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Prolonged activation of CXCR4 hampers the release-regulating activity of presynaptic NMDA receptors in rat hippocampal synaptosomes

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ABSTRACT

We investigated the impact of the prolonged exposure of rat hippocampal synaptosomes to CXCL12 (3 nM) on the NMDA-mediated release of [^{3}H]D-aspartate ([^{3}H]D-Asp) or [^{3}H]noradrenaline ([^{3}H]NA). Synaptosomes were stimulated twice with NMDA/CXCL12 and the amount of the NMDA-evoked tritium release (S1 and S2) quantified to calculate the S2/S1 ratio. The S2/S1 ratio for both transmitters was drastically decreased by 3 nM CXCL12 between the two stimuli (CXCL12-treated synaptosomes) in a AMD3100-sensitive manner. The phosphorylation of the GluN1 subunit in Ser 896 was reduced in CXCL12-treated synaptosomes, while the overall amount of GluN1 and GluN2B proteins as well as the GluN2B insertion in synaptosomal plasmamembranes were unchanged. We conclude that the CXCR4/NMDA cross-talk is dynamically regulated by the time of activation of the CXCR4s. Our results unveil a functional cross-talk that might account for the severe impairments of central transmission that develop in pathological conditions characterized by CXCL12 overproduction.

1. Introduction

Receptor-receptor interaction implies the colocalization of receptors that interact one each other to control neuronal functions (Pittaluga et al., 1997). At the presynaptic level, receptor-receptor cross-talk can promote synaptic plasticity (Musante et al., 2011) by favouring transmitter release (Olivero et al., 2018), but it can be also deleterious, i.e. if involving receptors that could be detrimental to CNS (i.e. the chemokine and the glutamate receptors, Meucci et al., 1998; Kaul et al., 2006).

Recently (Di Prisco et al., 2016), we described an “agonist-like” functional cross-talk linking the CXCR4 receptor (CXCR4) and the NMDA receptor (NMDAR) in rat hippocampal glutamatergic and noradrenergic terminals. This conclusion relied on the finding that CXCL12, the chemokine that preferentially binds CXCR4, cannot modify on its own the release of glutamate and noradrenaline, but it significantly potentiates the NMDA-evoked overflow of both transmitters when concomitantly added to the NMDA agonist.

Data exist in literature showing that the time of exposure of receptors to the respective ligands tightly regulates the receptor-receptor interaction outcome. For instance, the acute (90 s) activation of nicotinic receptors (nAChR) co-localized with NMDARs in dopaminergic terminals potentiates the NMDA-evoked dopamine release, but the facilitation turns to inhibition when the exposure to nicotine is prolonged during time (Salamone et al., 2014). The so far available data concerning the CXCR4/NMDA receptor cross-talk was obtained by exposing acutely (90 s) the two receptors to the respective agonists. However, since in pathological conditions CXCL12 is overproduced and long lasts in the biophase because of the lack of efficient mechanism(s) of removal (Li and Ransohoff, 2008), it seemed of interest to investigate the impact of the prolonged activation of CXCR4 on the NMDAR-mediated release efficiency. Our study unveiled a switch from facilitation to inhibition of the CXCL12-mediated control of the NMDAR-induced release when the exposure of synaptosomes to CXCL12 long-lasted during time. We speculate that our finding add new insights to the comprehension of the deleterious mechanisms at the basis of central disorders associated to inflammation and/or viral infection.

Abbreviations: [^{3}H]NA, [^{3}H]noradrenaline; [^{3}H]D-Asp, [^{3}H]D-aspartate; CNS, central nervous system; gp120, glycoprotein 120; HIV-1, human immunodeficiency virus 1; NMDAR, N-methyl-D-aspartate receptor; PKC, protein kinase C

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2. Materials and methods

2.1. Animal welfare and ethical statement

Adult rats (Sprague Dawley, 200–250 g) were obtained from Charles River (Calco, Italy) and housed in the animal facility of DIFAR, (authorization for animal utilization n. 484 of 2004, June, 8th) at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light-dark schedule (light 7.00 a.m. - 7.00 p.m.). Food and water were freely available. The experimental procedures were in accordance with the European legislation (Directive of September 22, 2010, no. 2010/63/EU), the ARRIVE guidelines and the Italian legislation (L.D. n. 26/2014) and approved by the Italian Ministry of Health (n° 29823–10). All efforts were made to minimize animal suffering and to reduce the number of animals to produce reliable results.

2.2. Synaptosomes preparation and release experiments

The animals were killed by decapitation, the hippocampi removed and synaptosomes prepared as previously described (Salamone et al., 2014). Synaptosomes were resuspended in a physiological solution having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4, and incubated for 15 min at 37 °C in the presence of [^{3}H]D-Asp (final concentration: 3 nM) or [^{3}H]NA (final concentration: 5 nM, in the presence of 0.1 μM 6-nitroquipazine and 0.1 μM GBR12909 to avoid false labelling of serotonergic and dopaminergic terminals). Noradrenaline, levo [7-^{3}H] and Aspartic acid, D-[2,3-^{3}H] were from Perkin Elmer (Boston, MA, USA). Identical synaptosomal aliquots were layered at the bottom of thermostated (37 °C) chambers and superfused at 0.5 ml/min with the physiological solution (Olivero et al., 2018). Two consecutive pulses (S1 and S2) of agonists (3 nM CXCL12 alone or in the presence of NMDA, Tocris Bioscience, Bristol, UK) were applied for 90 s starting from t = 39 (S1) and t = 69 min (S2). Fractions were collected according to the following scheme: two 3-min fractions (basal release, b) before (b1, $basal1_{S1}$, t = 36–39 min, $basal1_{S2}$, t = 66–69 min) and after (b2, $basal2_{S1}$, t = 45–48 min; $basal2_{S2}$, t = 75–78 min) the 6-min fractions ($peak_{S1}$, t = 39–45 min; $peak_{S2}$, t = 69–75 min; evoked release). When indicated, synaptosomes were exposed to CXCL12 (3 nM) alone or in the presence of AMD3100 (100 nM, Tocris Bioscience, Bristol, UK) from t = 48 min to t = 68 min. Fractions collected and superfused synaptosomes were then counted for radioactivity.

2.3. Biotinylation and immunoblotting

Synaptosomes were incubated in the absence or in the presence of 3 nM CXCL12 at 37 °C for 20 min under mild shaking. GluN2B synaptosomal surface levels subunit were evaluated by biotinylation and subsequent immunoblot analysis as already described (Salamone et al., 2014). The overall quantification of GluN1, GluN2B and phosphoGluN1 (Ser896) expression was carried out in control and CXCL12-treated synaptosomal lysates.

Proteins were separated by SDS-10% PAGE and transferred onto PVDF membranes (Salamone et al., 2014). After blocking with 5% non-fat dried milk in t-TBS (0.02 M Tris, 0.150 M NaCl and 0.05% Tween 20), membranes were incubated with rabbit anti-phosphoGluN1 (Ser896) antibody (1:4000, Abcam, ab75680), mouse anti-GluN1 antibody (1:4000, Millipore, MAB 1586), mouse anti-GluN2B antibody (1:4000, Abcam, ab93610) and mouse anti-β tubulin antibody (1:1000, Sigma, T8328) overnight at 4 °C. After extensive washes, membranes were incubated with appropriate horseradish peroxidase-linked secondary antibodies (1:20000). Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec, Cambridge, UK).

2.4. Data analysis and statistical procedures

The radioactivity into each sample was expressed as fractional efflux (Pittaluga et al., 1997). Drug effects were estimated by subtracting the neurotransmitter content into the fractions corresponding to the basal release from those corresponding to the evoked release. Analysis of variance was performed by ANOVA followed by Tukey-Kramer test. Student's t-test was used for direct comparisons. Data were considered significant for $p < 0.05$ at least.

3. Results

3.1. Effect of the prolonged exposure to CXCL12 on the release-regulating NMDARs presynaptically located in rat hippocampal glutamatergic and noradrenergic terminals

The basal transmitter release from untreated synaptosomes decreases slightly although not significantly during time ([^{3}H]D-Asp, S1:3.04 ± 0.48, S2: 2.61 ± 0.36, n = 6, *n.s.*; [^{3}H]NA, S1: 3.37 ± 0.51, S2: 2.84 ± 0.41 n = 8, *n.s*, results express as fractional efflux) because of the continuous depletion of the radioactive vesicular store. Actually, the superfusion of the thin layer of synaptosomes assures the rapid removal of any substance released from the superfused particles, impeding its re-uptake and re-storage into vesicles (Pittaluga et al., 1997; Pittaluga, 2016).

Synaptosomes were exposed to CXCL12 (3 nM) alone or in the presence of NMDA (concentration as indicated, Fig. 1A, S1), then superfused for 20 min and re-challenged with the agonists (Fig. 1A, S2). CXCL12 (3 nM) failed to affect on its own the spontaneous release of the two radioactive tracers ([^{3}H]D-Asp, control, S1: 3.12 ± 0.44, S2: 2.74 ± 0.38, +3 nM CXCL12, S1: 3.02 ± 0.36, S2: 2.56 ± 0.42, n = 6, *n.s.*; [^{3}H]NA, control, S1: 3.45 ± 0.56, S2: 2.77 ± 0.56, +3 nM CXCL12, S1: 3.27 ± 0.15, S2: 2.39 ± 0.41, n = 8, *n.s*). Differently, the exposure of synaptosomes to NMDA/3 nM CXCL12 elicits the release of both tritiated transmitters in S1 and S2 (Fig. 1B and C). However, because of the continuous depletion, the NMDA/3 nM CXCL12-evoked tritium release of [^{3}H]D-Asp and of [^{3}H]NA in S2 was significantly reduced when compared to that in S1 (Fig. 1 B and C).

The exposure of synaptosomes from S1 to S2 to a medium containing 3 nM CXCL12 did not cause significant changes in the basal release of [^{3}H]D-Asp or [^{3}H]NA in S2 ([^{3}H]D-Asp: S2: 2.28 ± 0.41 n = 6, *n.s.*; [^{3}H]NA: S2: 2.41 ± 0.31, n = 8, *n.s*). The CXCL12 treatment between S1 and S2, however, further reduced the 30 μM NMDA/3 nM CXCL12-evoked [^{3}H]D-Asp overflow in S2 (Fig. 1B, untreated synaptosomes, S2/S1: 0.61 ± 0.06; CXCL12-treated synaptosomes, S2/S1: 0.19 ± 0.05, $p < 0.05$, n = 6, corresponding to 68.78 ± 3.23% of reduction of release efficiency in S2). Similarly, the 100 μM NMDA/3 nM CXCL12-evoked release of [^{3}H]NA in S2 was significantly diminished by the CXCL2 from S1 to S2 (Fig. 1C; untreated synaptosomes, S2/S1: 0.46 ± 0.07; CXCL12-treated synaptosomes, S2/S1: 0.18 ± 0.05, $p < 0.05$, n = 8, corresponding to 60.87 ± 6.57% of reduction of release efficiency).

The concomitant presence of the CXCR4 antagonist AMD3100 (100 nM) from S1 to S2 significantly prevented the CXCL12-induced changes in S2 tritium release (Fig. 1B and C; [^{3}H]D-Asp, CXCL12/AMD3100-treated synaptosomes, S2/S1: 0.42 ± 0.06, $p < 0.05$, n = 3; [^{3}H]NA, CXCL12/AMD3100-treated synaptosomes, S2/S1: 0.45 ± 0.08, $p < 0.05$, n = 3).

3.2. The prolonged exposure to CXCL12 does not modify the expression of GluN2B subunit in synaptosomal plasmamembranes

Activation of CXCR4 receptors inhibits the expression of the GluN2B subunits in neurons (Nicolai et al., 2010). Since the GluN2B subunits participates to the expression of NMDARs in the noradrenergic and in the glutamatergic terminals (Di Prisco et al., 2016), we quantified the

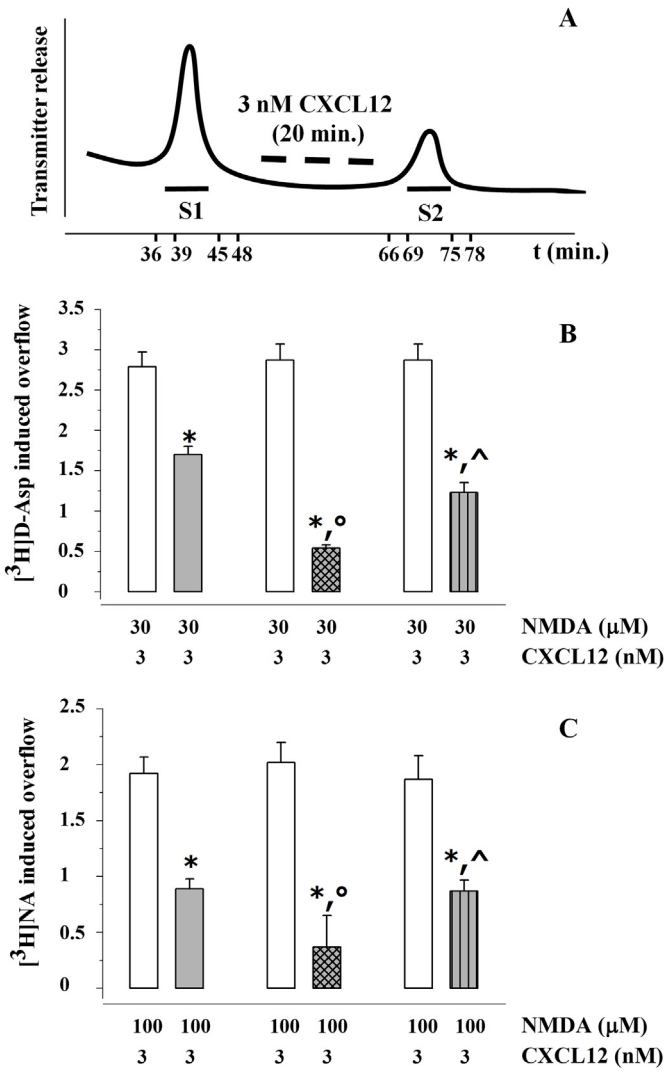

Fig. 1. Prolonged exposure to CXCL12 reduces NMDA-evoked releasing activity. A) Representative cartoon showing the pattern of the transmitters release in the multiple stimulation experiments during time. Effects of the prolonged exposure to CXCL12 on the NMDA-mediated release of [^{3}H]D-aspartate ([^{3}H]D-Asp, B) and [^{3}H]noradrenaline ([^{3}H]NA, C). Synaptosomes were exposed to NMDA/3 nM CXCL12 (empty bar, S1; grey bar, S2). When indicated, the physiological solution contained 3 nM CXCL12 (cross-hatched bars) or 3 nM CXCL12/100 nM AMD3100 (vertical-hatched bars) from S1 to S2. Results are expressed as induced overflow. Data are means ± SEM of six ([^{3}H]D-Asp release) and five ([^{3}H]NA release) experiments in triplicate. * $p < 0.05$ versus NMDA/3 nM CXCL12, S1; ° $p < 0.05$ versus NMDA/3 nM CXCL12, S2, in the absence of CXCL12 between the two stimuli; ^ $p < 0.05$ versus NMDA/3 nM CXCL12, S2, in the presence of CXCL12 between the two stimuli.

overall synaptosomal expression and the plasmamembranes surface levels of GluN2B subunit proteins in untreated and CXCL12-treated synaptosomes. The overall expression of the GluN2B subunit was comparable in both synaptosomal preparations (Fig. 2A and B). Furthermore, the exposure to CXCL12 (3 nM from S1 to S2) did not alter the density (Fig. 2D) of biotin-tagged GluN2B proteins (Fig. 2C, lane CXCL12) in plasmamembranes when compared to control (Fig. 2C, lane Control).

3.3. The prolonged exposure of synaptosomes to CXCL12 reduces the phosphorylation of GluN1 subunit

CXCR4 also control the phosphorylation in Serine 896 of GluN1 subunit [Ru and Tang, 2016, phospho-GluN1 (Ser896)]. The overall amount of GluN1 subunit and that of phospho-GluN1 (Ser896) were quantified in untreated and CXCL12-treated rat hippocampal synaptosomes. β-tubulin immunoreactivity was used as internal standard. The overall expression of the GluN1 subunit in CXCL12-treated synaptosomes was comparable to that in controls (Fig. 2A and B). Fig. 2 shows that exposure of synaptosomes to CXCL12 (3 nM) significantly decreased (Fig. 2F) the expression of the phospho-GluN1 (Ser896) (Fig. 2E, lane CXCL12) in synaptosomal lysates when compared to control (Fig. 2E, lane Control).

4. Discussion

The role of CXCL12 and CXCR4 receptors in the homeostasis of the mature brain still represents a matter of discussion. By acting at CXCR4 receptors, CXCL12 increases the intracellular mobilization of Ca^{2+} ions, it worsens the NMDA neurotoxicity and enhances the NMDA-mediated excitatory postsynaptic currents, well consistent with a CXCR4-mediated amplification of the NMDA signalling (Meucci et al., 1998; Kaul et al., 2006; Zhou et al., 2017). In the meanwhile, activation of CXCR4 receptors was shown to inhibit the expression of GluN2B subunits and to reduce the phosphorylation of the GluN1 proteins (Nicolai et al., 2010; Ru and Tang, 2016), best consistent with a CXCR4-induced down-regulation of the NMDARs.

Recently, we demonstrated that the acute exposure of synaptosomes to CXCL12 amplifies the NMDA-mediated releasing activities through a PKC-dependent pathway (Di Prisco et al., 2016). Now, we show that facilitation turns to inhibition when the exposure of synaptosomes to CXCL12 long lasts during time. In a whole, these observations led us to reconsider our previous conclusion, and to propose the chemokine-mediated control of the NMDA-induced transmitter release as a plastic event that dictates the role of NMDA at chemical synapses.

NMDARs exist both synaptically and extrasynaptically and, depending on their location (extrasynaptic versus synaptic), these receptors affect differently neuronal survival and plasticity (Hardingham and Bading, 2010). In general, extrasynaptic NMDARs preferentially contribute to maladaptive responses triggered by toxic events, while synaptic NMDARs, including the presynaptic release-regulating ones, promote neuroprotection. Our results suggest that CXCL12 can control the balance between the synaptic and the extrasynaptic NMDARs. In particular, while the brief activation of CXCR4 increases the synaptic NMDARs, its prolonged activation silences these receptors, indirectly allowing the extrasynaptic NMDAR component to prevail.

GluN2B subunit participates to the presynaptic NMDARs assembly in both glutamatergic and noradrenergic terminals (Musante et al., 2011; Di Prisco et al., 2016). The expression of this subunit is tightly regulated by CXCR4, which decreases its expression by tuning the histone deacetylase-mediated control of the GluN2B gene (Nicolai et al., 2010). Such a neuronal adaptation, however, did not occur in isolated nerve endings. Actually, the overall amount of GluN2B proteins in CXCL12-treated synaptosomes, as well as their insertion in synaptosomal plasmamembranes, were unchanged when compared to controls.

Beside the GluN2B, also GluN1 subunits participate to the expression of the presynaptic release-regulating NMDARs under study. In 2016, Ru and Tang demonstrated that gp120Bal down-regulates the phospho-GluN1(Ser896) level through a CXCR4-dependent mechanism. Ser896 is one of the two serine residues within the intraterminal domain of the GluN1 subunit that, in the phosphorylated form, controls the surface expression of the NMDARs (Chen and Roche, 2007). Notably, the prolonged exposure of synaptosomes to CXCL12 reduced the phospho-GluN1 (Ser896) also in nerve terminals, an event that might interfere with the subunit clustering in synaptic membranes. However, since the overall expression of the GluN1 subunit was unchanged in lysates from CXCL12-treated synaptosomes when compared to control, the possibility that CXCR4 tunes the in-out trafficking of the NMDARs in rat hippocampal synaptosomes seems unlike.

The question therefore arises on how the prolonged exposure of

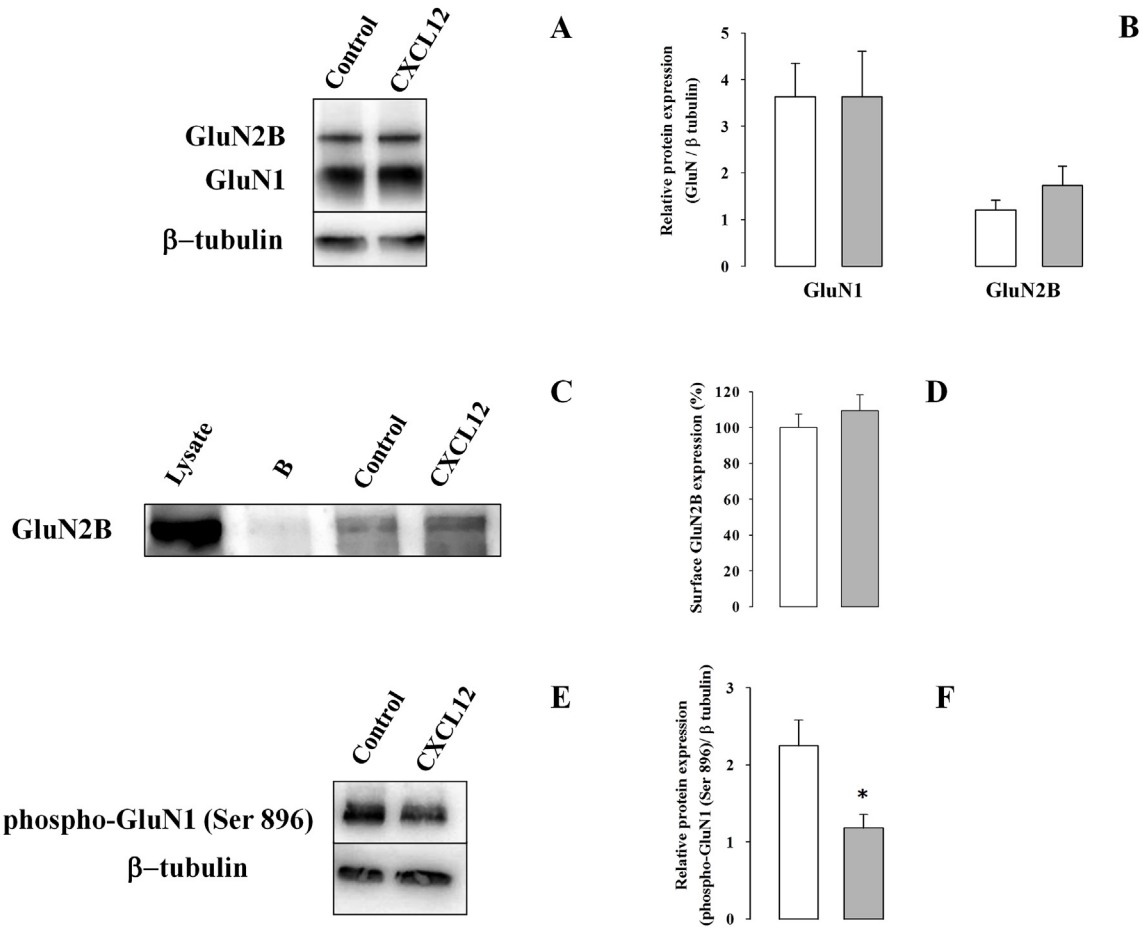

Fig. 2. Effect of the prolonged exposure to CXCL12 on the GluN1 and the GluN2B subunits in rat hippocampal synaptosomes. A) Analysis of the GluN1 and GluN2B subunit expression in control and 3 nM CXCL12-treated synaptosomal lysates; β−tubulin is the internal control. The blot is representative of three experiments. B) The graph shows the mean ± SEM of the GluN1 and GluN2B expression relative to β-tubulin; data are expressed as percentage of the GluN/β-tubulin; C) Analysis of the GluN2B expression in control and 3 nM CXCL12-treated (CXCL12) synaptosomal membranes. Non-biotinylated synaptosomes (B) were used to verify the specificity of neutravidin pull-down. Synaptosomal lysate (Lysate) is a positive control. The blots are representative of three experiments. D) The graph shows the mean ± SEM of surface GluN2B intensity; data are expressed as percentage of surface GluN2B expression. E) Western blot analysis of phospho-GluN1 (Ser 896) in control and 3 nM CXCL12-treated synaptosomal lysates. β−tubulin is the internal control. The blot is representative of five experiments. F) The graph shows the mean ± SEM of the phopsho-GluN1(Ser896) expression relative to the respective amount of β-tubulin; data are expressed as percentage of the phopsho-GluN1(Ser896)/β-tubulin; * $p < 0.05$ versus control.

synaptosomes to CXCL12 can down-regulate the NMDARs. In 2016 Ru and Tang proposed that the great influx of Ca^{2+} ions elicited by the CXCR4-induced overstimulation of NMDARs could activate cytosolic phosphatases to dephosphorylate GluN1 subunits. Interestingly, this cascade of events might occur also in hippocampal synaptosomes, since phosphatase represents in these terminals a rapid mechanism of protection against the hyper-activation of NMDARs (Goebel et al., 2005). Once dephosphorylated, GluN1 could trigger compensatory changes in the NMDAR subunit composition (i.e. GluN3 subunits replacing the GluN1 ones) that might account for a decreased sensitivity of the NMDARs to glutamate (Chen and Roche, 2007). The possibility that such an event could occur in synaptosomes is attractive and seems well consistent with the fact that the overall expression of GluN2B and of GluN1 subunits is unmodified in CXCL12-treated synaptosomes. Further studies are however required to verify the hypothesis.

To conclude, our observations add new insights to the current knowledge of the role of CXCR4 in controlling NMDA-mediated events in the CNS. The CXCR4/NMDAR interaction here described accounts for impaired glutamatergic and noradrenergic transmission in CNS observed in pathological conditions characterized by CXCL12 overproduction. In particular, the prolonged activation of CXCR4 can downregulate synaptic NMDARs, which are neuroprotective, favoring the maladaptative effects exerted by extrasynaptic NMDARs.

Conflicts of interest

None.

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