The CXCL12/CXCR4 signaling is required for DPP4 regulation of spontaneous inhibitory postsynaptic currents (sIPSCs)

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Research

Keywords: Febrile Seizures; Dipeptidyl peptidase 4; CXCL12;CXCR4;

Posted Date: February 20th, 2020

DOI: https://doi.org/10.21203/rs.2.24023/v1

Abstract

Background: Previous data suggested that dipeptidyl peptidase-IV (DPP4) involved in the occurrence of febrile seizure (FS), but its potential mechanism remains to be determined. Here, we investigated whether DPP4 regulated gamma-aminobutyric acid (GABA) mediated spontaneous inhibitory postsynaptic currents (sIPSCs) via the downstream C-X-C Motif Chemokine Ligand 12 (CXCL12)/ C-X-C chemokine receptor type 4 (CXCR4) signaling in cultured hippocampal neurons submitted to hyperthermia(39.5-40°C). Methods: Whole cell patch- clamp method was used to test sIPSC in vitro after DPP4 inhibition or CXCL12 administration. The level of CXCL12 and CXCR4 was tested using western blot analysis. The effect of CXCR4 antagonist AMD3100 (5 mg/ml, i.c.v) on seizures were tested using electroencephalogram (EEG) in a FS model. Results: We found that pharmacological DPP4 inhibitor sitagliptin (Sita,100μM) treatment or siRNA-mediated DPP4 knockdown enhanced the mean amplitude and frequency of sIPSCs in vitro. DPP4 knockdown with siRNA increased protein level of CXCL12 and CXCR4. Furthermore, CXCL12 (10 nM) treatment enhanced inhibitory transmission by increasing the mean frequency and amplitude of sIPSCs in vitro. AMD3100 administration decreased seizure severity by increasing hippocampal GABA content in vivo. Conclusions: Our data suggest that CXCL12/CXCR4 signaling is required for DPP4 regulation of sIPSCs, supporting that DPP4 played a key role in the pathogenesis of FS.

Background

Febrile convulsions (FS) are a common type of pathological brain activity in childhood, and longer seizure onsets are accompanied by serious clinical outcomes[1–3]. It is an important task to explore and develop new drug targets for the prevention and treatment of febrile convulsions.

Some data indicated that dipeptidyl peptidase-IV (DPP4) involved in the occurrence of febrile seizure, and DPP4 inhibition increased miniature inhibitory post-synaptic currents (mIPSCs), however, its underlying molecular mechanism remains unclear[3]. Previous data indicated that DPP4 inhibition could increase glucagon-like peptide-1(GLP-1) and GLP-1 receptor (GLP-1R) levels to effect FS development in rats[3]. Increasing evidence suggest that immune inflammatory factors play an important role in the pathogenesis of FS. Chemokines (chemotactic cytokines) are a class of small (7–11 kD), secreted cytokines which mediate leukocyte mobilization to sites of inflammation in the periphery[4]. CXCL12 and its receptor C-X-C chemokine receptor type 4 (CXCR4) are well characterized on neurons and glia in the hippocampus, which is also critical for neuronal migration/patterning during development. CXCL12 and CXCR4 levels are increased in a rat model of neuropathic pain (NP), and CXCL12/CXCR4 signaling plays a key role in regulation of NP[5]. The CXCL12-CXCR4 axis is involved in the recovery of neurotransmission of the injured nerve[6]. It has been shown that CXCR4 antagonist AMD3100 suppresses the long-term abnormal structural changes of newborn neurons in the intraventricular kainic acid model of epilepsy, which provided morphological evidences that AMD3100 might be beneficial for treating chronic epilepsy[7]. CXCL12/CXCR4 signaling regulates GABA and glutamate synaptic activity at serotonin

neurons in the rat dorsal raphe nucleus[8].We propose a hypothesis that DPP4 regulates GABA mediated synaptic transmission via the CXCL12/CXCR4 signaling.

In the present study, we investigated whether DPP4 regulates sIPSCs via the CXCL12/CXCR4 signaling in primary cultured neurons. It has been demonstrated that DPP4 inhibition by sitagliptin (Sita) or siRNA enhances neurotransmission via the CXCL12/CXCR4 signaling, suggesting that DPP4 plays a key role in FS.

Methods

Animals

Sprague-Dawley (SD) rats (male and female, n = 48, P14) were obtained from the Animal Experimental Center of Zhengzhou University. All experimental procedures complied with the Committee on the Ethics of Animal Experiments of Zhengzhou University (Permit No: SCXK 2010-0002).

Primary neuronal culture

Hippocampal neurons were isolated from the brains of neonatal SD rats (male and female, n = 12) based on described previously[9]. Immunofluorescence staining for microtubule-associated protein 2 (MAP2, 1:200, Abcam, Cambridge, MA, USA) was used to test the purity of the hippocampal neuronal population. The cells were maintained for 14 days before recordings.

Generation of experimental FS *in vivo*

Experimental FS model were generated in rats using hot water bath immersion as previously described[3].The seizures were scored according to the Racine scale[10]. Animals exhibiting stage 3 seizures (forelimb clonus) were regarded as exhibiting seizure behavior. CXCR4 antagonist AMD3100 (5 mg/ml, Selleckchem Co, Houston, USA) was administrated by i.c.v in rats.

Western blot analysis

The isolated hippocampal neurons maintained on 14 day were collected, and the protein content was determined using a BCA Protein Quantitation Kit (Thermo Fisher, USA) following the instructions. β-actin was used as a control for DPP4, CXCL12 and CXCR4, then the blots were incubated overnight with primary antibodies against DPP4 (1:2000, ab28340, Abcam, Cambridge, Massachusetts, USA), CXCL12 (1:1000, ab9797, Abcam, Cambridge, Massachusetts, USA) and CXCR4 (1:1000, Abs143375, Shanghai, China). The data were analyzed using Image J software (National Institutes of Health, MD, USA).

Enzyme-linked immunosorbent assay(ELISA)

Hippocampal tissues of the rats from the control, FS and FS+ AMD 3100 groups were immediately collected to measure the GABA according to ELISA kits (GABA: MyBioSource; San Diego, CA) according to

the manufacturer's instructions. A VICTOR™ X5 Multilabel Plate Reader (PerkinElmer, USA) was used to analyze the data.

Electroencephalogram (EEG) recording

The rats were deeply anesthetized with 10% chloral hydrate (i.p., 300 mg/kg). Using stereotaxic guidance, the rats were surgically implanted with an injection guide cannula and recording electrodes, as previously described[11, 12]. Rats from the FS+ AMD 3100 group were administered AMD 3100 (5mg/ml, i.c.v). EEG changes were recorded for a period (15-20 min) in the rats with febrile convulsions induced by the required conditions. The recorded EEG was exported, and the data were analyzed in LabChart 7.2(AD Instruments).

siRNA assay

siRNA targeting DPP4 and its nonspecific oligonucleotide were synthesized by GenePharma Co. (Shanghai, China) according to previous report[3]. Neurons were maintained on 10-14 days and transfected with siRNA mediated DPP4 (5'-GCUGAUAGCGCGAGAACUUTT-3'/5'-AAGUUCUCGCGCUAUCAGCTT) following to the manual instructions.

Electrophysiological recordings

The spontaneous inhibitory postsynaptic currents (sIPSCs) in the cultured hippocampal neurons (10-14 DIV) were recorded using the whole cell patch technique. The patch pipette (3–6 MΩ) was filled with internal solutions containing 145 mM CsCl, 5 mM NaCl, 10 mM HEPES, 5 mM EGTA, 4 mM Na_2ATP, 0.3 mM Na_2GTP and 5 mM QX-314. D (-)-2-amino-5-phosphonopentanoic acid (D-APV, 50 μM; Tocris Cookson, Ellisville, MO), 6-cyano-7-nitroquinoxaline-2, and 3-dione (CNQX, 10 μM; Tocris Cookson) were diluted immediately prior to use and acutely applied by bath superfusion (PH:7.20, osmolarity: 300–310 mosm/liter). The temperature (39.5°C) was monitored using a small thermistor placed directly in the recording chamber with continuous perfusion of a standard external solution at a rate of 2 ml/min[3]. Sita and CXCL12(10 nM, PeproTech, USA) were added into perfusion bath before recording. The currents were measured using a patch-clamp amplifier (Axon 700B, Axon Instruments, Foster City, CA, USA) and analyzed using Clampfit software (Axon Instruments).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed with the GraphPad Prism software (version 6). Two-tailed unpaired Student's t-test was used to examine significant differences between the two groups. Comparisons involving multiple groups were examined by one-way ANOVA. EEG data were analyzed using LabChart 7.2. Values of $p<0.05$ were considered statistically significant.

Results

DPP4 inhibition by Sita enhanced sIPSCs *in vitro.*

To determine the role of DPP4 in GABAergic synaptic transmission, we examined the effects of DPP4 inhibition by Sita(100 μM) on sIPSCs in primary cultured neurons submitted to hyperthermia(39.5-40°C). sIPSCs were recorded under voltage- clamp mode (Vm=-60 mv). Representative sIPSC traces were recorded from cultured neurons among Con, hyperthermia (Hyp) and Hyp+Sita groups (Fig.1A). Quantitatively, Sita increased the mean peak amplitude and burst frequency of sIPSCs compared to those in the Hyp group, respectively (Fig. 1B, C). Sita increased decay time constant without effect on rise time compared to those from hyp group (Fig. 1D, E). Furthermore, a significant decrease in interevent interval (ISI) was observed after sita treatment (Fig. 1F). The recorded sIPSCs were eliminated completely by bicuculline (20 μM) administration.

DPP4 knockdown by siRNA administration enhanced sIPSCs *in vitro.*

To further confirm these results, the cultured neurons were transfected to establish FAM-siRNA-mediated DPP4 knockdown to examine the effect of DPP4 on sIPSCs. Representative sIPSC traces were recorded from the cultured neurons under an inverted fluorescence microscope (Fig.2A). Quantitatively, DPP4 knockdown increased the mean peak amplitude and burst frequency of sIPSCs compared to those in the control siRNA group (Fig. 2B and C). In addition, DPP4 knockdown had no effect on decay time and rise time constant compared to those from hyp group (Fig. 2D, E). A significant decrease in ISI was observed after DPP4 knockdown (Fig. 2F). These results indicate that inhibition of DPP4 induces a strong GABAergic inhibitory effect on seizures.

DPP4 knockdown with siRNA increased level of CXCL12 and CXCR4.

Some chemokines including CXCL12 have previously been shown to be DPP4 substrates[13]. It has been shown that CXCL12/CXCR4 enhanced GABA and glutamate synaptic activity at serotonin neurons in the rat dorsal raphe nucleus[8]. The increase in tonic inhibition was prevented by CXCR4 antagonist[14]. We investigate the effect of DPP4 knockdown on level of CXCL12 and CXCR4 *in vitro*. Western blot results show that DPP4 knockdown decreased DPP4 protein level (Fig.3A,B) and increased level of CXCL12 (Fig.3C) and CXCR4 (Fig.3D) *in vitro* compared to those from sicontrol group. The results above indicate that DPP4 inhibition activates the CXCL12/CXCR4 signaling to regulate inhibitory neurotransmission.

CXCL12 administration enhanced sIPSCs *in vitro.*

To examine the effect of CXCL12/CXCR4 signaling on inhibitory transmission, sIPSCs were recorded after CXCL12 (10 nM) administration in primary cultured neurons submitted to hyperthermia(39.5-40°C). We found that CXCL12 administration enhanced inhibitory transmission by increasing the mean frequency and amplitude of sIPSCs (Fig.4A-C) compared to that from hpy group. The data above show that CXCL12/CXCR4 signaling involved in DPP4 regulation of inhibitory transmission *in vitro*.

CXCR4 antagonist AMD3100 decreased seizure severity *in vivo.*

To further confirm that CXCL12/CXCR4 signaling involved in DPP4 regulation of inhibitory transmission, CXCR4 antagonist AMD3100 (5 mg/ml, i.c.v) was administrated in rats. The results of EEG recording

showed that AMD3100 treatment attenuated neuronal discharge in a FS model (Fig.5A,B). Furthermore, AMD3100 treatment in rats decreased significantly seizure severity (Fig.5C) and bursts number of 4 stage (Fig.5D) compared to those from FS group. ELISA result indicated that AMD3100 treatment elevated GABA content *in vivo* compared to that from FS group (Fig.5E). These data suggest that CXCL12/CXCR4 signaling regulates seizures via the GABAergic transmission.

Discussion

It has been demonstrated that DPP4 inhibitor Sita treatment suppressed the neuronal firing activity in cultured neurons, supporting that DPP4 inhibition produced a seizure-suppressive effect[3]. The results obtained in this study suggested that the CXCL12/CXCR4 signaling was involved in DPP4 regulation of sIPSCs (Fig.6).

In the present study, our data show that a large increase in mean frequent and amplitude of sIPSCs was observed in primary cultured neurons treated with Sita or siRNA-DPP4(Fig.1.2). The inhibition of DPP4 may act presynaptically to promote GABA release from presynaptic nerve terminals, which was consistent with above results[3]. Whether DPP4 affects the Hyp -induced post-synaptic $GABA_A$ receptors decrease needs to be explored in the future. Some papers have addressed the role played by inflammatory cytokines in the regulation of GABAergic transmission [15-17]. The inhibition of DPP4 by Sita reduces inflammatory responses and chronic immune cell activation [18-20]. We speculate that the reduced inflammatory responses may be involved in the DPP4 regulation of GABAergic transmission.

Chemokines are small molecules called "chemotactic cytokines" and regulate many processes, and then chemokines and their receptors are anti-inflammatory factors in autoimmune conditions[21]. CXCL12 is a homeostatic chemokine, which acts as an anti- inflammatory chemokine during autoimmune inflammatory responses[22]. Inflammatory processes in brain tissue have been described in human epilepsy of various etiologies and in experimental models of seizures[23], and anti- high mobilitygroupbox 1(HMGB1) mAb prevented the *blood-brain-barrier* permeability, reduced HMGB1 translocation while inhibiting the expression of inflammation-related factors[24]. Recently, It has been demonstrated that CXCL12α potently stimulates the functional recovery of damaged neuromuscular junctions via interaction with CXCR4[25]. CXCR4 antagonist AMD3100 reverses the neurogenesis promoted by enriched environment and suppresses long-term seizure activity in adult rats of temporal lobe epilepsy[26].These data above show CXCL12/CXCR4 pathway plays a key role in epilepsy generation. It has also been shown that CXCL12/CXCR4 enhances GABA and glutamate synaptic activity at serotonin neurons in the rat dorsal raphe nucleus[8]. CXCL12 was known to be cleaved by DPP4, and CXCL12 was identified as the substrate of DPP4[13]. Western blot results indicated that DPP4 knockdown elevated the protein level of CXCL12 and CXCR4 (Fig.3). Electrophysiological results suggest that CXCL12 administration enhanced GABAergic transmission by increased mean frequent and amplitude of sIPSCs *in vitro* (Fig.4). Furthermore, CXCR4 antagonist AMD3100 treatment attenuated neuronal discharge and decreased significantly seizure severity in a FS model (Fig.5). It has been reported that DPP4-GLP-1-GLP1R signaling involved in several intracellular pathways and DPP4 inhibition or the GLP-1R stimulation

may have applications for treating inflammatory diseases[27]. Although DPP-4 inhibitors have attractive clinical features, the benefits from GLP-1 signaling may be undermined by their actions to enhance CXCL12[28]. In stroke, DPP4 inhibitor linagliptin did not occur via GLP-1R[29]. Whether DPP4 regulates FS via the CXCL12/CXCR4 signaling has been not determined. Our data suggested that CXCL12/CXCR4 signaling pathway was required for DPP4 regulation of GABAergic transmission in seizures. The association between CXCL12/CXCR4 signaling and GLP-1/GLP-1R signaling in FS development needs to be investigated in the future.

Conclusions

DPP4 inhibition and siRNA administration enhanced mean sIPSCs and elevated CXCL12 and CXCR4 protein levels. Furthermore, CXCL12 administration enhanced mean sIPSCs, and CXCR4 antagonist AMD3100 treatment attenuated neuronal discharge and decreased significantly seizure severity in a FS model. We provide evidence that CXCL12/CXCR4 signaling was involving in DPP4 inhibition enhancing the downstream sIPSCs, supporting that DPP4 playing a key role in FS development.

Abbreviations

DPP4: dipeptidyl peptidase-IV; FS: Febrile seizure; sIPSCs: spontaneous inhibitory postsynaptic currents; GABA: gamma-aminobutyric acid; CXCL12: C-X-C Motif Chemokine Ligand 12; mIPSCs :miniature inhibitory post-synaptic currents; CXCR4: C-X-C chemokine receptor type 4; Sita: sitagliptin; SD: Sprague-Dawley; MAP2: microtubule-associated protein 2; HMGB1: high mobilitygroupbox 1; BBB: *Blood-Brain-Barrier;* HP: holding potential; Hpy: hyperthermia; GABA: gamma-aminobutyric acid; EEG: electroencephalogram; ELISA: enzyme-linked immunosorbent assay; GLP-1: glucagon-like peptide-1; GLP-1R: glucagon-like peptide-1 receptor;

Declarations

Acknowledgements

We wish to thank prof. liecheng Wang from the anhui medical university for patch calmp technique.

Authors' contributions

ZYS, CZG and HYD contributed to the study design and analyses of data. HYD and ZYS performed the patch clamp and wrote the first draft, YZS participated in the subsequent drafts. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Sprague-Dawley (SD) rats were obtained from the Animal Experimental Center of Zhengzhou University. All animals experimental procedures were approved by the Committee on the Ethics of Animal

Experiments of Zhengzhou University (Permit No: SCXK 2010-0002).

Availability of data and materials

All data generated or analyzed in this study are included in this published article. All data are also available from the corresponding author on reasonable request.

Funding

This study was supported by Medical Technology Research and Development Program of Henan Province (Grant No. 2018020500) and Doctoral Development Program of Henan Provincial Cancer Hospital (Grant No. D982).

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

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Figures

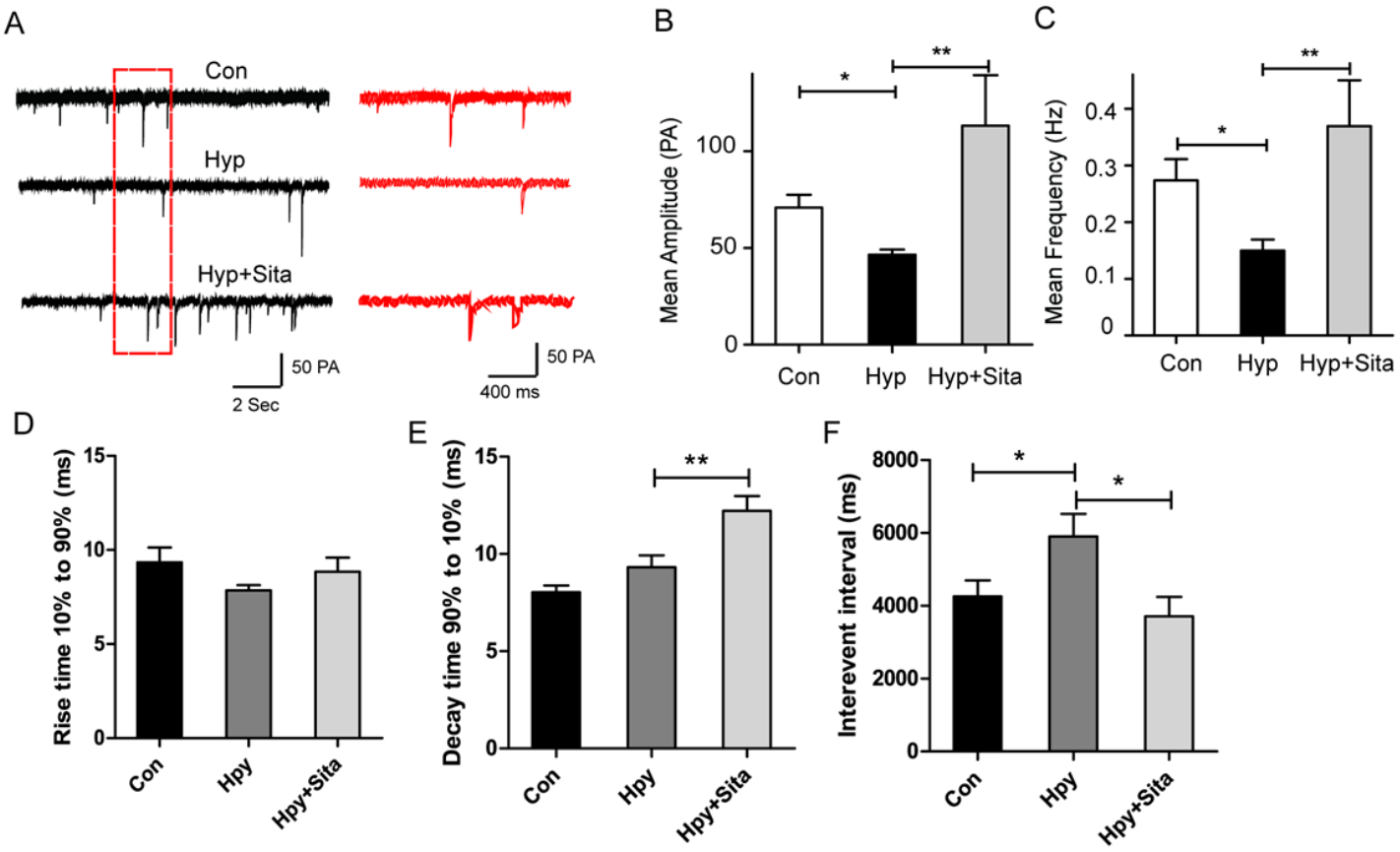

Figure 1

DPP4 inhibitor Sita increases sIPSCs in vitro. sIPSCs were recorded in extracellular fluid containing D-APV (50 μM) and CNQX (10 μM). Recording was performed using a CsCl-QX-314-based electrode solution at a holding potential (HP) of -60 mV. Traces show representative sIPSCs recorded in the cultured hippocampal neurons submitted to hyp (39.5-40°C). (A). The Sita (100 μM) treatment significantly increased the mean amplitude (B) and frequency (C) of sIPSCs. Rise time (D), decay time constant (E) and interevent interval (F) were calculated using Clampfit software. Con: Control; Hpy: hyperthermia; *p<0.05, **p<0.01, n=8. P values were calculated by one-way ANOVA.

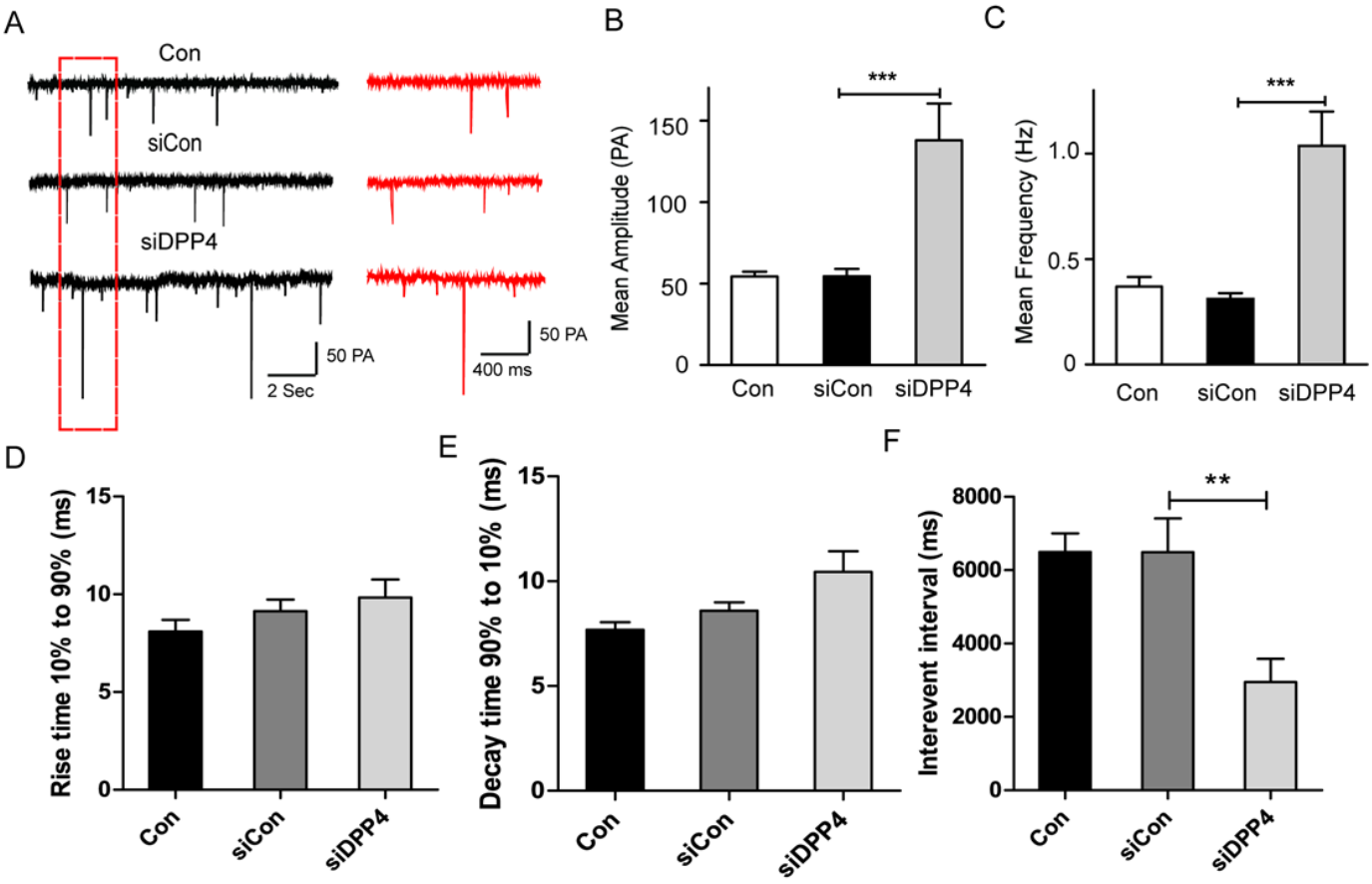

Figure 2

DPP4 knockdown increases sIPSCs in vitro. Traces show representative sIPSCs recorded in the cultured hippocampal neurons (A). DPP4 knockdown significantly increased the mean amplitude (B) and frequency (C) of sIPSCs. Rise time (D), decay time constant (E) and interevent interval (F) were calculated using Clampfit software. Con: control; siCon: siRNA control; siDPP4:siRNA DPP4; **p<0.01, ***p<0.001, n=6. P values were calculated by one-way ANOVA.

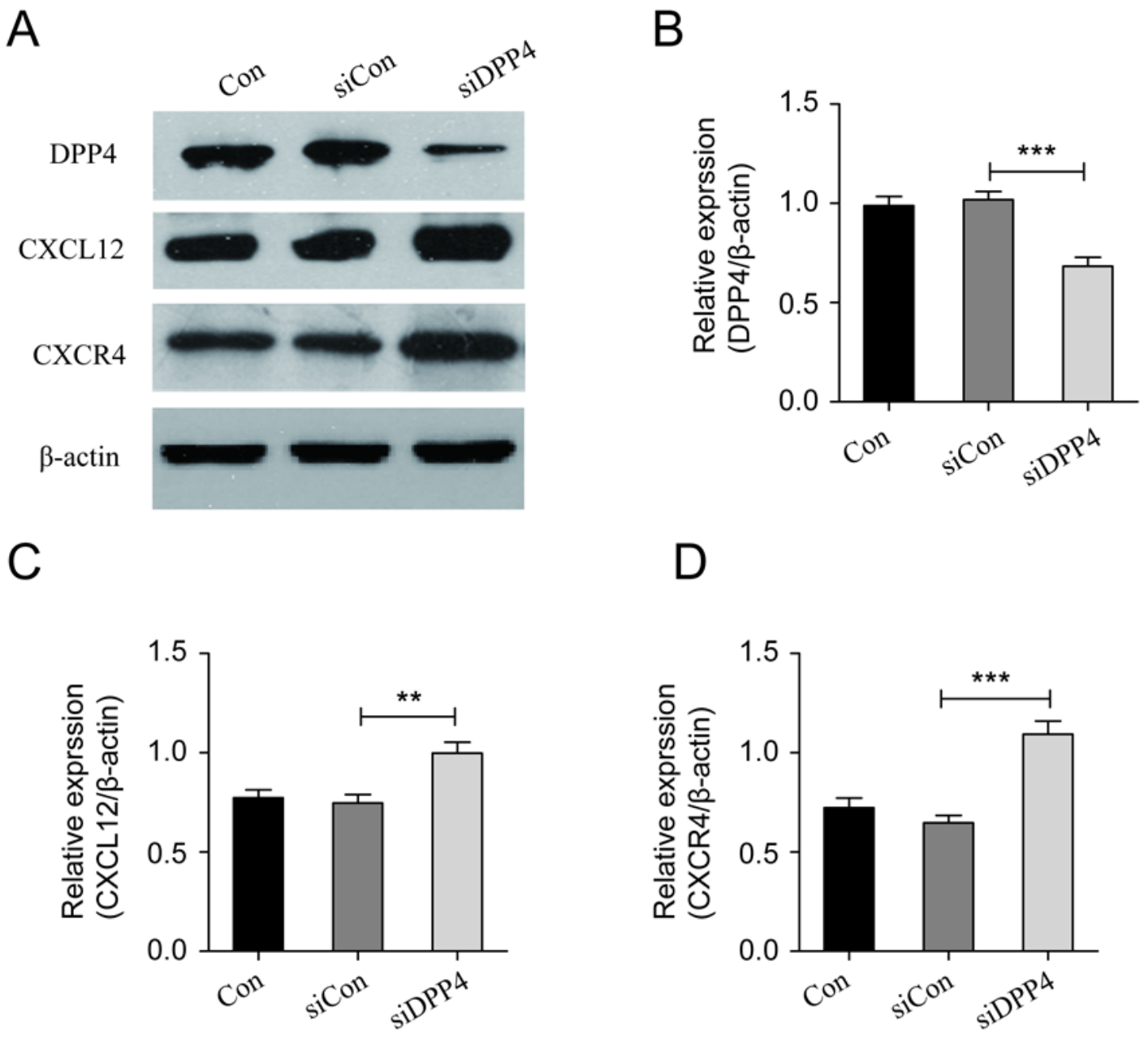

Figure 3

DPP4 knockdown increased the protein level of CXCL12 and CXCR4 in vitro. The proteins level of DPP4, CXCL12 and CXCR4 were measured using a western blot assay. A significant decrease in DPP4 level was observed after siDPP4 treatment in vitro (A,B). DPP4 knockdown increased the protein level of CXCL12 and CXCR4(C,D). Con: control; siCon: siRNA control; siDPP4:siRNA DPP4; All data are expressed as the mean ± SEM. P values were calculated by one-way ANOVA, **P < 0.01, ***P < 0.001, n =6.

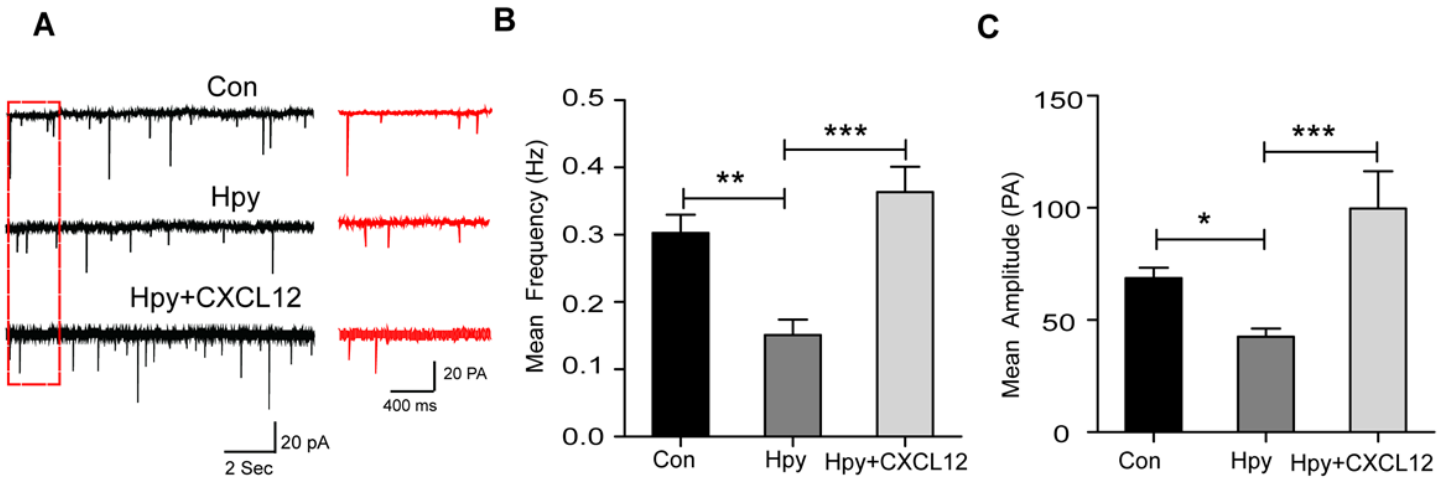

Figure 4

CXCL12 treatment increases sIPSCs in vitro. sIPSCs were recorded in extracellular fluid containing D-APV (50 μM) and CNQX (10 μM). Recording was performed using a CsCl-QX-314-based electrode solution at a HP of -60 mV. Traces show representative sIPSCs recorded in the cultured hippocampal neurons submitted to hyp (39.5-40°C)(A). The CXCL12 (10 nM) treatment significantly increased the mean frequency (B) and amplitude (C) of sIPSCs. *p<0.05, **p<0.01, ***P < 0.001, n=6. P values were calculated by one-way ANOVA.

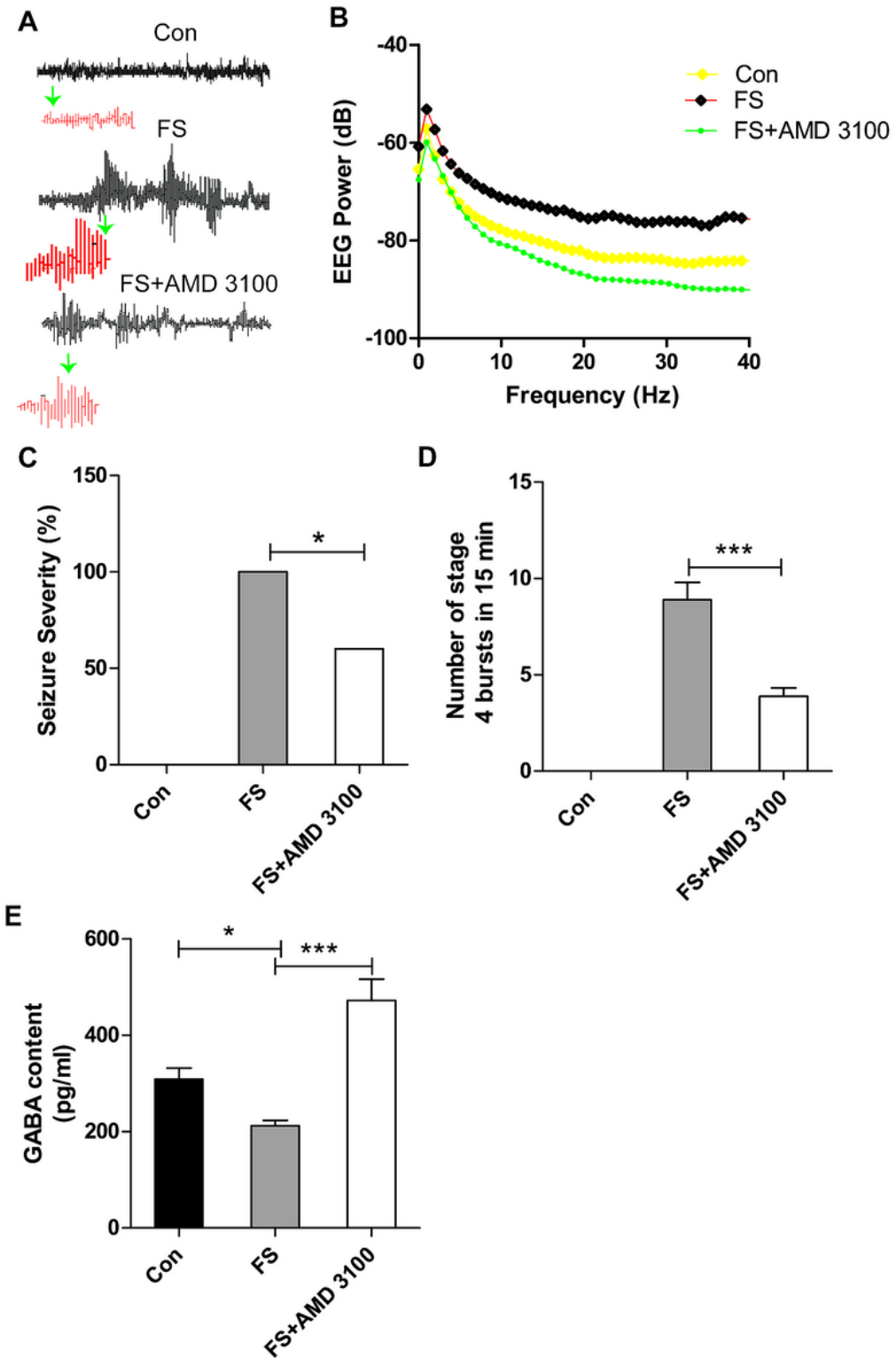

Figure 5

CXCR4 antagonist AMD3100 decreased seizure severity in vivo. CXCR4 antagonist AMD3100 (5 mg/ml, i.c.v) was administrated in rats. The results showed that AMD3100 treatment decreased neuronal firing frequency (A) and EEG power (B).Furthermore, the effect of AMD3100 on seizure severity (C), burst number of 4 stage (D) and GABA content (E) in hippocampus of rats were examined. *p<0.05, ***P < 0.001, n=12 per group. P values were calculated by one-way ANOVA.

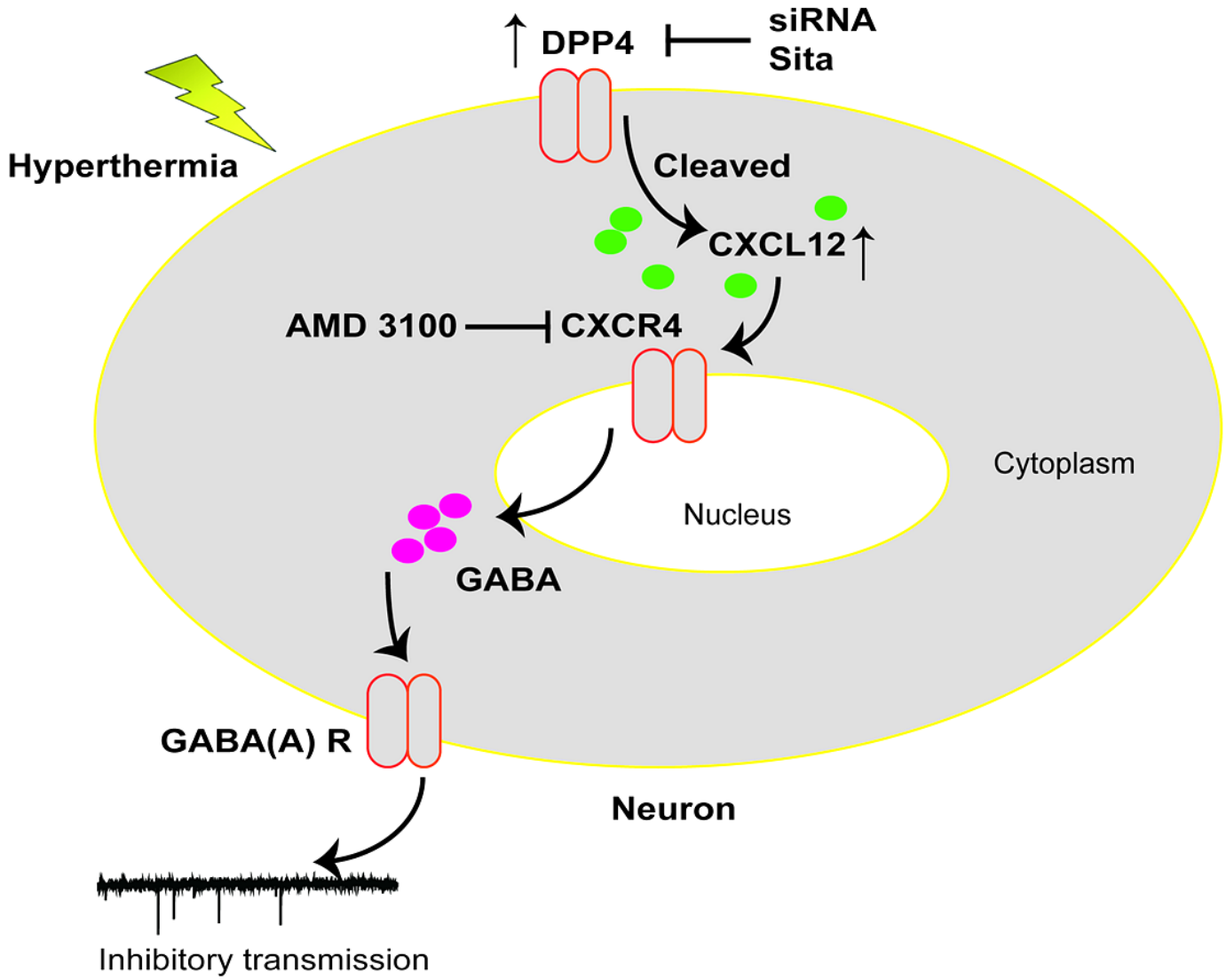

Figure 6

The scheme of the involvement of CXCL12/CXCR4 pathway in DPP4 regulation of FS. DPP4 inhibition by sita and siRNA elevated CXCL12 and CXCR4 levels, and CXCR4 antagonist decreased seizure severity, companied by increasing GABA content. Our data show that CXCL12/CXCR4 pathway is required for DPP4 regulation of sIPSCs.