CXCL12/CXCR4 signaling pathway regulates cochlear development in neonatal mice

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Abstract. Chemotactic cytokines (chemokines) are a highly conserved class of secreted signaling molecules that are important in various cellular processes. CXC chemokine ligand 12 (CXCL12) and its receptor, CXC chemokine receptor 4 (CXCR4) have been previously reported to be crucial for the establishment of neural networks in different neuronal systems. However, it is unclear whether the CXCL12/CXCR4 signaling pathway regulates the development of the cochlea. The current study investigated the effects of the CXCL12/CXCR4 signaling pathway on cochlear development in neonatal mice. The expression levels of CXCL12 and CXCR4 were detected using immunofluorescence, reverse transcription-quantitative polymerase chain reaction and western blot analysis demonstrating that CXCL12 and CXCR4 expression were significantly increased during cochlear development in neonatal mice. Treatment of spiral ganglion neurons with CXCL12 significantly decreased the protein expression levels of caspase-3 and cleaved caspase-3, indicating that CXCL12/CXCR4 signaling increased cell survival of spiral ganglion neurons. Furthermore, CXCL12 treatment significantly increased the number and length of neurites extending from spiral ganglion neurons. By contrast, the *in vitro* effects of CXCL12 were significantly abrogated by AMD100, a CXCR4 antagonist. Additionally, inhibiting CXCL12/CXCR4 signaling in neonatal mice significantly reduced the cell number and altered the morphology of spiral ganglion neurons *in vivo*. Thus, the present study indicates that the CXCL12/CXCR4 signaling pathway is important during the development of cochleae in neonatal mice.

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Abbreviations: CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; MAP2, microtubule-associated protein 2; BDNF, brain-derived neurotrophic factor

Key words: CXC chemokine ligand 12, CXC chemokine receptor 4, spiral ganglion neurons, cochlea

Introduction

The auditory sense is regulated by a specialized mechanosensory epithelium (the organ of Corti) in the cochlea, consisting of hair cells and nonsensory supporting cells (1), which extends along the length of the coiled cochlear duct in the ventral region of the inner ear (2). During early development, processes refine the afferent innervation of the sensory hair cells, leading to a precise pattern of innervation (3). Cochlear hair cells release neurotrophic factors, which participate in cochlear development, and the survival and maintenance of spiral ganglion neurons depend on neural activity and neurotrophic support (4). The cochlear spiral ganglion involves primary afferent bipolar neurons that propagate auditory signals from hair cells of the organ of Corti to the central auditory system (4). However, the signaling pathway that regulates cochlear prosensory specification requires further investigation (5). Thus, it is necessary to identify the signaling pathways that induce the precise and unique responses in cochlear prosensory cells.

Chemotactic cytokines (chemokines) are a large family of small protein ligands that are classified into four subfamilies, CXC, CC, CX3C and C chemokines, based on the location and organization of the first two cysteine residues (6). CXC chemokine ligand 12 (CXCL12), also termed stromal cell-derived factor 1, and its cognate receptor, CXC chemokine receptor 4 (CXCR4), are crucial for neurogenesis and regulate essential processes during the establishment of neural networks in neuronal systems, including neuronal migration, cell positioning and axon guidance (6-8). A previous study demonstrated that CXCL12 and CXCR4 are involved in regulating cerebellar granule cell migration (9). Additionally, the CXCL12/CXCR4 signaling pathway is critical for the formation of appropriate assemblies of γ-aminobutyric acid-ergic interneurons in the cerebral cortex and the pontine nuclei in the pons (10,11). Furthermore, in the spinal cord, the CXCL12/CXCR4 signaling pathway regulates the precision of initial sensory and motor axon trajectories of sensory and motor neurons (12). However, it is unclear whether the

CXCL12/CXCR4 signaling pathway participates in cochlear development.

The present study analyzed the expression of CXCL12 and CXCR4 during cochlear development in neonatal mice. It was demonstrated that CXCL12 and CXCR4 expression was significantly increased during cochlear development. Treatment of spiral ganglion neurons with CXCL12 *in vitro* significantly increased cell survival and neurite growth of spiral ganglion neurons, whereas blocking CXCL12/CXCR4 signaling impaired the above-mentioned processes. Furthermore, inhibiting CXCL12/CXCR4 signaling reduced the cell number and altered the morphology of spiral ganglion neurons *in vivo*.

Materials and methods

Mice and tissue sample collection. A total of 24 female neonatal C57BL/6J mice (age, 3 weeks; weight, 25-30 g), provided by the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China), were housed under a 12-h light/dark cycle at 24±1˚C and 45±5% humidity, with *ad libitum* access to food and water. The animal procedures were revised and approved by the Institutional Animal Care and Use Committee of Fourth Military Medical University. The mice were sacrificed by an intramuscular injection of ketamine (100 mg/kg; Yongnuo Pharmaceutical Co., Ltd., Yichang, China), after which cochleae were quickly and carefully removed, and the round window and vestibular membranes were opened then soaked in 4% paraformaldehyde solution (Sangon Biotech Co., Ltd., Shanghai, China) overnight at 4˚C. Cochlear tissue slices were stored at -20˚C until analysis.

Cell culture. Cochlear cartilage was removed with fine forceps, and the spiral ganglion tissue was separated and transferred to ice-cold Hanks' balanced salt solution (Sangon Biotech Co., Ltd.). The modiolus was cultured in Hibernate-A medium, containing 2% B27 and 0.1% penicillin (100 U/ml) and 0.1 mg/ml streptomycin (all Thermo Fisher Scientific, Inc., Waltham, MA, USA) as described previously (13). To assess the effect of CXCL12 on the spiral ganglion, spiral ganglion neurons were treated in medium containing 100 ng/ml CXCL12 (Sigma-Aldrich, St. Louis, MO, USA) with or without 20 μg/ml AMD3100 (CXCR4 antagonist; Sigma-Aldrich). The cells were maintained in a humidified 5% CO_2 incubator at 37˚C. The expression level of CXCR4 in spiral ganglion neurons was detected following incubation at 37˚C for 7 days. Microtubule-associated protein 2 (MAP2) was also detected after the 7-day incubation. To evaluate spiral ganglion neuron survival and neurite outgrowth in the stripe assays, the maintenance medium was supplemented with 30 ng/ml recombinant brain-derived neurotrophic factor (BDNF; Sigma-Aldrich) for a further 4 days of incubation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and treated with DNase (Sigma-Aldrich) to remove contaminating genomic DNA. cDNA was synthesized from 5 μg total RNA using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The primers used in the present study were as follows: Forward, 5'-GTTTGCTTTGGAGCTTCTCG-3' and reverse, 5'-GCTCTGGTGGAAGGTTGCTA-3' for CXCL12; forward, 5'-GCCATGGCTGACTGGTACTT-3' and reverse, 5'-GATGAAGGCCAGGATGAGAA-3' forCXCR4; and forward, 5'-ACCCATCACCATCTTCCAGGAG-3' and reverse, 5'-GAAGGGGCGGAGATGATGAC-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). qPCR was performed using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on the 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 94˚C for 4 min, followed by 35 cycles at 94˚C for 20 sec, 55˚C for 30 sec and 72˚C for 20 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (14). GAPDH served as the internal control for normalization of gene expression.

Western blot analysis. Proteins from the spiral ganglion neurons were extracted by homogenization in lysis buffer containing protease inhibitors (both Beyotime Institute of Biotechnology, Haimen, China). Protein concentration was measured using a Bradford protein assay kit. The proteins (40 μg) were separated by 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked with 3% skimmed milk for 1 h at room temperature, then incubated with rabbit anti-CXCR4 (1:800; cat. no. ab2074; Abcam, Cambridge, UK), anti-MAP2 (1:500; cat. no. M3696; Sigma-Aldrich), anti-caspase-3 (1:500; cat. no. sc-98785; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-cleaved caspase-3 (1:200; cat. no. ab2302; Abcam) and anti-β-actin (1:1,000; cat. no. sc-130656; Santa Cruz Biotechnology, Inc.) polyclonal antibodies overnight at 4˚C. Subsequently, the membranes were washed three times for 15 min each with Tris-buffered saline containing Tween-20, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary polyclonal antibody (1:2,000; cat. no. bs-0295G-HRP; BIOSS, Beijing, China) for ~1 h at 37˚C. The blots were visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Little Chalfont, UK) and the ChemiScope 5300 Pro gel imaging system (Clinx Science Instruments Co., Ltd., Shanghai, China). Protein band intensities were quantified using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and data were obtained by normalization to the control group.

ELISA. Freshly isolated spiral ganglion specimens were obtained from the mice. The protein extract was centrifuged and the concentration of CXCL12 in the supernatant was determined using a commercially available CXCL12 ELISA kit (cat. no. CSB-E04723m; Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's protocol.

Quantification of neurite outgrowth. Neurite outgrowth from the spiral ganglion neurons was assessed according to the number and lengths of the processes. Neurite outgrowth was observed from digital images of 7-10 randomly selected fields (magnification, x20), as previously described (15). The entire length of the longest process from each neuron was

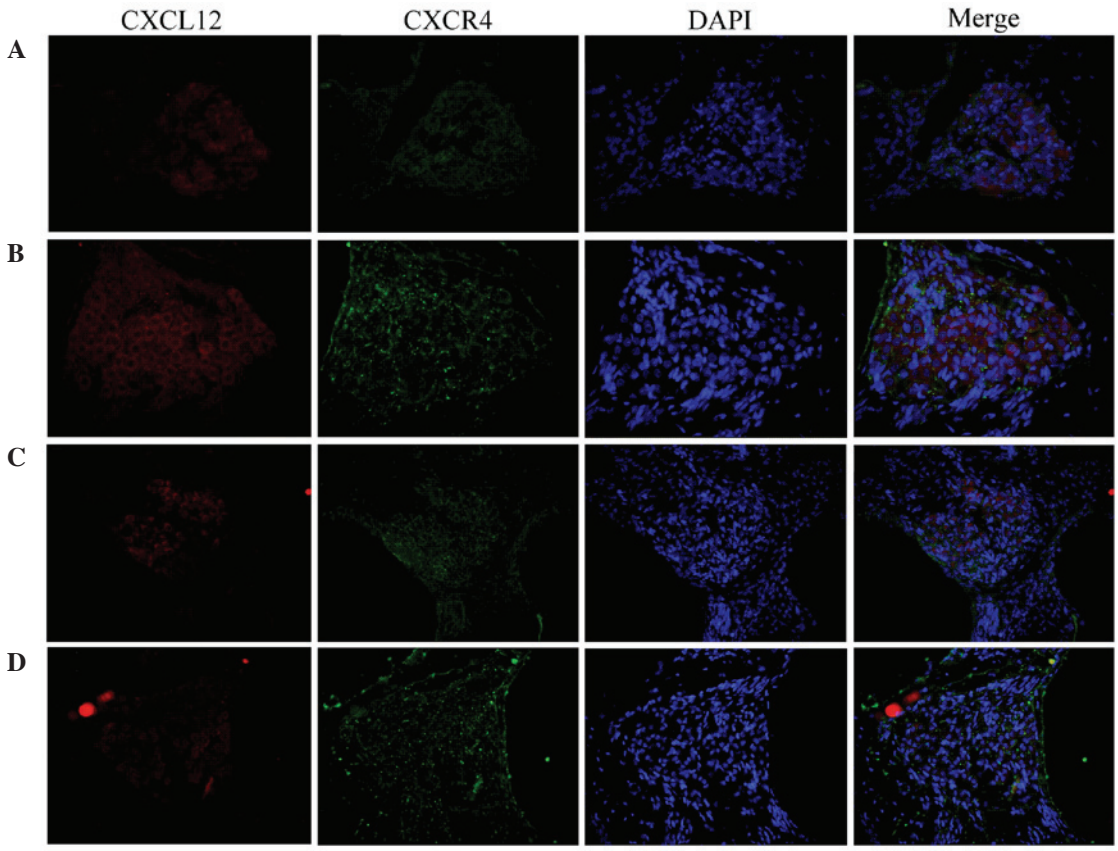

Figure 1. Immunofluorescence of CXCL12 and CXCR4 in spiral ganglion neurons of neonatal mouse inner ear. CXCL12 and CXCR4 expression in spiral ganglion neurons were detected by immunostaining at (A) P0, (B) P7, (C) P14 and (D) P21. Nuclei were stained with DAPI and merged images of red, green and DAPI staining are shown. Images were captured under a fluorescence microscope (magnification, x40). CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; DAPI, 4'-6-diamidino-2-phenylindole dihydrochloride; P, postnatal day.

determined using a TE300 Nikon semi-automatic microscope (Nikon Corporation, Tokyo, Japan). The mean neurite length per cell (the maximal possible distance along a neurite) and the mean number of neurites per cell were measured.

Immunohistochemistry. To prepare samples for immunohistochemical staining, mice were sacrificed by an intramuscular injection of ketamine (100 mg/kg) 48 h after intraperitoneal injection of AMD3100 (2.5 mg/kg), and the spiral ganglion was isolated as described previously (16). The spiral ganglion samples were fixed with 4% paraformaldehyde, then embedded in paraffin (Sigma-Aldrich) and cut into 5-μm serial sections using a microtome (Leica RM2016; Leica Microsystems GmbH, Wetzlar, Germany). Spiral ganglion sections were incubated overnight at 4˚C with a rabbit polyclonal neuron-specific enolase antibody (1:250; cat. no. ab53025; Abcam). Sections were then incubated with biotinylated goat anti-rabbit IgG (1:1,000; cat. no. bs-0295G-Bio; BIOSS) for 40 min at 37˚C, followed by incubation with pre-prepared streptavidin-biotinylated alkaline phosphatase complex (Beyotime Institute of Biotechnology) for 20 min. The sections were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich). For immunofluorescence detection, the cochleae were sectioned at a 10-μm thickness across the modiolus. The sections were permeabilized with 0.5% Triton X-100 (Sangon Biotech Co., Ltd.) for 5 min, blocked with 5% goat serum (Gibco; Thermo Fisher Scientific, Inc.) for 1 h, and then incubated with polyclonal rabbit anti-CXCL12 (1:100; ab2074; Abcam) and anti-CXCR4 antibodies overnight at 4˚C. Subsequently, the sections were washed three times for 5 min each with phosphate-buffered saline and then incubated for 1 h at room temperature with the appropriate secondary antibodies (1:1,000), as follows: CXCR4, Alexa Fluor 488-conjugated goat anti-rabbit polyclonal antibody (cat. no. ab150077; Abcam); and CXCL12, Alexa Fluor 647-conjugated donkey anti-rabbit polyclonal antibody (cat. no. ab150075; Abcam). Subsequently, the nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (1 μg/ml; BIOSS) for 10 min at room temperature. The fluorescence was observed with an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan), and the fluorescence intensity was quantified using ImageJ 1.48u software (https://imagej.nih.gov/ij/).

Statistical analysis. All results from the current study are presented as means ± standard deviation. Statistical differences were assessed using Student's t-test or one-way analysis of variance and $P<0.05$ was considered to indicate a statistically significant difference.

Results

CXCL12 and CXCR4 are upregulated in spiral ganglion neurons during cochlear development. To investigate whether

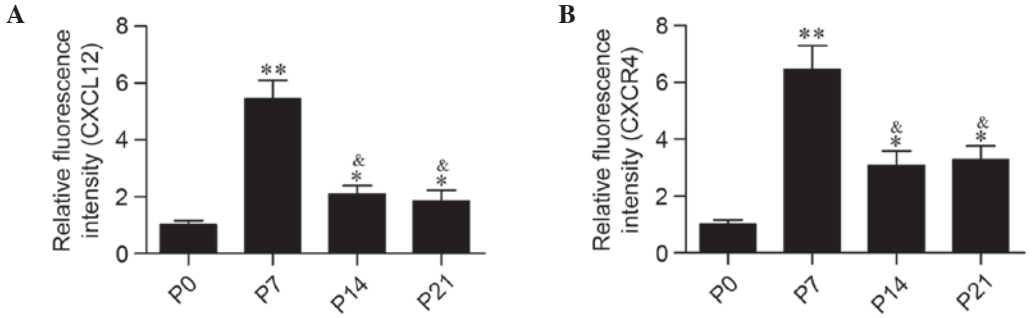

Figure 2. Quantitative analysis of relative fluorescence intensity. Relative fluorescence intensity of (A) CXCL12 and (B) CXCR4 in the different groups, following normalization with the P0 group. Fluorescence intensity was quantified using ImageJ software. *P<0.05 and **P<0.01 vs. P0; $^{\&}$P<0.05 vs. P7. Data are presented as the mean ± standard deviation. CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; P, postnatal day.

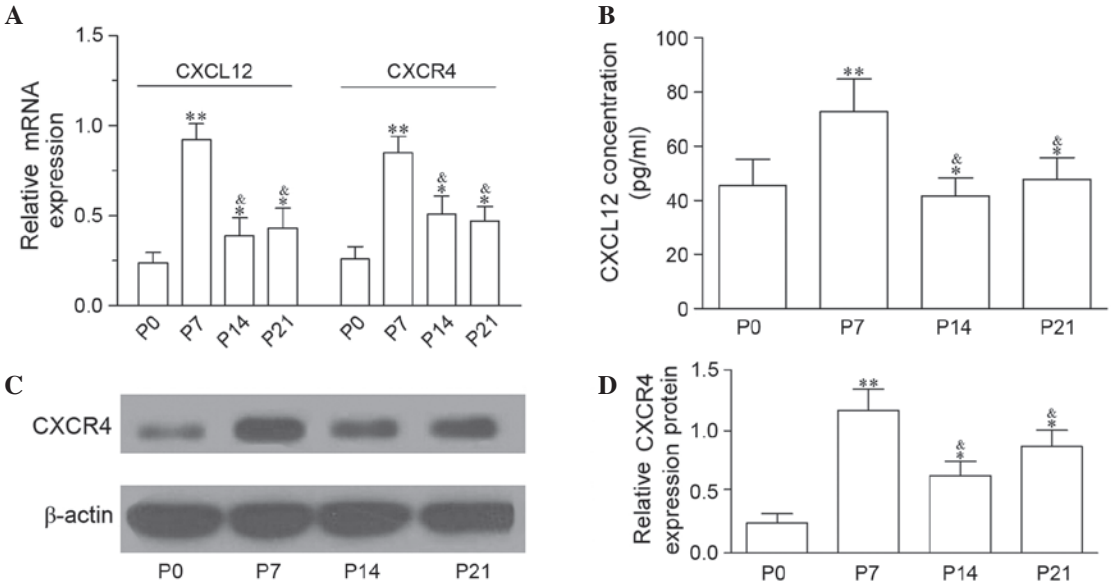

Figure 3. mRNA and protein expression levels of CXCL12 and CXCR4 in spiral ganglion neurons of neonatal mice. (A) Relative expression levels of CXCL12 and CXCR4 mRNA on P0, P7, P14 and P21 detected by reverse transcription-quantitative polymerase chain reaction analysis. (B) Concentration of CXCL12 protein was quantified by enzyme-linked immunosorbent assay in the different groups. (C) Protein expression of CXCR4 in spiral ganglion neurons of neonatal mice detected by western blot analysis. (D) Relative protein expression was quantified using Image J software. *P<0.05 and **P<0.01 vs. P0; $^{\&}$P<0.05 vs. P7. Data are presented as the mean ± standard deviation. CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; P, postnatal day.

CXCL12/CXCR4 is important in the development of cochleae, the expression levels of CXCL12 and CXCR4 in the cochlea of neonatal C57BL/6J mice were assessed using immunofluorescence (Fig. 1) and the relative intensities were quantified (Fig. 2). Compared with postnatal day 0 (P0), the protein expression levels of CXCL12 and CXCR4 were significantly increased in spiral ganglion neurons on P7 (P<0.01). Furthermore, the protein expression levels of CXCL12 and CXCR4 at P14 and P21 were decreased compared with P7 (P<0.05), but remained significantly increased compared with P0 (P<0.05; Fig. 2).

To further confirm the expression pattern of CXCL12 and CXCR4 in spiral ganglion neurons, the mRNA and protein expression levels of CXCL12 and CXCR4 were analyzed by RT-qPCR, and western blotting and ELISA, respectively. The results demonstrated that the mRNA expression levels of CXCL12 and CXCR4 were significantly increased on P7 (P<0.01), P14 and P21 (P<0.05) compared with P0, reaching a peak on P7 (Fig. 3A). Consistent with these findings, the protein concentration of CXCL12 (Fig. 3B) and levels of CXCR4 (Fig. 3C and D) were also significantly increased on P7 (P<0.01), P14 and P21 (P<0.05). Overall, the results of the present study indicated that CXCL12 and CXCR4 may participate in the development of the cochlea.

CXCL12 enhances the expression of CXCR4 in spiral ganglion neurons. CXCL12 and its receptor, CXCR4 are vital for central nervous system development by mediating cell migration, enhancing precursor cell proliferation and inducing neuronal circuit formation (17). Thus, the current study measured the expression of CXCR4 in the presence of CXCL12 to confirm that the CXCL12/CXCR4 signaling pathway may be involved in the development of spiral ganglion neurons. AMD3100, a CXCR4 antagonist, inhibits the CXCL12/CXCR4 signaling pathway (18). As demonstrated in Fig. 4A-C, compared with the control, the mRNA and protein expression levels of CXCR4 were significantly increased following treatment with CXCL12. By contrast, AMD3100 treatment reversed the effect of CXCL12 as the CXCR4 mRNA (P<0.05) and protein

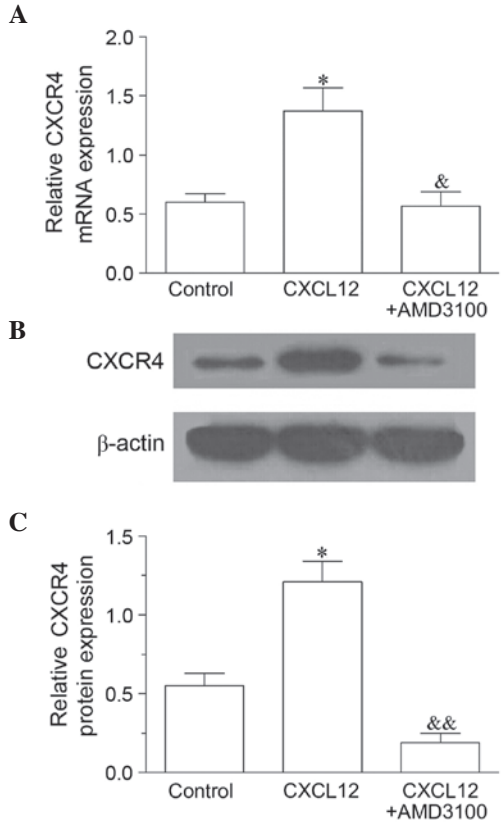

Figure 4. CXCL12 enhances the expression of CXCR4 in spiral ganglion neurons. (A) Reverse transcription-quantitative polymerase chain reaction analysis of CXCR4 mRNA expression in spiral ganglion neurons. Spiral ganglion neurons were treated with 100 ng/ml CXCL12, with or without 20 μg/ml AMD3100 for 7 days. *P<0.05 vs. control group; $^{\&}$P<0.05 vs. CXCL12-treated group. (B) Western blot analysis of CXCR4 protein expression in different treatment groups. (C) Relative protein expression level of CXCR4 was quantified using Image J software. *P<0.05 vs. control group; $^{\&\&}$P<0.01 vs. CXCL12-treated group. Data are presented as the mean ± standard deviation. CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4.

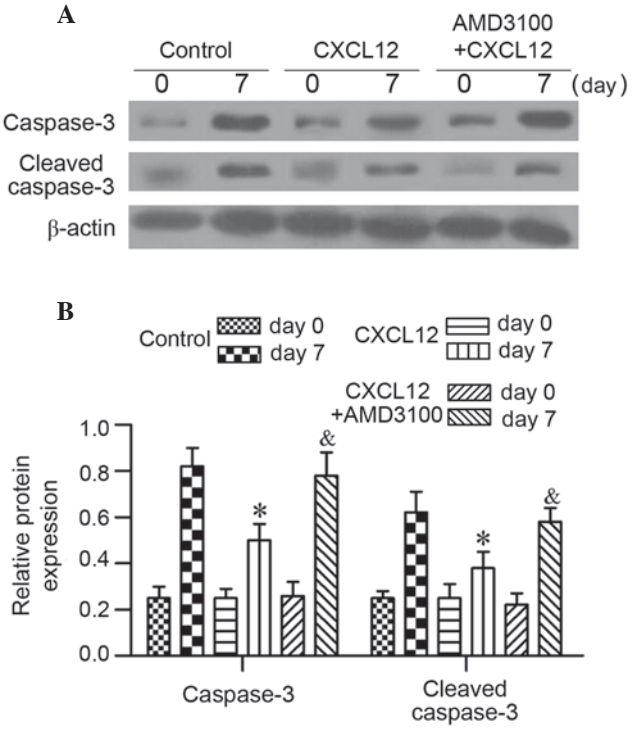

Figure 5. CXCL12/CXCR4 signaling inhibits the apoptosis of spiral ganglion neurons. (A) Western blot analysis of caspase-3 and cleaved caspase-3 protein expression in spiral ganglion neurons. Spiral ganglion neurons were treated with 100 ng/ml CXCL12 with or without 20 μg/ml AMD3100 for 7 days. (B) Relative protein expression levels of caspase-3 and cleaved caspase-3 were quantified using Image J software. *P<0.05 vs. control, day 7; $^{\&}$P<0.05 vs. CXCL12-treated group, day 7. Data are presented as the mean ± standard deviation. CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4.

(P<0.01) levels were significantly decreased by AMD3100 compared with CXCL12 treatment only (Fig. 4A-C).

Blocking CXCL12/CXCR4 signaling enhances spiral ganglion neuron apoptosis. To verify that CXCL12/CXCR4 is important for the regulation of cochlear development, CXCL12/CXCR4 signaling was inhibited by AMD3100 and the effect on spiral ganglion neuron apoptosis was evaluated. The present study demonstrated that treatment with CXCL12 significantly decreased the protein expression levels of caspase-3 and cleaved caspase-3 compared with the control (P<0.05). However, compared with CXCL12 treatment, the effect was significantly abrogated by AMD3100 (P<0.05; Fig. 5). The results indicated that CXCL12/CXCR4 signaling may inhibit cell apoptosis of spiral ganglion neurons.

Blocking CXCL12/CXCR4 signaling impairs the neurite growth of spiral ganglion neurons in vitro. To further confirm that CXCL12/CXCR4 signaling is important for neuronal circuit formation in the spiral ganglion, the current study analyzed its effect on neurite growth *in vitro*. The protein expression levels of the neurite marker, MAP2, were examined by western blot analysis. The results demonstrated that the protein expression level of MAP2 in spiral ganglion neurons treated with CXCL12 was significantly increased on day 7 compared with the control group (P<0.05). However, AMD3100 treatment abolished this effect, as the level of MAP2 was significantly reduced when compared with CXCL12 treatment (Fig. 6A and B). Furthermore, neurite outgrowth from the spiral ganglion neurons was evaluated by measuring the number and length of the neurites. When spiral ganglion neurons were treated with CXCL12, the number of neurites per cell increased compared with the control group (P<0.05; Fig. 6C). Similarly, the average neurite length in cells treated with CXCL12 was significantly longer compared with the control group (P<0.05; Fig. 6D). By contrast, compared with CXCL12 treatment only, the effects of CXCL12 on neurite number and length were significantly abrogated by AMD3100 treatment (P<0.05; Fig. 6C and D).

Blocking CXCL12/CXCR4 signaling impairs the number and alters the morphology of spiral ganglion neurons in vivo. To further confirm that the CXCL12/CXCR4 pathway is involved in the development of spiral ganglion neurons, the present study investigated the effect of CXCL12/CXCR4 inhibition on spiral ganglion neurons *in vivo*. The results demonstrated that spiral ganglion neurons were closely arranged with obviously stained cell bodies in the control group (Fig. 7A). By contrast, the number of spiral ganglion neurons was markedly

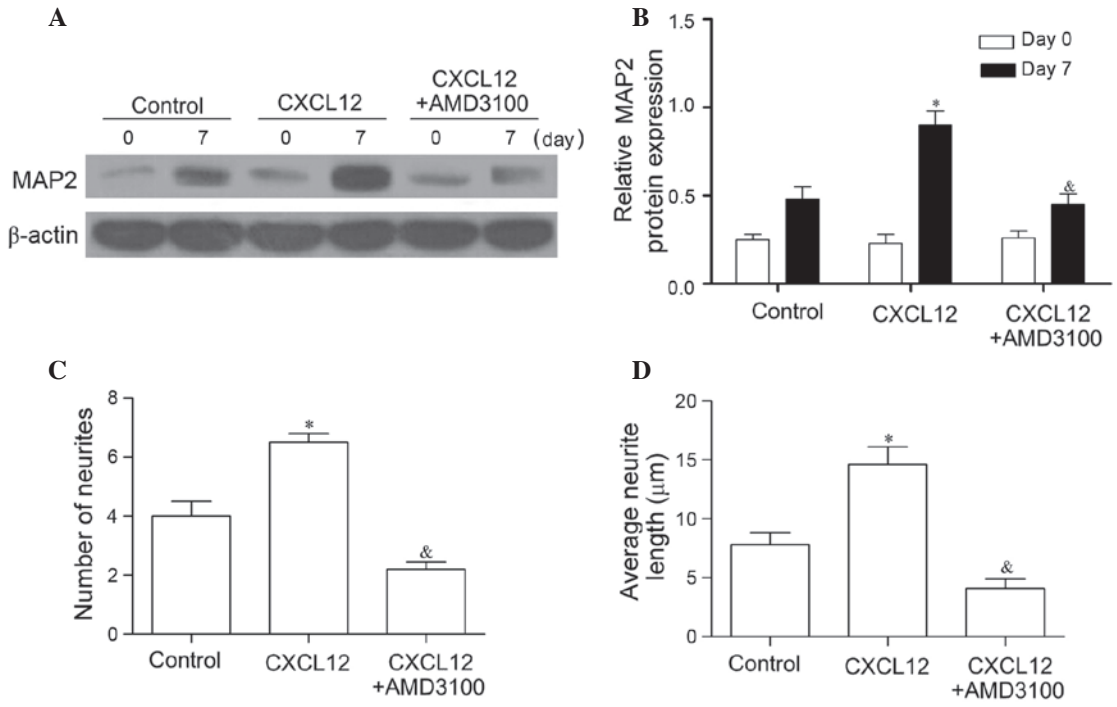

Figure 6. CXCL12/CXCR4 regulates the growth of dendrites in spiral ganglion neurons *in vitro*. (A) Western blot analysis of MAP2 protein expression in spiral ganglion neurons treated with CXCL12 in the presence or absence of 20 μg/ml AMD3100 for 7 days. (B) Relative protein expression level of MAP2 was quantified using Image J software. *P<0.05 vs. control, day 7; $^{\&}$P<0.05 vs. CXCL12-treated group, day 7. (C) The mean number of dendrites per cell in the various treated groups. The mean number of dendrites were calculated on day 7. *P<0.05 vs. control; $^{\&}$P<0.05 vs. CXCL12-treated group. (D) The mean length of dendrites per cell in the various treatment groups. *P<0.05 vs. control; $^{\&}$P<0.05 vs. CXCL12-treated group. Data are presented as the mean ± standard deviation. MAP2, microtubule-associated protein 2; CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4.

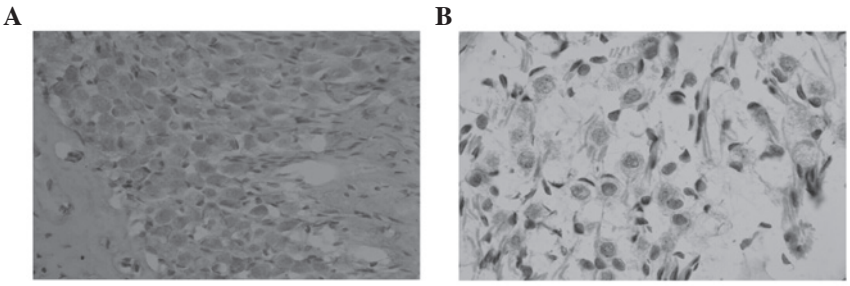

Figure 7. Photomicrographs illustrating spiral ganglion neurons (magnification, x40). Spiral ganglion neurons were stained by neuron-specific enolase antibody in the (A) control and (B) CXCL12/CXCR4-blocked groups. The mice were administered with CXCR4 antagonist, AMD3100, by intraperitoneal injection for 48 h. The spiral ganglion was then isolated and detected by immunohistochemistry using neuron-specific enolase antibody. The spiral ganglion neurons exhibited a normal morphology in the control group, but were reduced in number and had a fuzzy cell morphology in AMD3100-treated cells. Mice treated with phosphate-buffered saline served as the control. CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4.

reduced and the cells were irregularly arranged with fuzzy cell morphology in mice treated with AMD3100 (CXCR4 antagonist; Fig. 7B).

Discussion

In the present study, elevated CXCL12 and CXCR4 levels in the cochleae of neonatal C57BL/6J mice indicated that the CXCL12/CXCR4 signaling pathway is vital in the process of auditory sense formation. In addition, it was observed that treatment with CXCL12 significantly increased spiral ganglion neuron survival and neurite growth. This result was consistent with a previous observation on dissociated spiral ganglion neurons (19). Phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt signaling participates in mediating the effects of BDNF on spiral ganglion neurons *in vitro*, including neuronal survival and neurite extension (20). However, certain neurotrophins exert opposing effects on spiral ganglion neurons and alter the balance of competing signals to influence neurite formation. For example, activation of Ras-related C3 botulinum toxin substrate 1 (Rac)/cell division cycle 42 (cdc42)/c-Jun N-terminal kinases (JNK) signaling by BDNF may reduce the generation of neurites (20), which conflicts with previous findings indicating that rat sarcoma viral oncogene homolog or mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signaling was the primary mediator of spiral ganglion neuron outgrowth *in vitro*; the inhibition of which blocked the effects of neurotrophin-3 on spiral ganglion neurons (21). Previous studies

have demonstrated that these effects on spiral ganglion neurons are mediated by various signaling pathways, with neurite number being increased by the p38 and Akt signaling pathway, but inhibited by the Rac/cdc42/JNK signaling pathway. In the current study, neurite number and length were increased when CXCL12/CXCR4 was activated, indicating that the CXCL12/CXCR4 signaling pathway is involved in the development of spiral ganglion neurons.

CXCL12 and CXCR4 are constitutively expressed in the developing and mature central nervous system, stimulating a series of downstream signaling pathways (22). During early developmental stages, the expression of CXCR4 assists with the survival and proliferation of cortical neuronal precursors. In the mature central nervous system, the expression levels of CXCL12 and CXCR4 vary across a wide range of differentiated cells (23,24). In the hippocampus, neurogenesis and expression of CXCR4 persist throughout life (25). Additionally, following stroke, neuronal CXCR4 is upregulated in areas of neurogenesis (25). These observations indicate that CXCR4 possesses proliferative potential in neuronal cells. The present study demonstrated that following activation of the CXCL12/CXCR4 signaling pathway in spiral ganglion neurons, cell apoptosis was significantly decreased. However, inhibiting CXCL12/CXCR4 signaling significantly reversed the effect of CXCL12. These results suggest that the CXCL12/CXCR4 signaling pathway is important for the regulation of spiral ganglion neuron survival.

Notably, CXCL12 and CXCR4 modulate synapse formation and function. Normal neural function is required for connectivity, neurites must find their targets and dendrites must achieve normal morphology. A previous study demonstrated that activation of CXCR4 regulates the synaptic transmission of cerebellar and hippocampal granule cells *in vitro*, and induces transmission at nociceptive synapses (26). Furthermore, activation of CXCR4 is involved in the formation of parallel fiber synapses between cerebellar granule cells and Purkinje cells, in which CXCL12 modulates calcium transients (27-29). In the present study, the neurite marker, MAP2 was detected by western blot analysis, confirming that the CXCL12/CXCR4 signaling pathway was involved in neuronal circuit formation of spiral ganglion neurons. In addition, the *in vivo* morphology of spiral ganglion neurons was observed to demonstrate that the CXCL12/CXCR4 signaling pathway was involved in synapse formation.

A previous study demonstrated that the CXCL12 expression level is increased in the auditory nerve following cochlear injury (30). Another study provided further insight into the association between CXCL12 and transplanted stem cells following spiral ganglion neuron degeneration (31). Upregulation of CXCL12 in the host microenvironment and activation of CXCR4 in donor stem cells improved the migration efficiency of transplanted cells in the injured region of rat cochleae. However, the precise function of the CXCL12/CXCR4 signaling pathway in the development of the cochlea in neonatal mice remains to be determined.

In conclusion, the present study demonstrated that the expression levels of CXCL12 and CXCR4 were increased in spiral ganglion neurons during cochlear development. Treatment with CXCL12 increased cell survival and dendrite growth in spiral ganglion neurons, an effect that was blocked by the CXCR4 antagonist, AMD3100, implying that CXCL12/CXCR4 signaling is critical for spiral ganglion neuron growth. Furthermore, inhibition of CXCL12/CXCR4 signaling significantly reduced the number and altered the morphology of spiral ganglion neurons *in vivo*. Collectively, the current study suggests that the CXCL12/CXCR4 signaling pathway is important in cochlear development of neonatal mice.

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