# A Self-Assembling Peptide Reduces Glial Scarring, Attenuates Post-Traumatic Inflammation and Promotes Neurite Outgrowth of Spinal Motor Neurons

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**Abstract**

**Study Design.** Self-assembling Peptide Gel (SPG-178) provide new evidence for the role of a scaffold for treatment of the spinal cord through induction of neuroprotective factors.

**Objective.** To verify the reproducibility of SPG-178 as scaffold following spinal cord injury, we examine the characteristics of SPG-178 and protective effect on neural cells in vitro and vivo.

Summary of Background Data. The CNS extracellular matrix (ECM) may play a role in maintenance of the neuronal network by inhibiting axonal growth and suppressing formation of additional inadequate synapses. In this study, we show increased expression of NGF, BDNF, NT-4, TrkA and TrkB in SPG-178-promoted neurite outgrowth of motor neurons in vitro, and decreased inflammation and glial scar with use of SPG-178 in vivo.

**Methods.** We examined the effect of a self-assembling peptide, SPG-178, as a scaffold for neurite outgrowth of spinal motor neurons in vitro. An in vivo analysis was performed to evaluate if the SPG-178 scaffold attenuated or enhanced expression of various genes following spinal cord injury model rats.

**Results.** Expression of NGF, BDNF, NT-4, TrkA and TrkB increased in SPG-178-promoted neurite outgrowth of motor neurons in vitro. In vivo, SPG-178 increased expression of GDNF and NGF, and decreased glial scar.

**Conclusion.** This study provides new evidence for the role of SPG-178 as a scaffold in the spinal cord and suggests that this peptide is a neuroprotective factor that may serve as an alternative treatment for neuronal injuries.

**Key Words:** Spinal cord injury, Scaffold, Neurite outgrowth, Glial scar, Fibrous scar

**Level of Evidence:** 5

Key Ponits

**• A self-assembling peptide reduced glial scarring, attenuates post-traumatic inflammation.**
**• A self-assembling peptide promoted neurite outgrowth of spinal motor neurons.**
**• SPG-178 as a scaffold in the spinal cord was a neuroprotective factor that may serve as an alternative treatment for neuronal injuries.**
**• SPG-178 was a potential biomaterial for reconstruction of the injured spinal cord.**

Mini Abstract (50 words)



This study provides new evidence for the role of SPG-178 as a scaffold in the spinal cord and suggests that this peptide is a neuroprotective factor that may serve as an alternative treatment for neuronal injuries.

## Introduction

Neurons in the adult mammalian CNS do not spontaneously regenerate after injuries. Thus, repair of spinal cord injury (SCI), a traumatic injury of the spinal cord that often leads to morbidity and other severe consequences, is a challenging task for clinicians. The CNS extracellular matrix (ECM) may play a role in maintenance of the neuronal network by inhibiting axonal growth and suppressing formation of additional inadequate synapses. We developed a self-assembling peptide, SPG-178 (Self-assembling Peptide Gel, amino acid sequence #178; [$CH_3CONH$]-RLDLRLALRLDLR-[$CONH_2$]; R = arginine, L = leucine, D = aspartic acid, A = alanine), as a scaffold and potential therapeutic agent for SCI. The stability of the peptide solution/hydrogel at neutral pH (the isoelectric point, at which a protein has a zero net charge and reaches minimum solubility) contributes to the biocompatibility of the scaffold and provides an additional benefit for the sterilization procedure [1]. In this study, we show increased expression of NGF, BDNF, NT-4, TrkA and TrkB in SPG-178-promoted neurite outgrowth of motor neurons in vitro, and decreased inflammation and glial scar with use of SPG-178 in vivo. These results provide new evidence for the role of SPG-178 as a scaffold for treatment of the spinal cord through induction of neuroprotective factors.

## Materials and Methods

### Primary culture of spinal cord neurons

Primary cultures of mouse spinal cord neurons were prepared using the method described by Jiang et al. with minor modifications[2]. Briefly, spinal neurons were obtained from C57BL/6NCrl mice on embryonic day 13.5. The spinal cord was rapidly dissected from the embryo. Slices were digested in pre-warmed 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) at 37°C for 18 min in a conical flask agitated by hand every 5 min. The mixture was then centrifuged for 5 min at 378 x g. The supernatant was removed and the tissue was resuspended in DMEM containing 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum (Invitrogen), and triturated 15-20 times with a fire-polished Pasteur pipette. Cells were plated onto culture plates coated with poly-L-lysine (MW 30,000-70,000; Sigma, St. Louis, MO, USA) at a density of 15000 cells/well on 4-well dishes. Four hours later, the medium was replaced with serum-free neurobasal medium (Invitrogen) supplemented with 2% B27 supplement (Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.5 mM glutamine (Invitrogen). On the second day, 5 mM cytosine-β-D-arabinofuranoside (Sigma) was added to the medium for 24 h to inhibit non-neuronal cell division. The

cultures consisted of >90% neurons, as identified by immunocytochemical staining, and these cultures were utilized for the in vitro experiments described below after 2 days.

*Neurite outgrowth assay.*

Four-well chamber slides (Nunc, Roskilde, Denmark) were coated with 20 µg/ml poly-L-lysine (600 µl) (Sigma), 20 µg/ml laminin (600 µl) (Sigma), or SPG-178 (30 µl) (0.8% w/v hydrogel; Menicon Co.) and left overnight at 4°C. At 48 h after seeding, the neurons were fixed with 4% paraformaldehyde/PBS and stained with antineuron-specific β-tubulin (Covance) to visualize neurites. Neurite lengths were measured for at least 100 neurons with neurites longer than twice the cell body diameter. The distance of neurite outgrowth was quantified by orphometric image analysis (integrated optical density, OD) on fluorescent photomicrographs using Image J software (National Institutes of Health, Bethesda, MD, USA).

Animals

A total of 72 C57BL/6NCrl mice (female, 8 weeks old) were used in the study. Animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and all efforts were

made to minimize suffering. All animal procedures were approved by the Institutional Animal Care and Use Committee of [redacted] for the use of laboratory animals.

Spinal cord injury model

Adult female C57BL/6NCrl mice (8 weeks old) were housed under a 12 h light-dark cycle in standard cages with access to food and water ad libitum. The mice were anesthetized with an intraperitoneal injection of somnopentyl (60 mg/kg, Kyoritsu Seiyaku). After laminectomy at the 10th thoracic spinal lamina, the dorsal surface of the dura mater was exposed. Dorsal spinal cord hemisection was then performed at spinal T10 with a fine scalpel. Using a stereotaxic frame and glass capillary needle connected to a Hamilton microsyringe, 5 µl of 0.8% SPG-178 hydrogel (treatment group) or saline (control group) was injected into the dorsal gap of the spinal cord after SCI. The muscles and skin layers were then sutured.

Quantitative real-time PCR

Quantitative RT-PCR analysis of total RNA was performed on cells extracted with TRIzol reagent (Invitrogen) and purified with RNeasy columns (Qiagen, Valencia, CA,

USA). Expression levels of selected mRNAs were quantified using real-time RT-PCR. Differences in expression between groups were expressed using cycle time (Ct) values as relative increases. With the control as 100%, and assuming that the Ct value reflects the initial copy number and there was a 100% efficacy, a difference of one cycle is equivalent to a twofold difference in the copy number. Sequences of primers used for RT-PCR are listed in Table 1.

Immunocytochemistry and immunohistochemistry

The following antibodies were used in the assays below: Cy3-conjugated anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Sigma); anti-Iba1 polyclonal rabbit antibody (Wako); anti-type IV collagen polyclonal rabbit antibody (LSL); and fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (Sigma).

Immunocytochemistry was performed at room temperature. Cells were fixed with 4% paraformaldehyde in PBS for 30 min. After washing three times with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After three additional washes with PBS, the cells were blocked in 3% bovine serum albumin in PBS (blocking solution). Cells were incubated with the primary antibodies (diluted with blocking solution) for 60 min, followed by incubation with the fluorochrome-conjugated

secondary antibody (also diluted with blocking solution) for 60 min. After three more washes with PBS, coverslips were mounted on slides with FluorSave (Calbiochem).

For immunohistochemistry, mice were perfused transcardially under deep ether anesthesia with buffered 4% paraformaldehyde. Spinal cords were isolated, post-fixed in 4% paraformaldehyde overnight, and cryoprotected in buffered 30% sucrose during the following night. Tissues were cut into 20-µm sections with a cryostat and mounted on glass slides. Sections were blocked in blocking solution and then incubated with primary antibodies (diluted 1:100 with blocking solution) overnight at 4°C. After rinsing in PBS, the sections were incubated with the secondary antibody for 60 min at room temperature. Subsequently, the sections were rinsed in PBS, mounted with FluorSave, and observed using a BZ-9000 microscope (Keyence, Osaka, Japan) fitted with the appropriate filters.

Statistical analysis

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA), using an unpaired two-tailed Student t test for single comparisons and one-way ANOVA with a post hoc Bonferroni test for multiple comparisons. In all analyses, significance was accepted at $p < 0.05$.

## Results

### In vitro assays

We first examined the effects of SPG-178 on neurite outgrowth. SPG-178, laminin, and PLL all enhanced neurite growth in primary cultured spinal cord motor neurons (Fig. 1A), with laminin and SPG-178 producing significant 1.7- and 1.6-fold increases, respectively, compared with the control (Fig. 1B). There was no significant difference between the laminin and SPG-178 groups. We next investigated whether SPG-178 influenced primary motor neuron neurotrophic function. Real-time PCR was used to quantify expression of key genes involved in nerve regeneration. In the SPG-178 group, levels of mRNAs for NGF (2 and 7 days), BDNF (2 days) and NT-4 (2 and 7 days), and for the TrkB (2 days) and TrkA (7 days) receptors, were significantly increased compared to the control group ($p<0.05$) (Fig. 2A-D).

### In vivo experiments

An in vivo analysis was performed to evaluate if the SPG-178 scaffold attenuated or enhanced expression of various genes following SCI. At 7 days after injection, the SPG-178 group had higher levels of mRNAs for GDNF and NGF compared to the

control (saline) group (Figure 3A). This suggests that after 1 week the scaffold may have already played a role in regeneration of damaged nervous tissue [3]. However, there was no significant difference in receptor mRNA levels between the two groups (Figure 3B). We also examined several genes involved in matrix remodeling and found that mRNAs for MMP-2 and -14 were significantly upregulated in the SPG-178 group compared to the control group ($p<0.05$) (Fig. 3C).

Glial scar formation

Accumulation of GFAP-positive reactive astrocytes with typical changes of hypertrophy, process extension and increased expression of intermediate filaments appeared around the SCI lesion in the SPG-178 and control groups. However, there was less accumulation of these cells in control mice, based on the GFAP-positive area in a region of 600 μm width around the lesion core being significantly smaller in control mice from 14 to 56 days after SCI (Fig. 4AB). These astrocytes eventually migrated centripetally to the lesion epicenter and gradually compacted the $Iba1^+$ inflammatory cells, contracting the lesion area from 14 to 28 days after SCI. $Iba1^+$ cell accumulation in the lesion was significantly reduced in the SPG-178 group from 14 to 28 days after SCI, but there was no difference between SPG-178 and control mice at 56 days after

injury (Fig. 5AB). Clear expression of collagen IV, a marker for glial scarring that appears in the later stages of scarring [4], was present in the injured area at 14 days after injury and the scar area was significantly reduced in SPG-178 mice (Fig. 6AB).

## Discussion

In this study, we found that injection of SPG-178 leads to expression of neuropathic factors, decreased inflammation, and reduced glial scarring. The SPG-178 peptide solution (2.4 mM) is transparent and able to form a stable hydrogel at neutral pH when triggered by an increase in salt concentration. The stability of the peptide solution/hydrogel at neutral pH contributes to the biocompatibility of the scaffold. The solution can also be sterilized with an autoclave, which is advantageous in the sterilization procedure. Insignificant degradation of the SPG-178 peptide was detected by MALDI-TOF MS and no change in gelation behavior was caused by autoclaving [1]. Among the neurotrophic factors examined, SPG-178 significantly increased the mRNA levels for NGF (2 and 7 days), BDNF (2 days), NT-4 (2 and 7 days), and the TrkA (7 days) and TrkB (2 days) receptors (Fig. 2A-D). BDNF, NGF, and NT-4 mRNA is extensively distributed in motor-related neurons in the brain, such as neurons in the cerebellum, basal ganglia, brain stem, and even the spinal cord [5,6]. Trk A B are the Trk

family of receptor tyrosine kinase and is activated by BDNF , NGF, and neurotrophin-4[7]. It was considered that binding of those neurotrophic factors to membrane receptors activated by SPG-178 promoted neurite outgrowth.

At 7 days after injection, the SPG-178 group had higher mRNA levels for GDNF and NGF compared to the control group (Figure 3A), which suggests that the scaffold plays a role in regeneration of damaged nervous tissue within the first week after injection. Spinal cord injury (SCI) typically leads to formation of scar tissue that can be categorized into glial and fibrotic components. Treatment with SPG-178 led to repair of injured tissue while reactive astrocytes formed a physical barrier against inflammatory cells. This barrier is commonly referred to as a glial scar. The process of reactive gliosis involves migration of reactive astrocytes and completion of the glial scar. Most studies on CNS injury have shown that the glial scar formed in part by reactive astrocytes hinders axonal regeneration. In mice lacking both GFAP and vimentin, reduced astroglial reactivity resulted in improved sprouting of axons and functional restoration after SCI [8]. However, reactive astrocytes are also important for supporting repair of the blood-brain barrier, since they prevent infiltration of $CD45^+$ leukocytes and protect neurons and oligodendrocytes, as shown by the selective ablation of dividing astrocytes using ganciclovir and GFAP-TK transgenic mice [9,10]. Furthermore, Okada et al. showed

that Stat3 signaling in reactive astrocytes has an important role in repair of injured tissue and recovery of motor function [11]. These results seem to be in conflict with one another, but consideration of the timeframes in which these events were observed suggests a possible phase-dependent role of reactive astrocytes [11,12]. Kawaja et al. found that reactive astrocytes do not necessarily form scars [13]. In the present study, collagen IV expression in the injured area, which could impede axon regeneration, was significantly reduced in mice treated with SPG-178, despite significant accumulation of reactive astrocytes. In our study, SPG-178 also seems to have that reactive astrocytes have a pivotal role in the repair of injured tissue and the recovery of motor function. The fibrotic scar tissue is thought to play multiple roles including inhibition of axon regeneration and limiting infiltration of immune cells into the spinal cord parenchyma [14-16]. In this study, histological sections of the injury site showed a reduction in the fibrotic scar. The reduction in fibrotic scar after SPG-178 is associated with increased expression of neurotrophic factors after SCI because of permissive environment. Taken together, our data indicate that SPG-178 injection after spinal cord transection provided a permissive environment for nerve regeneration.

MMP-2 is a metalloproteinase involved in neuroinflammation and remodelling of the neural ECM. After SCI, MMPs are upregulated and initially involved in disruption of the blood-spinal cord barrier[17], after which they participate in regenerative processes such as angiogenesis and axonal sprouting/regrowth [18,19]. MMP-14 is a proteolytic activator of MMP-2, and mRNAs for both these proteins were upregulated by SPG-178.

In summary, our study showed that SPG-178 has a neuroprotective effect in the injured CNS in vivo and in cultured neurons in vitro, and is a potential biomaterial for reconstruction of the injured spinal cord. The mechanisms through which SPG-178 exerts these properties remain to be verified, but these results suggest that SPG-178 could serve as an effective alternative treatment for neuronal injuries.

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Table 1



| | Forward | Reverse |
|---|---|---|
| Gap43 | GGCTCTGCTACTACCGATGC | GGCTTGTTTAGGCTCCTCCT |
| MAP2 | CTGGACATCAGCCTCACTCA | AATAGGTGCCCTGTGACCTG |
| GDNF | CTTGGGTTTGGGCTATGAAA | ACAGGAACCGCTGCAATATC |
| BDNF | TTGTTTTGTGCCGTTTACCA | GGTAAGAGAGCCAGCCACTG |
| NGF | AAGCTGCAGACACTCAGGAT | CGTATCTATCCGGATAAACC |
| NT-3 | CTCATTATCAAGTTGATCCA | CCTCCGTGGTGATGTTCTATT |
| NT-4 | CCCTGCGTCAGTACTTCTTCGAGAC | CTGGACGTCAGGCACGGCCTGTTC |
| TrkA | GTGCTCAATGAGACCAGCTTC | CTTCAGTGCCCTTGACAGCCAC |
| TrkB | CCTCCACGGATGTTGCTGAC | GCAACATCACCAGCAGGCA |
| TrkC | TGGACTGGATAGTCACTGG | TGGGTCACAGTGATAGGAG |
| MMP-2 | CAAGTTCCCCGGCGATGTC | TTCTGGTCAAGGTCACCTGTC |
| MMP-3 | GCTGCCATTTCTAATAAAGA | GCACTTCCTTTCACAAAG |
| MMP-9 | CTGGACAGCCAGACACTAAAG | CTCGCGGCAAGTCTTCAGAG |
| MMP-14 | CAGTATGGCTACCTACCTCCAG | GCCTTGCCTGTCACTTGTAAA |

Tables



| | Forward | Reverse |
|---|---|---|
| Gap43 | GGCTCTGCTACTACCGATGC | GGCTTGTTTAGGCTCCTCCT |
| MAP2 | CTGGACATCAGCCTCACTCA | AATAGGTGCCCTGTGACCTG |
| GDNF | CTTGGGTTTGGGCTATGAAA | ACAGGAACCGCTGCAATATC |
| BDNF | TTGTTTTGTGCCGTTTACCA | GGTAAGAGAGCCAGCCACTG |
| NGF | AAGCTGCAGACACTCAGGAT | CGTATCTATCCGGATAAACC |
| NT-3 | CTCATTATCAAGTTGATCCA | CCTCCGTGGTGATGTTCTATT |
| NT-4 | CCCTGCGTCAGTACTTCTTCGAGAC | CTGGACGTCAGGCACGGCCTGTTC |
| TrkA | GTGCTCAATGAGACCAGCTTC | CTTCAGTGCCCTTGACAGCCAC |
| TrkB | CCTCCACGGATGTTGCTGAC | GCAACATCACCAGCAGGCA |
| TrkC | TGGACTGGATAGTCACTGG | TGGGTCACAGTGATAGGAG |
| MMP-2 | CAAGTTCCCCGGCGATGTC | TTCTGGTCAAGGTCACCTGTC |
| MMP-3 | GCTGCCATTTCTAATAAAGA | GCACTTCCTTTCACAAAG |
| MMP-9 | CTGGACAGCCAGACACTAAAG | CTCGCGGCAAGTCTTCAGAG |
| MMP-14 | CAGTATGGCTACCTACCTCCAG | GCCTTGCCTGTCACTTGTAAA |

Figure legends

Figure 1

The effects of SPG-178 on neurite outgrowth. (A) SPG-178, laminin, and PLL all enhanced neurite growth in primary cultured spinal cord motor neurons. (B) Laminin and SPG-178 producing significant 1.7- and 1.6-fold increases, respectively, compared with the control.

Figure 2

Real-time PCR to investigate whether SPG-178 influenced primary motor neuron neurotrophic function. (A-D)In the SPG-178 group, levels of mRNAs for NGF (2 and 7 days), BDNF (2 days) and NT-4 (2 and 7 days), and for the TrkB (2 days) and TrkA (7 days) receptors, were significantly increased compared to the control group ($p<0.05$).

Figure 3

An in vivo analysis to evaluate if the SPG-178 scaffold attenuated or enhanced expression of various genes following SCI. (A) At 7 days after injection, the SPG-178 group had higher levels of mRNAs for GDNF and NGF compared to the control (saline) group. (B) There was no significant difference in receptor mRNA levels between the two groups. (C) Matrix remodeling (mRNAs for MMP-2 and -14) were significantly upregulated in the SPG-178 group compared to the control group ($p<0.05$).

Figure 4

(A, B) There was less accumulation of these cells in control mice, based on the GFAP-positive area in a region of 600 μm width around the lesion core being significantly smaller in control mice from 14 to 56 days after SCI. These astrocytes eventually migrated centripetally to the lesion epicenter and gradually compacted the $Iba1^+$ inflammatory cells, contracting the lesion area from 14 to 28 days after SCI.

Figure 5

(A, B) $Iba1^+$ cell accumulation in the lesion was significantly reduced in the SPG-178 group from 14 to 28 days after SCI, but there was no difference between SPG-178 and control mice at 56 days after injury.

Figure 6

(A, B) Clear expression of collagen IV, a marker for glial scarring that appears in the

later stages of scarring, was present in the injured area at 14 days after injury and the scar area was significantly reduced in SPG-178 mice.

Table 1

Primer sequences used in quantitative RT-PCR

Figure 1

Click here to download Figures Fig1.TIF

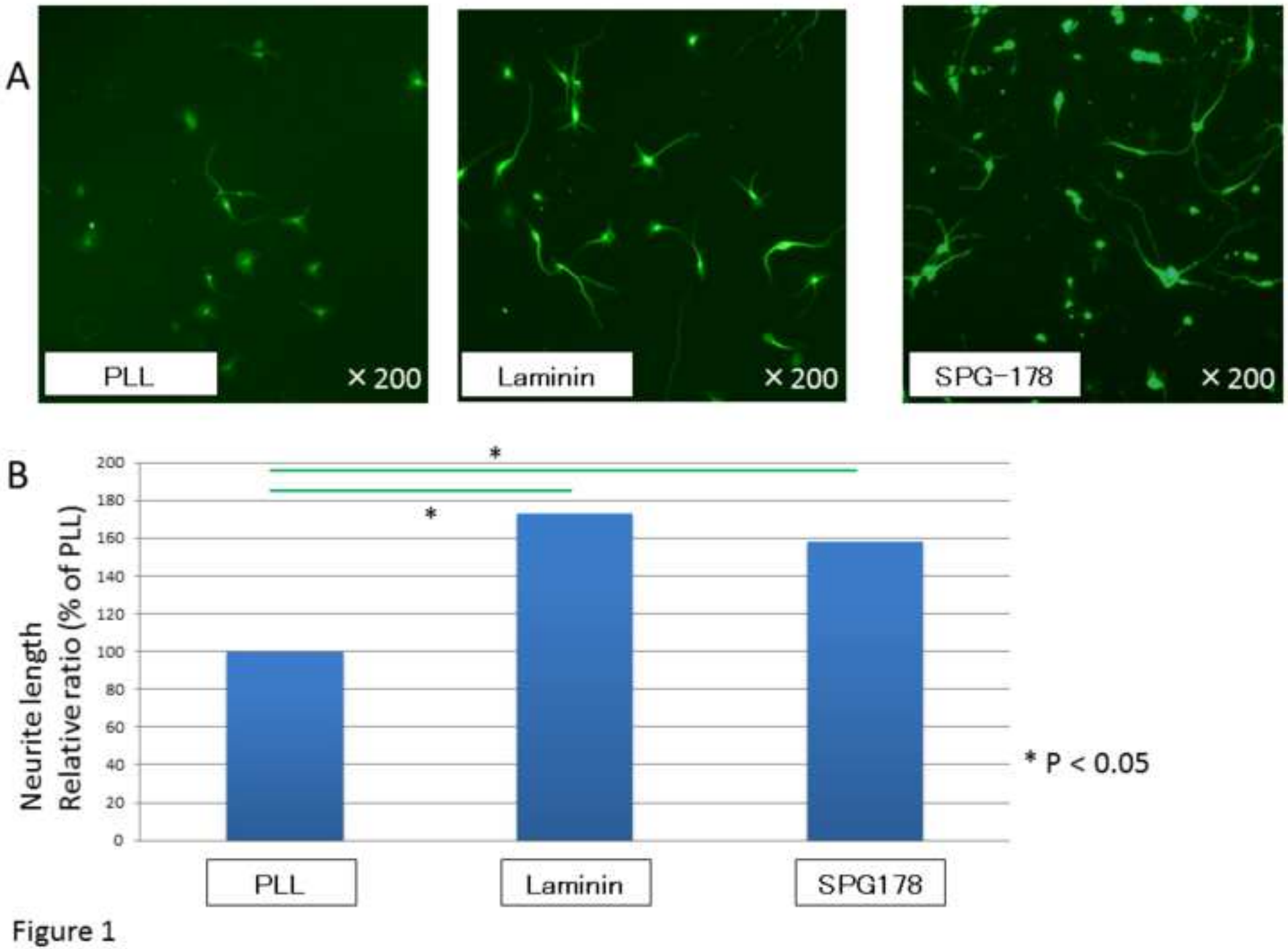


Figure 1

Figure 2

Click here to download Figures Fig2.TIF

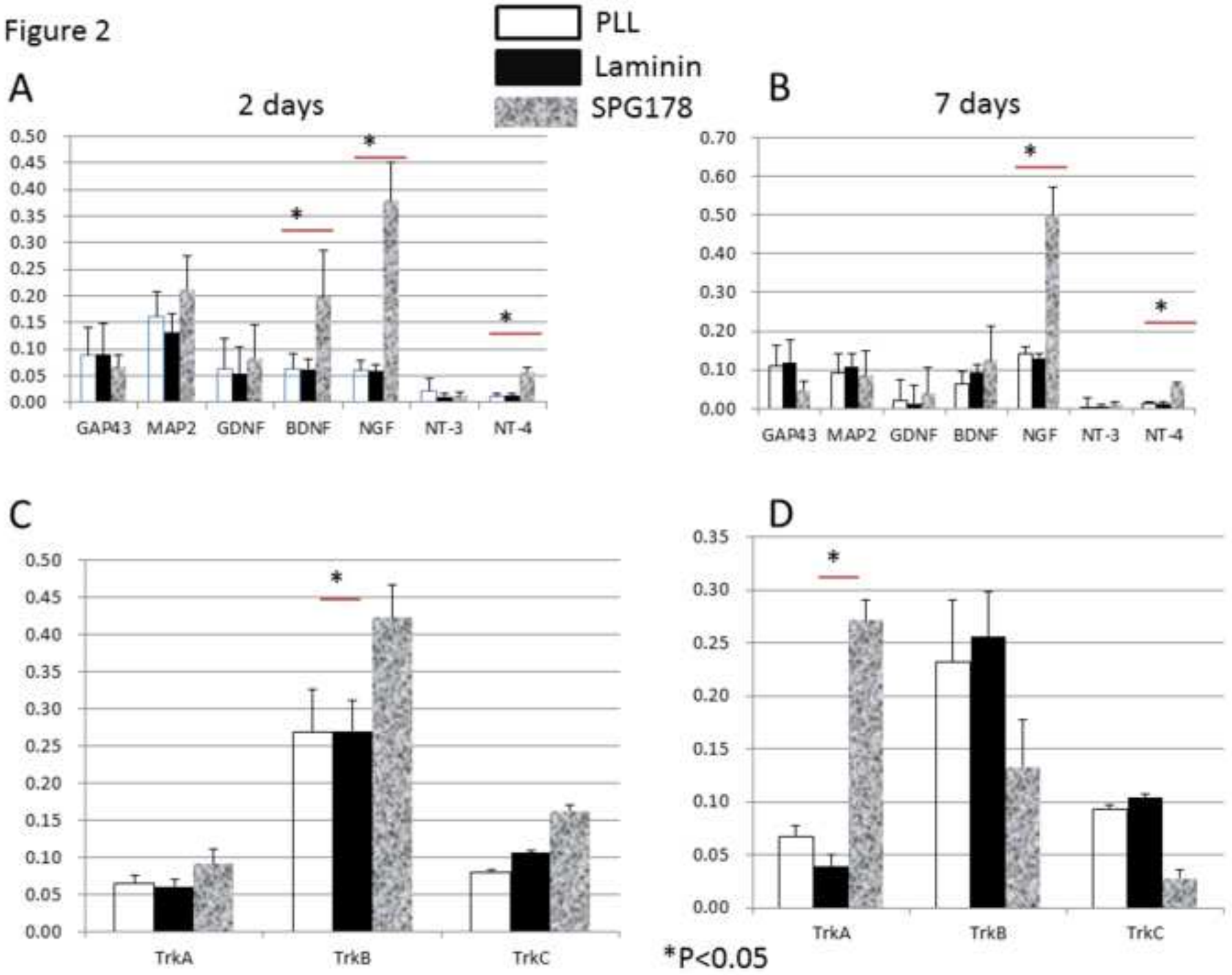

Figure 3

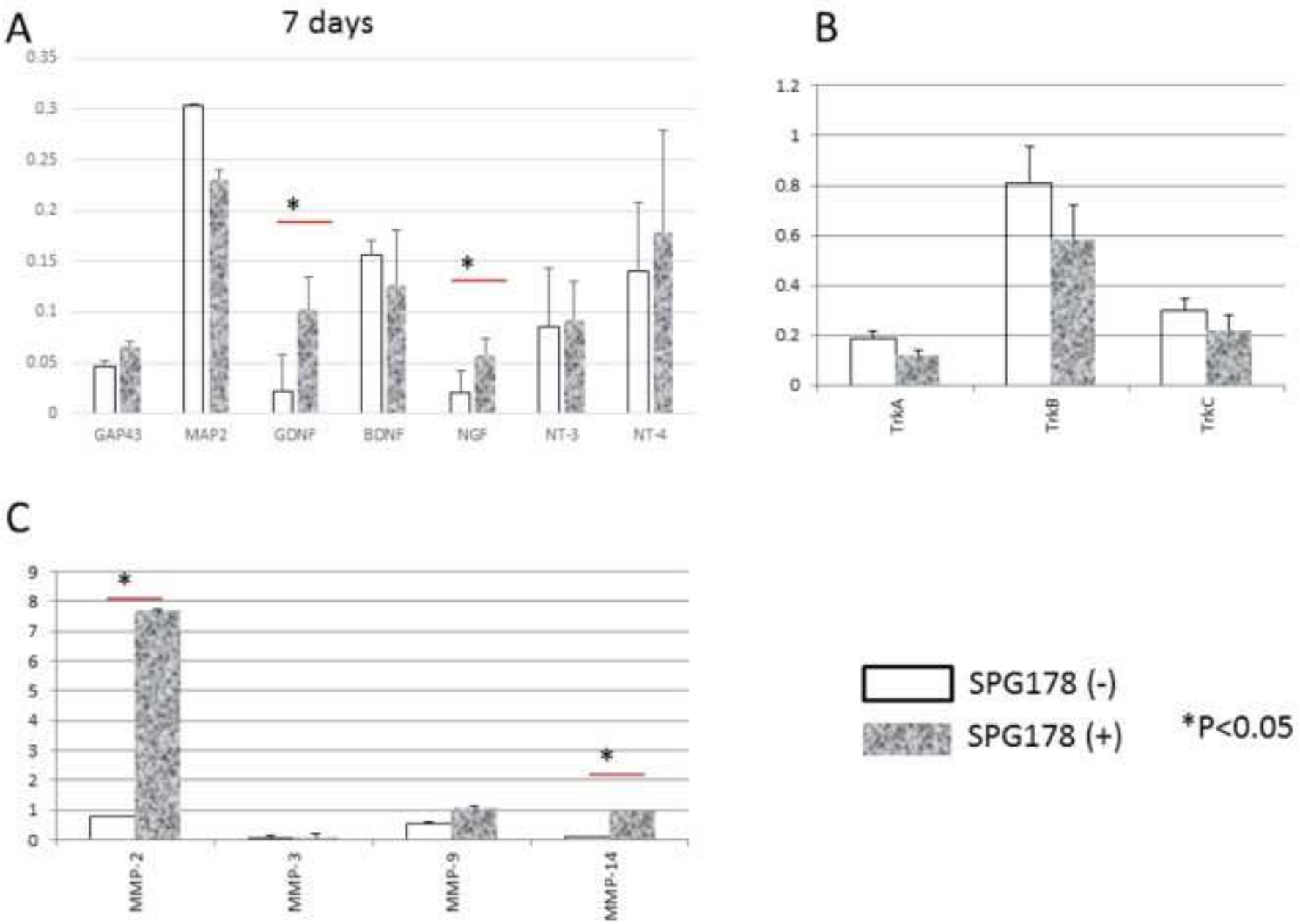

Figure 4



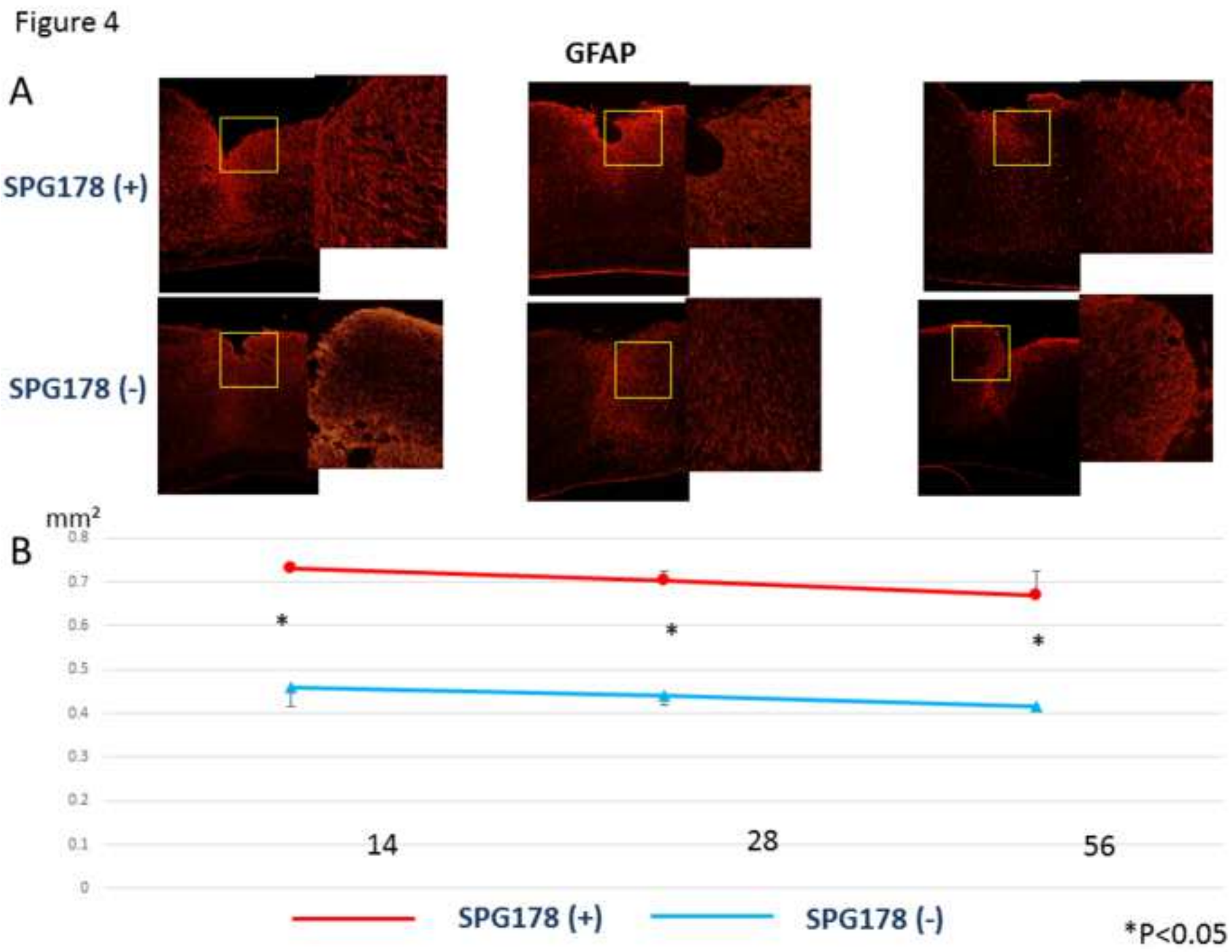

Figure 5



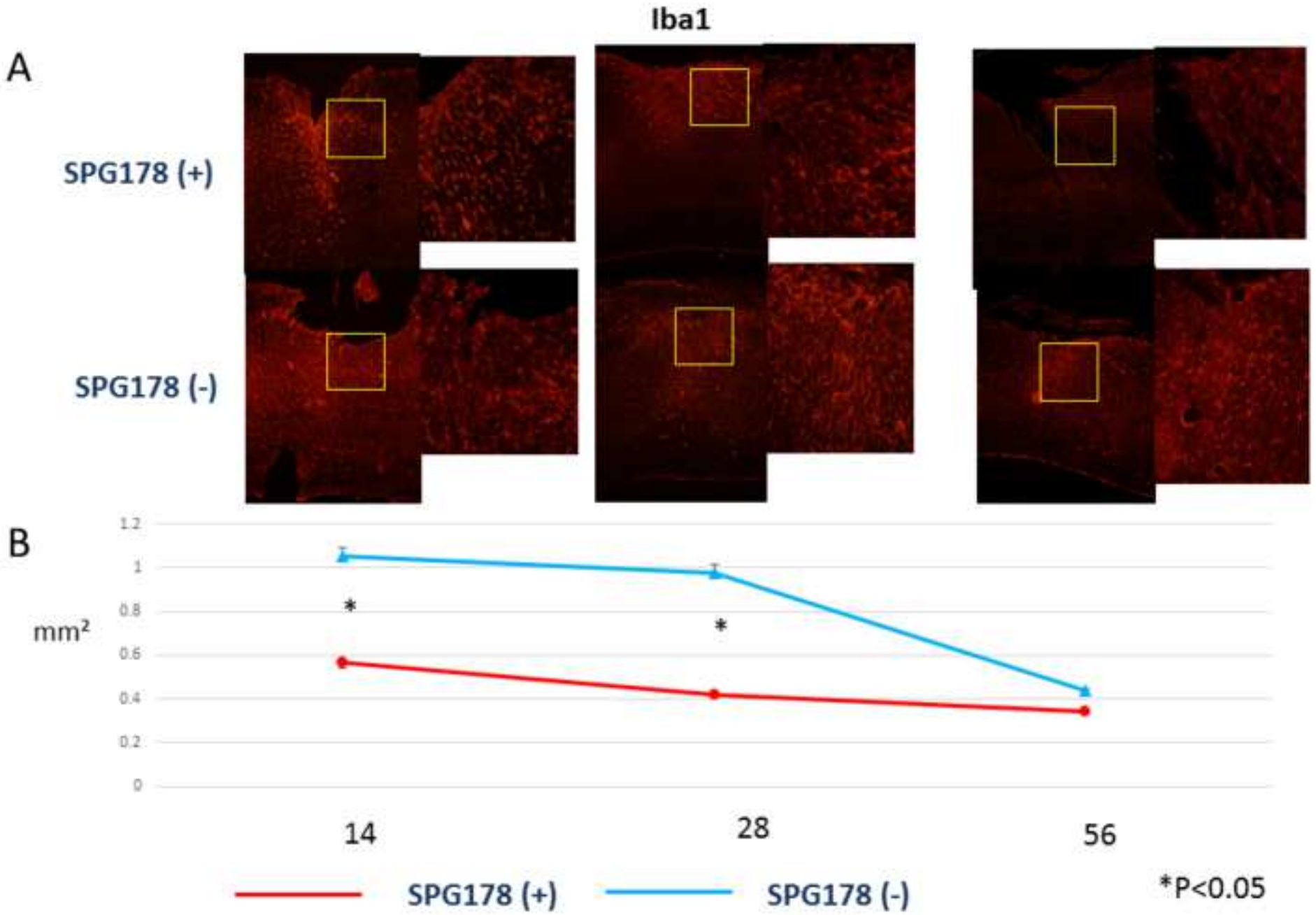

Figure 6