Annexin 1 exerts anti-nociceptive effects after peripheral inflammatory pain through formyl-peptide-receptor-like 1 in rat dorsal root ganglion

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Editor's key points

- Annexin 1 (ANXA1) and formyl-peptide-receptor-like 1 (FPR2/ALX) are present in both neuronal and glial dorsal root ganglia cells in rats.
- ANXA1 is up-regulated in dorsal root ganglia (DRGs) after inflammatory pain in rats.
- Intrathecal ANXA1 acts via FPR2/ALX and exerts anti-nociception at the level of DRGs after inflammation.
- This study adds to our understanding of the mechanisms of action of ANXA1 in inflammatory pain.

Background. Annexin 1 (ANXA1) has analgesic effects in inflammatory pain. We aimed to investigate the anti-nociceptive role of ANXA1, at the dorsal root ganglion (DRG) level, through an interaction with formyl-peptide-receptor-like 1 (FPR2/ALX).

Methods. Inflammatory pain was evoked by injecting complete Freund’s adjuvant (CFA, 50 μl) into the hindpaw of male Sprague–Dawley rats. The distribution of ANXA1 and FPR2/ALX in L4/5 DRGs was evaluated by immunofluorescence. The expression of ANXA1 was measured by western blot. The involvement of FPR2/ALX in the anti-nociception of ANXA1 was investigated by thermal (irradiant heat) and mechanical (von Frey filament) pain tests with intrathecal (i.t.) ANXA1-derived peptide (Anxa1 2–26), FPR2/ALX agonist 5(S)-6(R)-7-trihydroxyheptanoic-acid-methyl-ester (BML-111), and antagonist N-t-Boc-Phe-Leu-Phe-Leu-Phe (Boc1).

Results. ANXA1 and FPR2/ALX localized in the satellite glial cells and neurones in L4/5 DRGs. CFA treatment (n=20) increased ANXA1 expression in L4/5 DRGs within 7 days (P<0.01). I.T. Anxa1 2–26 (20 and 100 μg μl⁻¹) and BML-111 (10 and 100 nmol) reduced CFA-induced thermal and mechanical nociception within 48 h (n=40) (P<0.05). However, i.t. Boc1 10 μg intensified inflammatory pain (P<0.05) and reversed the anti-nociceptive effect of Anxa1 2–26 (n=25) (P<0.05). Moreover, ANXA1 expression increased in L4/5 DRGs after i.t. Anxa1 2–26 (20 μg μl⁻¹) (P<0.05) and BML-111 (10 nmol) (P<0.01) but decreased after i.t. Boc1 (10 and 100 μg) alone (P<0.01) or Boc1 (10 μg) co-injection with Anxa1 2–26 (20 μg μl⁻¹) (P<0.05).

Conclusions. Endogenous ANXA1 expression at the DRG level is involved in CFA-induced inflammatory pain, and i.t. ANXA1 20 μg μl⁻¹ produces its anti-nociceptive effect through FPR2/ALX.

Keywords: annexin 1; ganglia, sensory; inflammation; receptors, formyl peptide

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Annexin 1 (ANXA1, a 37 kDa protein) was originally found to be involved in the anti-inflammatory action of glucocorticoids1 and expressed in the mononuclear phagocyte system.2 Bolton and colleagues’3 study showed the presence of ANXA1 in the nervous system. Recently, the analgesic role of ANXA1 was confirmed in murines.4 Moreover, ANXA1 knockout (ANXA1⁻/⁻) mice were shown to be more sensitive to nociception compared with wild-type mice (ANXA1⁺/⁺),5 thereby confirming the participation of ANXA1 in nociceptive sensation.

FPR2/ALX, a member of the formyl-peptide receptor (FPR) family, is not only expressed in neutrophils and monocytes,6 but also in the nervous system.7 Perretti’s8 9 studies indicated that FPR2/ALX is able to interact with several endogenous molecules including both ANXA1 and its peptidomimetics. Moreover, the inhibition of formalin-induced nociception by ANXA1 peptidomimetics was shown to depend on activation of FPR family members.9 These results raised the possibility that FPR2/ALX mediates essential effects of ANXA1 in pain modulation.
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No studies to date have addressed the antinociceptive role of ANXA1 and FPR2/ALX at the level of dorsal root ganglia (DRGs). This study was undertaken to investigate a potential involvement of ANXA1 to inhibit nociception through FPR2/ALX in the DRGs in a rat model of inflammatory pain.

Methods
Animal model and surgery
Animal experiments conformed to the Animal Care Committee at Huazhong University of Science and Technology and the guidelines for pain research with laboratory animals. Male Sprague–Dawley rats weighing 250–300 g were housed in individual cages under conditions of a 12 h/12 h light/dark cycle and with food and water available ad libitum. Inflammation was induced by subcutaneously injecting 50 μl of complete Freund’s adjuvant (CFA) (Sigma, St Louis, MO, USA) into the dorsal surface of the rat’s left hindpaw as described previously. The injection was performed under anaesthesia by means of ether inhalation. For intrathecal (i.t.) catheterization, animals were anaesthetized with 10% chlorohydrate (0.35 g kg⁻¹ i.p.). Adequate anaesthesia was ascertained by no withdrawal response to a paw pinch. Implantation of i.t. cannulas was performed as described previously. A polyethylene-10 catheter was inserted rostrally between L5 and L6 vertebrae to reach the hindpaw as described previously. The injection was per- formed under anaesthesia by means of ether inhalation. For intrathecal (i.t.) catheterization, animals were anaesthetized with 10% chlorohydrate (0.35 g kg⁻¹ i.p.). Adequate anaesthesia was ascertained by no withdrawal response to a paw pinch. Implantation of i.t. cannulas was performed as described previously. A polyethylene-10 catheter was inserted rostrally between L5 and L6 vertebrae to reach the lumbar enlargement of the spinal cord. The animals were allowed a period of 5 days for post-surgical recovery.

Drugs
The Anxa12–26 (Ac-AMVSEFLQAWFIEENNEQYVQTVK) peptide was prepared by Boster Biological Technology, Ltd (Wuhan, China), by using solid-phase stepwise synthesis. The FPR2/ALX agonist 5(S)-6(R)-7-trihydroxyheptanoic-acid-methyl- ester (BML-111) and antagonist N-t-Boc-Phe-Leu-Phe-Leu-Phe (Boc1) were purchased from Calbiochem (San Diego, CA, USA). Other materials were obtained from Sigma-Aldrich S.r.l. (Milan, Italy). Anxa12–26, BML-111, and Boc1 were injected via the catheter in a volume of 10 μl followed by 10 μl of saline for flushing. When co-administered Boc1 with ANXA1, we applied the first single-drug injection combined with the equivalent vehicle volume of the second drug. Drugs for i.t. administration were freshly dissolved in saline each test day. BML-111 and Boc1 stock solutions were prepared by dissolving the compounds in dimethyl sulphoxide (DMSO); aliquots of this solution were used for subsequent dilution in saline (DMSO:saline 1:3, v/v). After baseline behavioural assessments, i.t. injections of either drug or vehicle were performed at 12 and 1 h before CFA injection. We chose these doses based upon our preliminary experiments.

Immunofluorescence and image analysis
Animals were deeply anaesthetized with 10% chlorohydrate 0.35 g kg⁻¹ and transcardially perfused with saline, followed by 4% paraformaldehyde (in 0.01 M PBS, pH 7.4). L4/5 DRGs were removed, post-fixed for 6 h, and treated with 30% sucrose (in 0.1 M PB, pH 7.4) overnight. Cryosections (12 μm) were cut and stored at −20°C. Mounted DRG sections were allowed to thaw at room temperature. Indirect immunofluorescence was used to detect ANXA1, FPR2/ALX, and glial fibrillary acidic protein (GFAP). For analyses of co-localization of ANXA1 with GFAP, FPR2/ALX with GFAP, and ANXA1 with FPR2/ALX, we used dual-labelling immunofluorescence. Donkey or goat serum was used for blocking for 1 h. Next, the antibodies to ANXA1 (rabbit, Santa Cruz Biotechnology, CA, USA, 1:100), FPR2/ALX (mouse, Sigma-Aldrich, St Louis, MO, USA, 1:200), and GFAP (chicken, Abcam, Cambridge, MA, USA, 1:2000) were incubated for 48 h. FITC- and TRITC-conjugated second antibodies were then incubated for 1 h. The sections were examined with a laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany) using an omnichrome air-cooled helium/neon laser tuned to produce beams at 488 and 568 nm. Five randomly selected sections were used. All immunoreactive positive profiles in a section were outlined, creating an artificial overlay. Measuring the average optical density of double-labelled cells was conducted on confocal images randomly taken from two view fields from each section; measuring was performed using NIH Image J software (Bethesda, MD, USA).

Nociceptive behavioural tests
Thermal hyperalgesia was assessed by using a plantar analgesia meter for paw stimulation (Ugo Basile, Italy) as described previously. Mechanical alldynia was inspected by using an electronic von Frey anaesthesiometer (Ugo Basile, Italy) applied to the plantar surface of the hindpaw. The paw withdrawal thermal latency (PWL) and paw withdrawal mechanical threshold (PWT) were recorded five times with a 5 min interval, for both left and right hindpaws, as the time (s) taken from the onset of radiant heat and hand- held force (g) transducer stimulation to withdrawal of the hindpaw. A mean value of these five consecutive measurements was taken for each paw.

Western blot analysis
At 0 (before CFA injection), 1, 7, and 14 days after CFA injection and after behavioural experiments (48 h after CFA treatment) with i.t. injection, animals were killed by decapitation and exsanguination. L4/5 DRGs were removed as previously described and immediately homogenized in ice-chilled lysis buffer (50 mM Tris–HCl, pH 7.5) containing 150 mM NaCl, 0.1% Nonidet P40, 0.1% cholic acid, 2 mg ml⁻¹ leupeptin, 1 mg ml⁻¹ pepstatin, 2 mM phenylmethylsulphonyl fluoride, and 2 mM EDTA. The homogenates were centrifuged at 15 000g for 10 min at 4°C to yield the total protein extract in the supernatant. The concentration of protein was measured as previously described. Equal amounts of samples (50 μg) were denatured and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis by using 12% running gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories,
Hercules, CA, USA). The PVDF membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 2 h and incubated with the primary antibody, rabbit polyclonal anti-ANXA1 (Santa Cruz Biotechnology, 1:500) and anti-β-actin (Sigma, 1:10 000) overnight at 4 °C. Specific reactive bands were detected using a peroxidase-conjugated goat anti-rabbit secondary antibody (1:8000; Jackson ImmunoResearch, Baltimore, MD, USA) for 1 h. The enhanced chemiluminescence method (ECL plus Western blotting detection reagents; Amersham Biosciences, Little Chalfont, UK) was used to reveal the protein bands. The optical density of each band was measured with a computer-assisted imaging analysis system (Quantity One, Bio-Rad, Hemel Hempstead, UK) and normalized to optical density of the housekeeping gene β-actin.

Data analysis and statistics

Data are expressed as mean (SD). Pre-CFA baseline measures were analysed by one-way analysis of variance (ANOVA) for radiant heat and von Frey tests. Post-drug time course measures for hyperalgesia and allodynia were analysed by ANOVA with repeated measures followed by the Newman–Keuls post hoc testing. The immunofluorescent results were analysed by Student’s t-test. Western blot results were analysed by one-way ANOVA followed by Dunnett’s multiple comparisons. P<0.05 was considered statistically significant.

Results

Localization of ANXA1 and FPR2/ALX in L4/5 DRGs

ANXA1-positive cells were detected throughout the L4/5 DRGs. We found ANXA1 to be localized in SGCs as indicated by the co-expression with GFAP and in neurones (Fig. 1A–C). Similarly, FPR2/ALX was abundantly expressed in SGCs and some of the DRG neurones (Fig. 1D–F). Double immunostaining revealed that ANXA1 was co-localized with FPR2/ALX in both neurones and SGCs of the L4/5 DRGs (Fig. 1G–I).

CFA injection induces up-regulation of ANXA1 expression in L4/5 DRGs

The unilateral CFA injection increased ANXA1 expression in L4/5 DRGs on both sides, with the most prominent increase being found in the ipsilateral DRG on day 1 post-CFA injection (Fig. 2A–I). The mean density of ANXA1 immunoreactivity in ipsilateral L4/5 DRGs was significantly increased [97.31 (8.36)] but not significantly on the contralateral side [70.74 (6.29)] when compared with the naive group [56.66 (4.72)] (Fig. 2K).

Western blot results reconfirmed the increase in ANXA1 protein after CFA treatment. In the ipsilateral DRGs, ANXA1 significantly increased on day 1 [2.08 (0.05)] and day 7 [2.09 (0.07)] post-CFA injection compared with naive animals [0.22 (0.06)] (Fig. 2J and L). Contralaterally, a significant increase in ANXA1 also occurred on day 1 [1.44 (0.04)] but recovered at 7 days [0.43 (0.03)] post-CFA injection compared with control [0.41 (0.04)] (Fig. 2J and L).
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Fig 2 Effects of CFA-induced inflammation on ANXA1 expression in L4/5 DRGs. ANXA1- and GFAP-immunoreactivity in DRGs are shown in naive (A and B) and 1 day post-CFA injection on the ipsilateral side (D and E) and the contralateral side (G and H). Overlaid pictures are shown in (C, F, and I). (J) Western blot analysis showed an increase in ANXA1 in the ipsilateral L4/5 DRGs within 14 days and significant increase on days 1 and 7 after CFA injection. A significant increase on the contralateral side was also observed in the CFA 1 day group. β-Actin served as a loading control. (K) The average optical density of double-labelled cells in L4/5 DRGs was elevated in the CFA 1 day ipsilateral group. Each bar represents mean (SD) of five rats. *P<0.05 (ipsilateral vs naive) (scale bar=50 μm). (L) Quantification of ANXA1 level on both sides of the L4/5 DRGs. Each bar represents mean (SD) of five rats. *P<0.05; **P<0.01 (post-CFA vs naive).
I.T. Anxa12–26 or BML-111 attenuates CFA-induced inflammatory nociception and increases ANXA1 expression in L4/5 DRGs

Both PWLs and PWTs were increased in rats receiving i.t. Anxa12−26 at 20 and 100 μg μl−1 compared with 5 μg μl−1 or vehicle only. When compared with vehicle [3.19 (0.62) s; 11.63 (2.07) g], Anxa12−26 (20 and 100 μg μl−1) significantly increased PWL from 4 [5.88 (1.06); 5.06 (0.27) s] to 24 h [6.74 (0.91); 7.33 (0.81) s] (Fig. 3A and B) and PWT from 4 [17.04 (2.39); 18.83 (1.66) g] to 36 h [20.67 (1.58); 21.63 (4.51) g] (Fig. 3C). Similarly, i.t. BML-111 (10 and 100 nmol) increased PWL in the ipsilateral hindpaw from 12 [7.25 (2.07); 8.43 (1.47) s] to 48 h [6.43 (0.46); 7.81 (1.24) s] and PWT from 12 [21.40 (3.87); 21.10 (2.42) g] to 36 h [19.88 (2.10); 22.67 (2.48) g] after CFA injection (Fig. 4A and B) compared with vehicle [5.49 (0.87) s; 13.54 (2.96) g]. Neither Anxa12−26 nor BML-111 significantly influenced the PWL and PWT of the contralateral hindpaw (Figs 3B and D, 4B and D). Western blot data revealed that i.t. 20 μg μl−1 Anxa12−26 or 10 nmol BML-111 significantly up-regulated [1.01 (0.05); 1.34 (0.10)] endogenous ANXA1 in the ipsilateral L4/5 DRGs at 2 days post-CFA injection when compared with vehicle [0.57 (0.06); 0.57 (0.08)] (Figs 3E and F, 4E and F). However, a slight but not significant decrease [0.39 (0.06); 0.41 (0.06)] in ANXA1 expression was observed after i.t. 100 μg μl−1 Anxa12−26 or 100 nmol BML-111. On the contralateral side, both Anxa12−26 and BML-111 had similar effects on ANXA1 expression, but to a smaller extent vs the ipsilateral side (Figs 3E and F, 4E and F).

I.T. Boc1 enhances CFA-induced inflammatory pain and down-regulates ANXA1 expression in L4/5 DRGs

I.T. Boc1 (10 and 100 μg) significantly decreased CFA-induced PWL and PWT from 24 [2.32 (0.40) s; 10.37 (2.59) g] to 48 h [3.11 (0.71) s; 9.34 (1.95) g] after CFA injection when compared with the vehicle treatment [4.12 (0.46) s; 14.99 (1.11) g] (Fig. 5A and C). In the contralateral hindpaw, PWL and PWT were not significantly altered (Fig. 5B and D). ANXA1 protein was significantly decreased [0.54 (0.03); 0.47 (0.08)] by i.t. 10 and 100 μg Boc1 in the ipsilateral DRG at 2 days after CFA injection when compared with vehicle [1.36 (0.13)] (Fig. 5E and F). On the contralateral side, the changes in ANXA1 expression were similar to those on the ipsilateral side; notwithstanding the smaller responses, they still differed significantly from the vehicle controls (Fig. 5E and F).

Effects of Boc1 on Anxa12−26 induced anti-nociception and endogenous ANXA1 up-regulation after CFA-induced inflammatory pain

Boc1 (10 μg) and Anxa12−26 (20 μg μl−1) were co-injected i.t. before CFA treatment. Consistently, i.t. Anxa12−26 (20 μg μl−1) produced obvious anti-nociceptive effect on both PWL and PWT. Co-injection with Boc1 significantly blocked Anxa12−26’s anti-nociceptive effect as measured with PWL from 8 [4.06 (1.04) s] to 36 h [4.47 (0.64) s] and PWT from 8 [18.34 (3.17) g] to 48 h [15.41 (3.13) g] after CFA injection (Fig. 6A and C) when compared with Anxa12−26 alone [6.85 (1.07) s; 23.42 (2.59) g]. In the contralateral hindpaw, PWL and PWT were not significantly changed (Fig. 6B and D).

I.T. co-administration of Boc1–Anxa12−26 significantly inhibited [0.90 (0.11); 0.61 (0.09)] Anxa12−26-induced up-regulation [1.29 (0.11); 1.05 (0.09)] of endogenous ANXA1, both in the ipsilateral and in the contralateral L4/5 DRGs at 2 days after CFA injection (Fig. 6E and F).

Discussion

In the present study, (i) subcutaneous injection of CFA into the rat hindpaw resulted in the up-regulation of ANXA1 in L4/5 DRGs. (ii) In the nociception tests, i.t. administration of the ANXA1-derived peptide Anxa12−26 caused an anti-nociceptive effect, and the FPR2/ALX agonist BML-111 displayed similar effects to Anxa12−26, whereas FPR2/ALX antagonist, Boc1, blocked the effects. (iii) ANXA1 expression was up-regulated in the L4/5 DRGs when FPR2/ALX was activated by i.t. Anxa12−26 or BML-111 at a medium dose while it was suppressed when FPR2/ALX was inhibited by i.t. Boc1.

So far, little was known about the specific distribution or function of ANXA1 and FPR2/ALX in DRGs. Our results show that ANXA1 is present in both neurones and SGCs in L4/5 DRGs. These data partially concur with Naciff’s finding that ANXA1 was distributed in the perinuclear, and the nucleolus of the rat DRG cellular bodies.17 In our study, ANXA1 was also observed in the SGCs surrounding the DRG neurones. This difference probably due to the disparities in the epitopes recognized by the antibodies and the protocols applied in both studies. Regarding the epitope recognition, post-translational modifications of ANXA1 in the SGCs, such as cross-linking, might mask immunoreactive epitopes. As for the protocols, Naciff chose 4-μm-thick paraffin-embedded brain sections and pH 2.7 buffer solution, whereas we used 12-μm-thick frozen DRG sections and pH 7.4 buffer solution. Evidence suggested that acidity, fixation conditions, and tissue handling may influence ANXA1 epitopes and result in conflicting cellular distribution.18 To provide indisputable expression profiles, further studies implementing different conditions and comprising different neural tissues are needed. The presence of FPR2/ALX in DRGs has not been reported previously. In our study, FPR2/ALX, like ANXA1, was seen in both neurones and SGCs in L4/5 DRGs. This parallel distribution of ANXA1 and FPR2/ALX in DRG is consistent with a possible role for FPR2/ALX in mediating effects of ANXA1. These data are consistent with Dalpiaz and colleagues6 and D’Acquisto’s19 findings that ANXA1 is expressed in parallel with FPR2/ALX in DRG and peripheral tissues, and that ANXA1 and FPR2/ALX are expressed in brain sections and pH 2.7 buffer solution, whereas we used 12-μm-thick frozen DRG sections and pH 7.4 buffer solution. Evidence suggested that acidity, fixation conditions, and tissue handling may influence ANXA1 epitopes and result in conflicting cellular distribution.18 To provide indisputable expression profiles, further studies implementing different conditions and comprising different neural tissues are needed. The presence of FPR2/ALX in DRGs has not been reported previously. In our study, FPR2/ALX, like ANXA1, was seen in both neurones and SGCs in L4/5 DRGs. This parallel distribution of ANXA1 and FPR2/ALX in DRG is consistent with a possible role for FPR2/ALX in mediating effects of ANXA1. These data are consistent with Dalpiaz and colleagues6 and D’Acquisto’s19 findings that ANXA1 is expressed in parallel with FPR2/ALX in neutrophils, monocytes, and T cells. ANXA1 was identified originally as being responsible for the anti-inflammatory actions of glucocorticoids2 released from neutrophils, mononuclear, and macrophage cells. Eberhard and colleagues10 also proved that ependymal cells, microglia, and neurones could synthesize and release ANXA1 in response to inflammation. Our finding shows the up-regulation of ANXA1 in the ipsilateral DRG after CFA.
Fig 3  Effects of i.t. Anxa1–26 on CFA-induced inflammatory nociception and endogenous ANXA1 expression in L4/5 DRGs. I.T. administration of Anxa1–26 (20 and 100 μg μl⁻¹) increased the PWLs (a) from 4 to 24 h and PWTs (c) from 4 to 36 h in the ipsilateral hindpaw after CFA injection but did not alter the PWLs and PWTs in the contralateral hindpaw (b and d). Each line represents means (SD) of five rats. *P<0.05 vs vehicle. (e) 20 μg μl⁻¹, but not 5 and 100 μg μl⁻¹ Anxa1–26, increased ANXA1 expression after CFA injection on both the ipsilateral and the contralateral sides, but the contralateral increase was smaller. (f) Quantification of ANXA1 levels on both sides of the L4/5 DRGs. Each bar represents mean (SD) of five rats. *P<0.05 vs vehicle.
Fig 4  Effects of i.t. BML-111 on CFA-induced inflammatory pain and endogenous ANXA1 expression in L4/5 DRGs. I.T. BML-111 (10 and 100 nmol) increased the PWLs from 12 to 48 h (a) and PWTs from 12 to 36 h (c) in the ipsilateral hindpaw after CFA treatment but did not change the PWLs and PWTs in the contralateral hindpaw (b and d). Each line represents means (SD) of five rats. *P<0.05 vs vehicle. (e) 10 nmol, but not 1 and 100 nmol, BML-111, increased ANXA1 expression after CFA injection both on the ipsilateral and the contralateral sides, but the contralateral increase was smaller. (f) Quantification of ANXA1 level on both sides of the L4/5 DRGs. Each bar represents mean (SD) of five rats. *P<0.05 vs vehicle.
Fig 5 Effects of i.t. Boc1 on CFA-induced inflammatory pain and endogenous ANXA1 expression in L4/5 DRGs. I.T. administration of Boc1 (10 and 100 μg) decreased both PWLs (A) and PWTs (C) in the ipsilateral hindpaw from 24 to 48 h after CFA injection but did not alter the PWLs and PWTs on the contralateral side (B and D). Each line represents means (SD) of five rats. *P<0.05 vs vehicle. (E) 10 and 100 μg Boc1 decreased endogenous ANXA1 expression in the ipsilateral L4/5 DRGs after CFA injection. (F) Quantification of ANXA1 level on both sides of the L4/5 DRGs. Each bar represents mean (SD) of five rats. **P<0.01 vs vehicle.
Fig 6 Effects of i.t. co-administered Boc1 and Anxa12–26 on CFA-induced inflammatory pain and endogenous ANXA1 expression in L4/5 DRGs.

I.T. co-administration of Boc1 (10 μg) and Anxa12–26 (20 μg μl⁻¹) reversed Anxa12–26-evoked anti-nociception in the ipsilateral hindpaw on thermal hyperalgesia from 8 to 36 h (A) and mechanical allodynia from 8 to 48 h (C) after CFA treatment but did not alter the PWLs and PWTs in the contralateral hindpaw or either hindpaw of naive and vehicle-treated rats (B and D). *P < 0.05 (Boc1 10 μg vs Anxa12–26 20 μg μl⁻¹ + Boc1 10 μg), #P < 0.05, ##P < 0.01 (Anxa12–26 20 μg μl⁻¹ vs naïve or vehicle). Each line represents means (SD) of five rats. (E) Anxa12–26 (20 μg μl⁻¹) significantly increased ANXA1 expression in both the ipsilateral and contralateral L4/5 DRGs after CFA injection. Co-administration of Boc1 (10 μg) with Anxa12–26 (20 μg μl⁻¹) significantly reduced ANXA1 expression. (F) Quantification of ANXA1 level on both sides of the L4/5 DRGs. Each bar represents mean (SD) of five rats. **P < 0.01 (Anxa12–26 20 μg μl⁻¹ vs Anxa12–26 20 μg μl⁻¹ + Boc1 10 μg), *P < 0.05 (Anxa12–26 20 μg μl⁻¹ vs naïve), ##P < 0.01 (Anxa12–26 20 μg μl⁻¹ vs vehicle).
treatment. By inference, the increased expression of ANXA1 in DRG might be the result of synthesis and release from neurones or glia. Our study does not provide direct evidence for whether FPR2/ALX mediates the inhibitory effects of ANXA1 on activated glial cells and the kind of cells involved in the up-regulation of ANXA1. As a next step, we will perform in vitro studies to further explore the contributions of both cell types, by, for example, establishing a system of co-cultured DRG neurones and SGCs. The up-regulation of ANXA1 was also seen in the contralateral DRG after unilateral CFA injection. This finding, consistent with von Banchet and colleagues' report that a unilateral peripheral inflammation can induce protein changes on bilateral DRGs, implies that the contralateral increase in ANXA1 may be secondary. A possible mechanism comprises the signalling molecules produced by neurones or glia that may diffuse into the systemic circulation and then reach the microenvironment of the contralateral DRG in the absence of blood–nerve barrier.

We found that i.t. Anxa12–26 alleviated CFA-induced inflammatory nociception, indicating the involvement of ANXA1 in nociceptive modulation, which accord with previous study that Anxa12–26 inhibits the hyperalgesic effects. ANXA1 was shown to be a novel endogenous ligand of FPR and received much attention as a putative endogenous anti-inflammatory agent. Walther and colleagues indicated that Anxa12–26 behaved as an FPR agonist and that FPR antagonists were able to inhibit Anxa12–26-induced effects. In our study, i.t. administration of the FPR2/ALX agonist BML-111 displayed similar effects as did Anxa12–26, whereas the antagonist Boc1 blocked the effects. These results support Pieretti and colleagues' finding that exogenous ANXA1 inhibit formalin-induced nociception associated with inflammatory processes through a mechanism that involves FPR. Interestingly, Boc1, when administered alone, aggravated CFA-induced nociception and down-regulated ANXA1 expression. The results might be explained by the concept that an 'endogenous ANXA1 tone' in the DRGs contributes to determining the threshold of inflammatory pain. This is in concordance with the similar endogenous tone in the cannabinoid systems. Moreover, ANXA1 null mice display an increased susceptibility to nociceptive pain. Together, these findings indicate a role for an endogenous ANXA1 tone in modulating nociception.

Curiously, the high dose of ANXA1 peptide and BML-111 did not increase but mildly decreased ANXA1 expression. We suggest that, as the expression of ANXA1 increases after Anxa12–26 and BML-111 application, the expression of FPR2/ALX might change, too. Studies reported that FPR2/ALX expression rapidly changes in the presence of high or low agonist concentrations. Our next work will investigate the effects of Anxa12–26 and BML-111 on FPR2/ALX expression in DRG. Hopefully, these new experiments will reveal why high dose of Anxa12–26 and BML-111 do not increase ANXA1 expression, despite the fact that, at the same dose, both Anxa12–26 and BML-111 are able to induce anti-nociceptive effects.

In conclusion, both ANXA1 and FPR2/ALX are distributed in neurones and SGCs in rat L4/5 DRGs. The ANXA1 expression was significantly increased after CFA-induced inflammation. I.T. ANXA1-derived peptide not only attenuated the nociception but also up-regulated ANXA1 expression. More importantly, this up-regulation can be mimicked by i.t. administration of the FPR2/ALX agonist and blocked by its antagonist. Taken together, our results indicate that ANXA1 may contribute to modulating CFA-induced inflammatory pain by acting on FPR2/ALX at the DRG level.

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Conflict of interest

None declared.

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References

9 Perretti M. The annexin 1 receptor(s): is the plot unravelling? Trends Pharmacol Sci 2003; 24: 574–9
11 Cook CD, Moore KL. Effects of sex, hindpaw injection site and stimulus modality on nociceptive sensitivity in arthritic rats. Physiol Behav 2006; 87: 552–62


18 McKanna JA, Zhang MZ. Immunohistochemical localization of lipocortin 1 in rat brain is sensitive to pH, freezing, and dehydration. J Histochem Cytochem 1997; 45: 527–38


26 Flower RJ, Blackwell GJ. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. Nature 1979; 278: 456–9

27 Walther A, Riehemann K, Gerke V. A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. Mol Cell 2000; 5: 831–40

