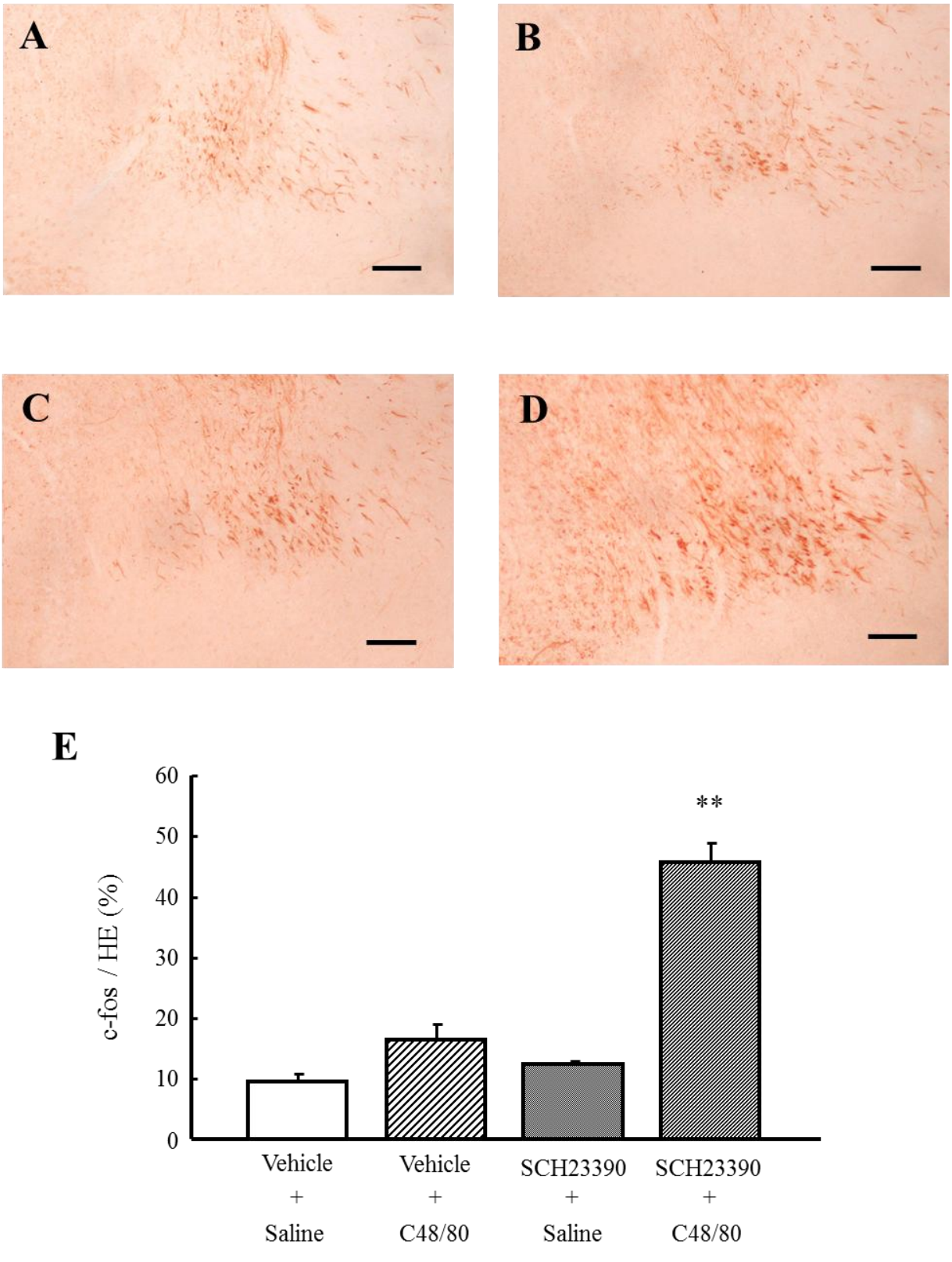
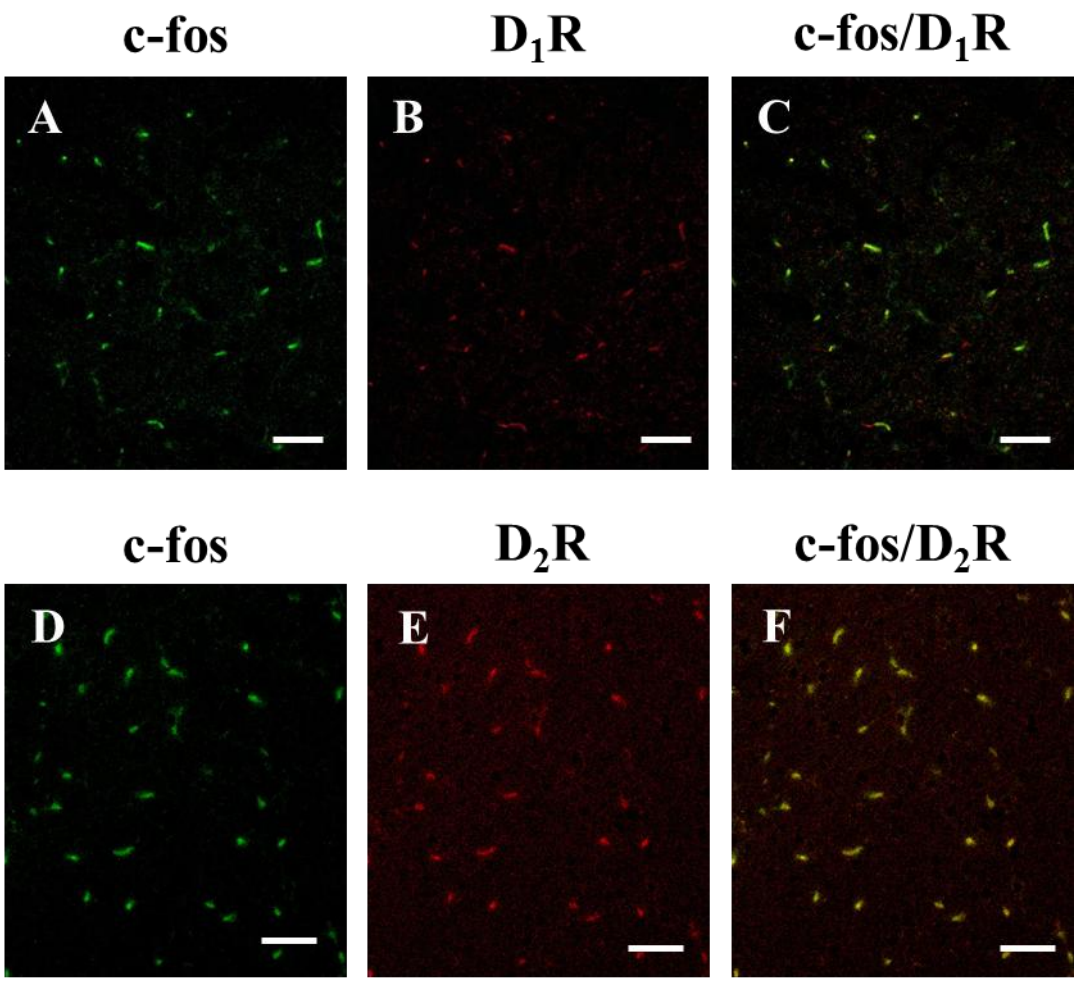


Fig. 3 Akimoto and Furuse



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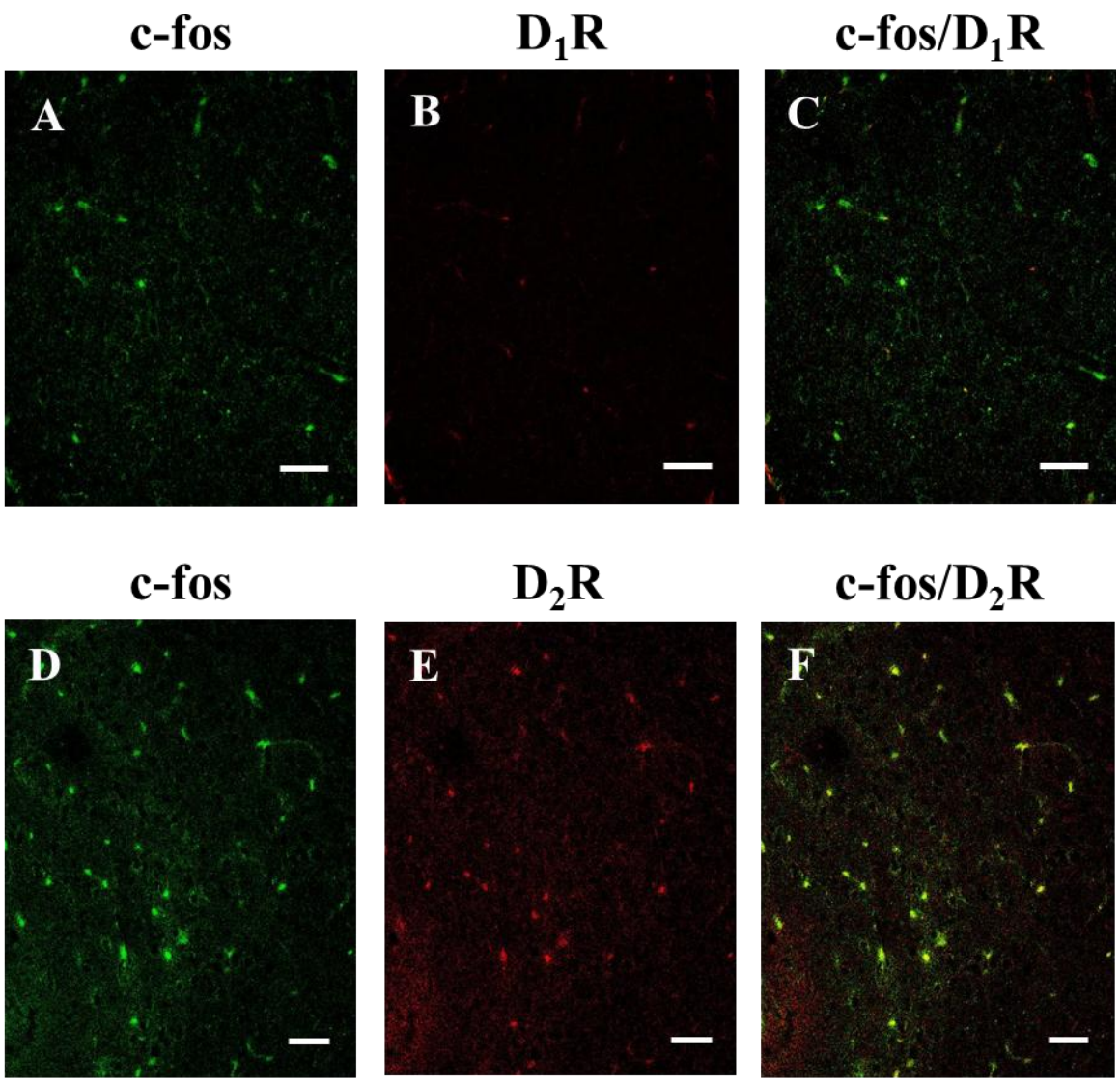

Fig. 5 Akimoto and Furuse

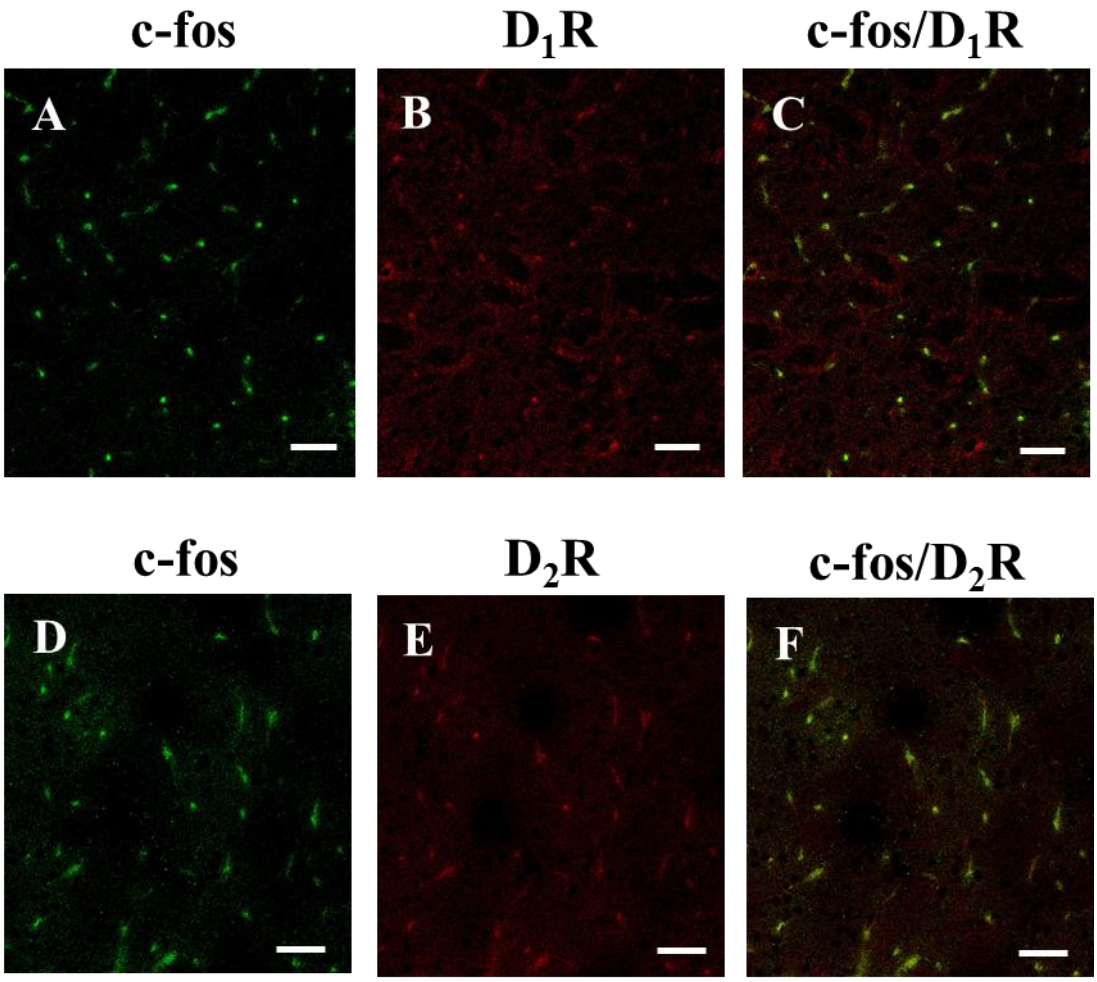

Fig. 6 Akimoto and Furuse