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# Long-term incubation with CXCL2, but not with CXCL1, alters the kinetics of TRPV1 receptors in cultured dorsal root ganglia neurons

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**Abstract:** CXCL1 and CXCL2 are homologous chemokines that can be upregulated in different pathological conditions, affecting among other targets, neuronal ionic channels or receptors. TRPV1 is a polymodal nociceptor expressed in both dorsal root and trigeminal ganglia neurons. According to existing data, short-term incubation with CXCL1 can reduce TRPV1 desensitization, however, the long-term modulatory effect of both CXCL1 and CXCL2 on this receptor is less known. In the present study we investigated the influence of overnight incubation with 1.5 nM CXCL1 or CXCL2 on the functioning of TRPV1 receptors expressed in cultured dorsal root ganglia neurons. Calcium imaging and patch-clamp recordings showed that under the same experimental conditions and at the same concentration, only CXCL2 significantly decreased the TRPV1 current and increased its desensitization rate, whereas CXCL1 had no effect. This study proposes a different contribution of CXCL1 and CXCL2 to the modulation of TRPV1-mediated processes, in spite of their highly homologous sequence.

**Key words:** CXCL1; CXCL2; TRPV1; dorsal root ganglia neurons

## INTRODUCTION

Chemokines are a large family of chemotactic cytokines that can be divided into four classes. The largest group is comprised of the CC chemokines that have two adjacent cysteines in their structure (CysCys); the second group is comprised of the CXC chemokines that have a single amino acid between two cysteine residues (CysXCys), the third group is the CX3C chemokines with 3 amino acids between two cysteines (CysXXXCys), and the last group has a single member, XC chemokine, also named lymphotactin [1]. CXCL1 and CXCL2 are two low-molecular-weight members of the ELR(+) CXC chemokine family, with 78% homology of their sequence [2], which act specifically through CXCR2, a G protein-coupled receptor [3].

CXCL1 (C-X-C motif) ligand 1), also known as GROα (growth related oncogene α), KC (keratinocyte derived chemokine) or CINC-1 (cytokine-induced neutrophil chemoattractant-1), is a chemokine with pleiotropic effects. It is known as a neutrophil chemoattractant [2], but it can also enhance tumorigenesis or reinforce senescence when it is secreted by a tumor (e.g. melanoma, ovarian, prostate) or senescent cells [4-7]. It can stimulate proliferation and remyelination during brain development when acting on oligodendrocyte progenitors [8], and it promotes pain, both inflammatory and neuropathic, when it is secreted by macrophages or astrocytes [9-12].

CXCL2 (C-X-C motif chemokine 2), also known as MIP-2 (macrophage inflammatory protein 2), Scyb2 (small inducible cytokine subfamily member 2) or GROβ (growth-regulated protein β) [13], is secreted by activated macrophages, neutrophils, microglia, astrocytes or endothelial cells [2,14]. Similar to CXCL1, it can exert diverse effects, such as stimulating axonal outgrowth [15], suppressing the growth of lung carcinoma [16], promoting neuropathic pain [17], modulating functional properties of the AMPA-type glutamate receptor GluR1 [18] or increasing apoptosis and neurodegeneration [19,20].

The transient receptor potential vanilloid 1 (TRPV1) is a polymodal nociceptor acting as a non-selective cation channel with a preference for calcium, which is highly expressed in dorsal root and trigemi-

nal ganglia neurons. It is directly activated by capsaicin, the active substance found in chili peppers, and other endogenous or exogenous agonists of natural, semisynthetic and synthetic origin [21], and can be modulated by cytokines and chemokines [22-25].

Previous studies have shown that long-term exposure (24 h) of dorsal root ganglia (DRG) neurons to CXCL1 can increase TTX-S $I_{Na+}$, TTX-R $I_{Na+}$ and potassium currents [26,27], while short-term exposure (4 h) reduces TRPV1 desensitization [28]. However, for CXCL2, there is little information about the short- and long-term effects on the abovementioned ionic channels or receptors involved in neuronal excitability. In the present paper we investigated the effects of overnight exposure to CXCL1 or CXCL2 on the functioning of TRPV1 receptors expressed in cultured DRG neurons. We hypothesized that since CXCL1 and CXCL2 are highly homologous, they could have similar effects on TRPV1. Using both calcium microfluorimetry and patch-clamp recordings, we observed that only CXCL2 significantly affected TRPV1 by reducing the current density and increasing the desensitization rate, while CXCL1 had no effect.

## MATERIALS AND METHODS

### Animals and cell culture

All experimental protocols are in agreement with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and the Romanian Law 43/2014 on the use of animals for scientific purposes, and were approved by the Ethics Committee of the Faculty of Biology, University of Bucharest. Adult male Wistar rats (100-150 g) were killed by inhalation of 100% $CO_2$ followed by decapitation, and all DRG were bilaterally removed, prepared as previously described [29] and plated on 24-mm coverslips or 35-mm Petri dishes, in DMEM (D5523) with F10 (N6635), 7.4 mM glucose, 1% GlutaMAX (Gibco) and 0.5% penicillin-streptomycin. The experimental groups contained n=25 animals for calcium imaging experiments, and n=10 animals for electrophysiology recordings. From 10 to 12 dishes were prepared from each animal; the dishes were subsequently divided into control or treated. Some of the control dishes were used for immunocytochemistry labeling. All experiments were conducted at 25°C, 24 h after plating the neurons. If not otherwise mentioned, reagents were from Sigma.

### Immunocytochemistry (ICC)

Immunostaining of cultured DRG neurons was performed as previously described [30]. The slides were visualized under an AxioObserver D1 Zeiss (Carl Zeiss, Germany) fluorescence microscope and processed with Image-J software. The primary antibodies were rabbit polyclonal anti-CXCR2 (1:200, Santa-Cruz Biotechnology, Heidelberg, Germany) and mouse monoclonal anti-TRPV1 (1:100, Sigma). The secondary antibodies were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (1:1500, Life Technologies, New York, USA).

### Calcium imaging

DRG neurons cultured on coverslips were incubated for 30 min at 37°C in standard extracellular solution (see Solutions below) containing 2 μM Calcium Green-1 AM and 0.02% Pluronic F-127 (both from Invitrogen), and left to recover for 30 min before use. Neurons were illuminated with an Optoscan monochromator (Cairn Instruments, Faversham, UK) and the fluorescence changes were recorded using Axon Imaging Workbench 4.0 (Indec Biosystems, Mountain View, CA, USA). The experimental protocol consisted in perfusion with extracellular solution (ES) for 60 s prior to the first capsaicin application (25-30 s, 1 μM), followed by 6-7 min wash with ES and the second capsaicin application. In calcium imaging, the washout period was longer due to the slow dynamics of the calcium signal.

### Electrophysiology

In DRG neurons, whole-cell patch-clamp recordings were performed using a WPC-100 patch clamp amplifier (E.S.F. Electronic, Göttingen, Germany), low-pass filtered at 3 kHz and digitized at 5-25 kHz through a DigiData 1322A interface with a Pclamp 9.2 software (both from Molecular Devices, Sunnyvale, CA, USA). The borosilicate glass pipettes (Harvard Apparatus, UK) were pulled to a final resistance of 3-6 MΩ using a vertical puller (WPI, Berlin, Germany). Giga-seal

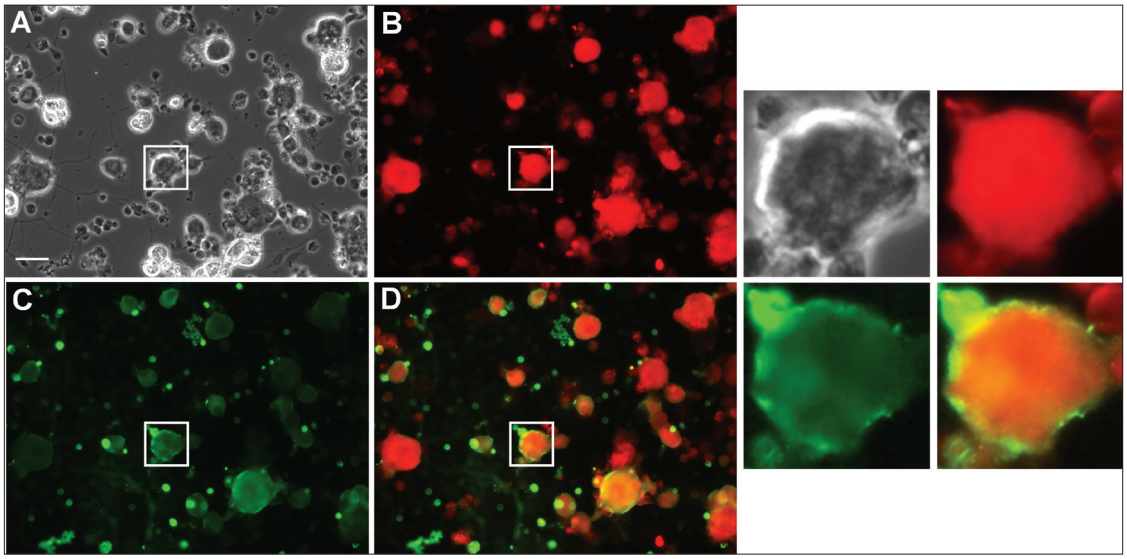


**Fig. 1.** Colocalization of CXCR2 and TRPV1 receptors in cultured DRG neurons. (**A**) Brightfield image of cultured neurons. Immunostaining for CXCR2 (**B**) and TRPV1 (**C**) receptors overlap in some neurons (**D**). The inset represents a magnified cell to detail the colocalization of the receptors. Scale bar: 30 μm

was reached from a holding potential of -80 mV and whole-cell configuration was subsequently followed by the compensation of the capacitive currents. The recordings were conducted only when the access resistance was lower than 10 MΩ. The experimental protocol was: 20 s of ES flow, 10-15 s of capsaicin application 1 μM, 120 s wash-out with ES and the second capsaicin application. In the electrophysiological recordings, the 2 min wash was enough to remove the effect of capsaicin on TRPV1 currents. All solutions had a flow rate of 2-3 ml/min.

### Solutions

Cultured DRG neurons were incubated overnight (20-24 h) with 1.5 nM CXCL1 or 1.5 nM CXCL2. The extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 7.4 glucose, pH 7.4 (at 25°C, adjusted with NaOH). The pipette solution contained (in mM): 140 CsCl, 5 EGTA/KOH, 10 HEPES, pH 7.4, at 25°C with CsOH.

### Statistical analysis

All data were given as means±SEM; statistical significance was tested using a two-tailed Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

## RESULTS AND DISCUSSION

According to literature data, DRG neurons express both CXCR2 and TRPV1 receptors [26,31]. An ICC staining on DRG neurons was performed to reconfirm the colocalization of CXCR2 and TRPV1 receptors as shown in Fig. 1.

To investigate the effects of long-term exposure to CXCL1 on TRPV1 functioning, cultured DRG neurons were incubated overnight with 1.5 nM CXCL1 and the response of TRPV1 to two consecutive applications of 1 μM capsaicin was tested using calcium imaging and patch-clamp techniques. We selected this concentration because it was previously described as efficient on $Na^+$ and $K^+$ channels in DRG neurons [26,27].

Calcium imaging recordings showed no change either for the $[Ca^{2+}]_i$ increase induced by the first capsaicin application compared to control conditions (ΔF/F0 for control =0.56±0.03, n=106, and after CXCL1=0.49±0.02 ΔF/F0, n=160, P>0.05) (Fig. 2A-C), or for the desensitization rate measured as the ratio between the response to the second capsaicin application and the first one ($\Delta F/F0_{2nd}/\Delta F/F0_{1st}$ for control =32.75±6.12%, n=33 and after CXCL1=28.98±3.29%, n=46, P>0.05) (Fig. 2D). Patch-clamp recordings reconfirmed the lack of effect on TRPV1 currents for both the first capsaicin application (current density

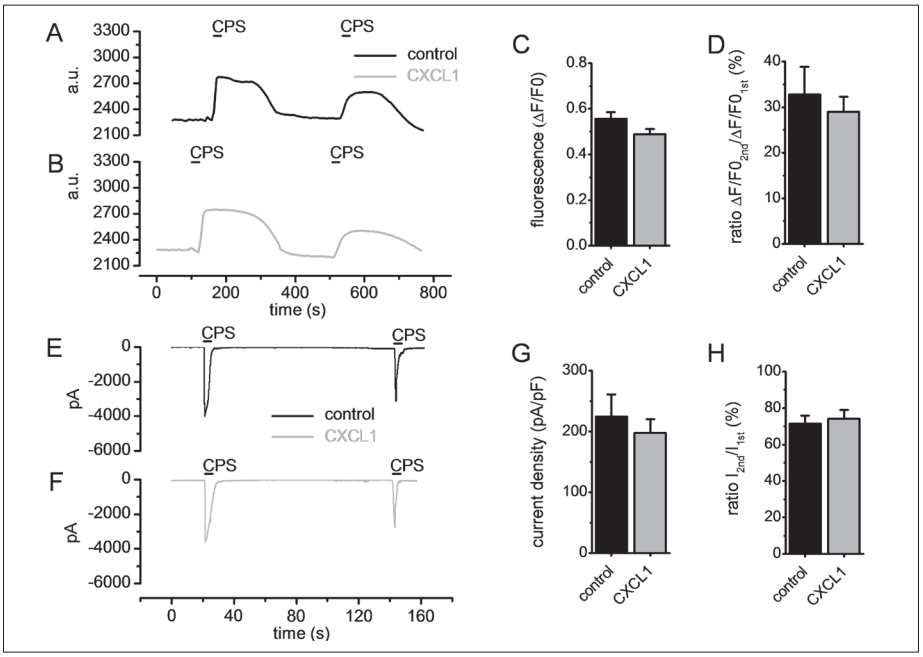


**Fig. 2.** The effect of CXCL1 on TRPV1 in cultured DRG neurons. Representative traces of $[Ca^{2+}]_i$ induced by two capsaicin applications in control conditions (**A**) and after CXCL1 application (**B**). Bar graphs representing mean ΔF/F0 after the 1st capsaicin application (**C**) and mean ratios of the second fluorescence change (ΔF/F0) to the first fluorescence change (**D**) under control conditions and after CXCL1 application. Representative traces of the TRPV1 current under control conditions (**E**) and after CXCL1 application (**F**). Bar graphs showing the mean current density (**G**) and desensitization rate (**H**) of the TRPV1 response.

for control conditions=225.0±36.18 pA/pF, n=17 and after CXCL1=197.5±22.80 pA/pF, n=16, P >0.05) (Figs. 2E-G) and the desensitization rate (for control conditions=71.51±4.33%, n=17 and after CXCL1=74.19±4.67 %, n=16, P >0.05) (Fig. 2H).

In a similar way, the effects of long-term exposure to 1.5 nM CXCL2 on TRPV1 functioning on cultured DRG neurons was tested using calcium imaging and patch-clamp techniques. Calcium imaging recording revealed that unlike CXCL1, incubation with CXCL2 significantly increased the desensitization rate ($\Delta F/F0_{2nd}/\Delta F/F0_{1st}$ for control=32.75±6.12 %, n=33 and after CXCL1=19.86±2.29 %, n=59, *P <0.05) (Figs. 3A, B and D), although it had no effect on the $[Ca^{2+}]_i$ increase induced by the first capsaicin application compared to the control (ΔF/F0 for control =0.56±0.028, n=106 and after CXCL2 =0.61±0.046, n=61, P>0.05) (Figs. 3 A, B and C). Even more, patch-clamp recordings, which allow a more refined analysis of the TRPV1 kinetics, revealed a significant decrease in current density, even after the first capsaicin application (for control =225.0±36.18 pA/pF, n=17 and after CXCL2=83.30±11.49 pA/pF, n=21, ***P<0.001) (Figs. 3 E, F and G) and a significant increase of desensitization rate (for control =71.51±4.33 %, n=17 and after CXCL2=48.62±6.89 %, n=21, *P<0.05) (Figs. 3 E, F and H).

This study revealed that under the same experimental conditions and at the same concentration, only CXCL2 significantly decreased the TRPV1 current and increased its desensitization rate, while CXCL1 had no effect. Our ICC experiments showed that TRPV1 and CXCR2 are colocalized in some DRG neurons. However, the signaling cascade between the two receptor types is still unclear.

Published data indicates that CXCL1 can increase DRG neuron excitability either in the short-term (4 h) by reducing TRPV1 desensitization, which consequently keeps the receptors active and able to respond to repetitive stimuli [28], or in the long-term (24 h) by upregulating $Na^+$ and $K^+$ currents [26,27]. The data from our study suggest that acute application (12 min) of CXCL1 has an activatory effect on TRPV1 from

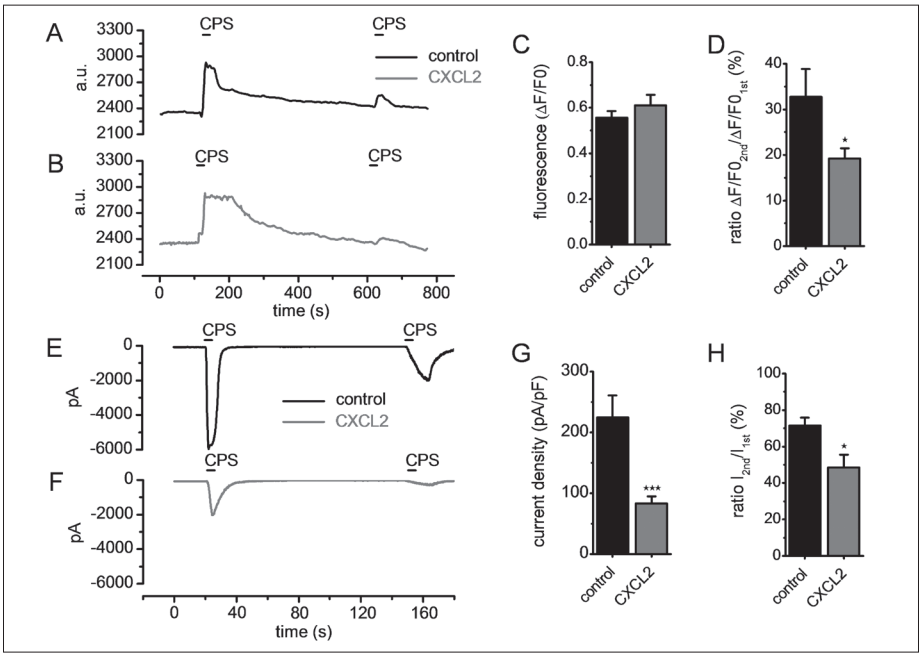


**Fig. 3.** The effect of CXCL2 on TRPV1 in cultured DRG neurons. Representative traces of $[Ca^{2+}]_i$ induced by two capsaicin applications under control conditions (**A**) and after CXCL2 application (**B**). Bar graphs representing mean ΔF/F0 after the 1st capsaicin application (**C**) and mean ratios of the second fluorescence change (ΔF/F0) to the first fluorescence change (**D**) under control conditions and after CXCL2 application. Representative traces of the TRPV1 current under control conditions (**E**) and after CXCL2 application (**F**). Bar graphs showing the mean current density (**G**) and desensitization rate (**H**) of the TRPV1 response.

DRG neurons via CXCR2 receptors and actin filaments (data not published). Together, these findings suggest that if it acts from 12 min to 4 h, CXCL1 can activate TRPV1, while in the long-term (overnight) the effect disappears. Even when separating the neurons into small (ϕ<25 μm) and large (ϕ>35 μm), the effect of CXCL1 after long-term incubation remained undetected through calcium imaging and patch clamp (data not shown). For CXCL2 there are no data for short-term effect, but for long-term it seems it significantly inhibits TRPV1 and promotes desensitization in all cultured DRG neurons analyzed.

It is not unusual that chemokines have a different time course of action on TRPV1 channels. The CCL2 chemokine increased the function and mRNA level of TRPV1 channels via the PI3K/Akt signaling pathway after 24 to 36 h incubation [22], while CCL3 increased TRPV1-mediated $Ca^{2+}$ influx in DRG neurons through a G protein-dependent signaling pathway after only 20 min of incubation [25]. Also, CXCL1 and CCL2 can trigger CGRP release from DRG neurons in a time-dependent manner up to 9 h [32].

A well-known characteristic of TRPV1 functioning is that its response can be sensitized or desensitized. Sensitization, mainly due to increased phosphorylation by PKC or PKA [33,34], is associated with higher or longer responses to specific agonists, which subsequently depolarize the neurons and increase excitability. Desensitization, due to dephosphorylation or calmodulin activation [35,36] is associated with reduced responses to consecutive applications of capsaicin. Because of this effect, capsaicin application on the skin is currently used for treating some pain states [37,38]. We previously showed that overnight exposure to hypoxia/hyperglycemia sensitizes TRPV1 by reducing the desensitization rate [30]. Any molecule that would increase TRPV1 desensitization and therefore reduce its ability to respond to subsequent stimuli would be a very good candidate for pain treatment. In our study, only CXCL2 increased the desensitization rate of TRPV1 expressed in cultured DRG neurons, while CXCL1 had no effect. These results suggest that even though the two chemokines are 78% homologous and bind to the same receptor, they can have quite

opposite effects, possibly due to the intracellular signaling pathway activated downstream of the CXCR2 receptor. Additional experiments are required to confirm if the inhibitory effects of CXCL2 really translate in reduced pain behavior.

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**Authors' contribution:** ATD – experiments and data analysis, AFT – data analysis and writing, VR – research coordination.

**Conflict of interest disclosure:** The authors declare that there is no conflict of interest.

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