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Research paper

# Substance P enhances endogenous neurogenesis to improve functional recovery after spinal cord injury

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ABSTRACT

Endogenous neural stem cells (NSCs) are the most promising sources for replacing cells lost after spinal cord injury (SCI). We have previously shown that substance P (SP), a neuropeptide, improves functional recovery after SCI and increases the numbers of cells in lesion sites, but how this occurs is unclear. Here, we investigate whether SP regulates the neurogenesis of resident NSCs as well as exerting a beneficial effect on functional improvement. We found that SP (5 nmol/kg) treatment markedly improved functional recovery and elicited robust activation of endogenous NSCs and boosted the number of $EdU^+$ proliferating cells differentiating into neurons, but it reduced astroglial differentiation in the lesion sites. Consistently, treatment with SP (10 nM) *in vitro* significantly increased the proliferation of NSCs *via* activating the Erk1/2 signaling pathway and promoted neuronal differentiation but not astroglial differentiation. These results suggest that SP may represent a potential therapeutic agent for SCI *via* enhancing endogenous neurogenesis.

## 1. Introduction

Spinal cord injury (SCI) causes the loss of multiple cell types at or close to the lesion site, resulting in tissue disruption, which leads to permanent functional impairments (Gregoire et al., 2015; Ke et al., 2006; Lindvall and Kokaia, 2010). Recently, stem cell therapy has been considered a promising strategy for SCI, and numerous studies have evaluated the beneficial effect of transplanting a variety of stem cell types in SCI animal models (Keirstead et al., 2005; Parr et al., 2007; Teng et al., 2002), but there are challenges in identifying the best source of stem cells and in overcoming the limitations and risks associated with transplantation strategies.

Endogenous neural stem cells (NSCs), located in the ependymal layer in central canal of the spinal cord (Bambakidis et al., 2008; Meletis et al., 2008), have been shown to exhibit self-renewal and neuronal differentiation *in vitro* (Johansson et al., 1999; Shihabuddin et al., 1997; Weiss et al., 1996). Thus, therapies using endogenous stem cells may not require exogenous stem cell sources, thereby avoiding the obstacle of immune rejection and the ethical and moral considerations associated with exogenous stem cells. However, several lines of evidence suggest that the ependymal NSCs could actively proliferate and migrate towards the lesion site, where the majority of cells differentiate into various glial cells but not neurons after SCI (Johansson et al., 1999). Therefore, optimization of the regenerative responses of endogenous NSCs, especially altering their specification, should provide new strategies for treating SCI.

Substance P (SP) is well known as an endogenous neuropeptide that is involved in cell proliferation and in the synthesis of cytokines and growth factors in multiple cell types (Ho et al., 1997; Kim et al., 2015; Payan and Goetzl, 1985). In a previous report, we showed that SP enhanced functional recovery from SCI *via* the anti-inflammatory modulation associated with the induction of M2 macrophages at the lesion

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site (Jiang et al., 2012). More importantly, we observed that neuron-like cells also increased in the lesion site of spinal cord after SP treatment, but the original source of those cells is not yet fully elucidated.

In this respect, we hypothesized that SP could exert its therapeutic effects by promoting endogenous neurogenesis after SCI. This study was undertaken to examine the effects of SP on NSCs proliferation and differentiation in adult mice after SCI and to elucidate its modulation of the differentiation of newborn NSCs, which contributes to the functional improvement after injury to the central nervous system.

## 2. Materials and methods

### 2.1. Animals

C57BL/6 mice were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China). All animal experiments were performed in accordance with the guidelines of the Sun Yat-sen University Institutional Animal Care and Use Committee.

### 2.2. Surgical procedure and SP administration

Adult male C57BL/6 mice (25–30 g, 8–9 weeks old) were used in this study. Basic surgical procedures and compression injury were performed as described previously (McDonough et al., 2013; Plemel et al., 2008). In brief, after exposing the spinal column at the level of T8–T11, laminectomy was performed on the 10th thoracic vertebra. The spinal cord was laterally compressed to a thickness of 0.35 mm for 15 s using one pair of modified forceps. The SCI mice were then i.v. injected with SP (5 nmol/kg) in PBS (Sigma-Aldrich, St Louis, Missouri, USA) daily for three consecutive days: immediately, 24 h, and 48 h after SCI respectively. The control group received i.v. injections of PBS at the same time points, as previously described (Jiang et al., 2013). To characterize the proliferative response in C57BL/6 mice, EdU (50 mg/kg) were i.p. injected daily for seven consecutive days, starting on the day of the injury, at the same time points in normal mice without SCI, and mice were killed 1 day after the final injection (Ke et al., 2006; Song et al., 2002).

### 2.3. Isolation and culture of NSCs from C57BL/6 mice

NSCs cultures were derived and maintained as described previously (Park et al., 2010), with minor modifications. Briefly, mouse embryos were removed at embryonic day (E) 11.5–12, and the cerebral cortices was mechanically dissected and dissociated to yield suspensions of single cells, which were cultured in a serum-free medium, containing DMEM/F12 medium (1:1; Gibco USA), 20 ng/ml EGF (Peprotech, USA), 10 ng/ml bFGF (Peprotech, USA), 2% B27 (Invitrogen, USA), 1% N2 (Invitrogen, USA), and 100 IU/ml penicillin/streptomycin (Invitrogen, USA). These cells were cultured at 37 °C under 5% $CO_2$ and propagated every other day.

### 2.4. Proliferation assay on cell and spinal cord sections

For cell counting, NSCs were seeded onto poly-D-lysine-coated 12-well plates at a density of $1 \times 10^5$ cells per well. Subsequently, the cells were trypsinized with 0.25% trypsin and counted daily for 4 consecutive days (Song et al., 2002).

NSCs proliferation was evaluated with Click-iT®EdU cell Fluor Cell Proliferation Assay Kit (Invitrogen, USA). The cells were labeled with 10 μM EdU for 24 h and then washed carefully to remove the dye and stained according to the manufacturer's instructions; nuclei were counterstained with DAPI.

To characterize the proliferative response in mice, the spinal cords were further fixed with 4% PFA and dehydrated in 30% sucrose at experimental time points after EdU injection. A 1.5 cm segment of the spinal cord, was embedded in OCT compound (Sakura Finetek, Japan), cut at 10 μm thick, and then stained with Click-iT®EdU cell Fluor Cell Proliferation Assay Kit (Invitrogen, USA), nuclei were counterstained with DAPI.

### 2.5. Western blot analysis

Injured spinal cords (8 mm) centered on the injury site were removed at the specific times and frozen immediately on dry ice. The spinal cord tissue were lysed and homogenized in RIPA buffer (Millipore, USA) containing protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and PhosSTOP (Roche, Mannheim, Germany). NSCs were lysed with ice-cold lysis buffer (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and PhosSTOP (Roche, Mannheim, Germany). Subsequently, 50 μg of total protein was separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated with primary antibodies targeting Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology, USA), p44/42 MAPK (Erk1/2) (Cell Signaling Technology, USA), and β-actin (Sigma-Aldrich, USA) was used as the loading control (Liu et al., 2015a,b; Hong et al., 2009). The results were confirmed using four independent experimental sets.

### 2.6. In vitro differentiation of NSCs

NSCs differentiation was induced by plating cells onto poly-D-lysine-coated 24-well plates in DMEM/F12 medium (1:1; Gibco, USA) supplemented with 2% FBS (Invitrogen, USA), 2% B27 (Invitrogen, USA), and 100 IU/ml penicillin/streptomycin (Invitrogen, USA). The cells were maintained in culture for 7 days and were fed every day (Song et al., 2002). Differentiation was subsequently confirmed by immunostaining and qPCR. The primers for qPCR were listed in Supplemental Table S1.

### 2.7. Immunofluorescence staining analysis

At specific time points, the spinal cords were further fixed with 4% PFA and dehydrated in 30% sucrose. A 1.5 cm segment of the spinal cord, centered at the injury epicenter, was embedded in OCT compound (Sakura Finetek, Japan). Either longitudinal sections or cross sections were cut at 10 μm thick. Cells were fixed in 4% PFA for 20 min at room temperature. For the immunofluorescence study, anti-Neurofilament L (Millipore, USA); anti-NeuN (Abcam, UK); anti-Nestin (Millipore, USA); anti-Tuj-1 (Abcam, UK); anti-GFAP (Abcam, UK); anti-NK-1 receptor (Abcam, UK) were applied. All images were obtained using a Zeiss LSM780 confocal microscope (Carl Zeiss, Jena, Germany). Nuclei were counterstained with DAPI. For cell counting, three longitudinal sections were obtained from depths of 560, 600 and 640 μm into the cord (from the dorsal side) in each animal, respectively. There were three injury sites, rostral, epicenter, and caudal, in each longitudinal section, and four random visual fields (400 x) of each injury site were counted.

For analysis of mouse spinal cords morphology, the collected tissues were fixed in 4% paraformaldehyde at 4° C overnight and then embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin and eosin (H & E).

### 2.8. Behavioral analysis

We evaluated behavioral functions using the BMS open-field locomotor rating scale, which was developed specifically for mice and allows achievement of scores ranging from 0 (complete paralysis) to 9 (normal mobility) (Ziv et al., 2006).