

Identification of BLyS, a non-myelin associated protein, as a functional ligand for Nogo-66 receptor

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Supplemental Materials

Supplemental materials include materials and methods, eight figures and legends.

Materials and Methods

RNA expression in chicken neurons

Chicken DRGs or RGCs at stage E7 and E12 were isolated. RNA was extracted using Trizol reagent (Invitrogen, Beijing, China). Expression of *NgR*, *NgRH1*, *NgRH2*, *BCMA* and *TACI* were detected using RT-PCR with primers shown in Table 1.

NF- kappa B luciferase assay

COS-7 cells were transfected with 30 ng *Renilla-luciferase*, 30 ng *ELAM-luciferase* reporter plasmids (kindly provided by Dr Douglas Golenbock, University of Massachusetts Medical School), 400 ng *p75/Lingo1* plasmid (*p75* and *Lingo1* genes are driven by two separate promoters), and 200 ng receptor genes (*TACI*, *NgR* or *GFP* control) in 24 well plates (Corning, Corning, NY). 4 hours after transfection, 1 µg/ml FLAG-AP-Nogo66 or BLyS (Peprotech, England, UK) was added to the culture medium in each well. After additional 20 hours, cells were lysed and luciferase activities were measured using a Dual-Luciferase Reporter (DLR) assay kit (Promega,

Madison, WI) and a TD 20/20 luminometer (Turner Design, Sunnyvale, CA).

Rat spinal cord injury model

We modified a spinal cord injury model as previously described (Scheff et al., 2003).

Adult male Wistar rats were anesthetized and suspended by attaching Adson forceps to the rostral T11 and caudal L1 vertebral bodies. A laminectomy was performed at T12 to expose the dorsal portion of the spinal cord. A spinal cord contusion was created using a blunt-force impactor, which caused severe and reproducible lesions of the injured spinal cord parenchyma. Overlying muscle layers were sutured and the skin was closed. The injured spinal cords were harvested on 7th day after injury. Spinal cords undergoing similar operations except for injury were used as negative controls.

Immunohistology

For SCI experiments, Wistar rats were anesthetized and perfused transcardially with heparinized saline (20 mU/ml) followed by a fixative solution containing 2% PFA and 3.75% acrolein in 0.1M PBS for 15 minutes. The spinal cords from rats were dissected free and post-fixed in the same fixative solution containing 15% sucrose overnight at 4 °C. They were then transferred to a 30% sucrose solution in PBS for 24 hours. The samples were frozen on dry ice and then transferred into embedding medium (Tissue-Tek, Miles Inc. Elkhart, IN) for histology section. 15 µm spinal cord transverse cross-sections were washed in PBS and subsequently permeabilized in PBS containing 0.3% Triton X-100, blocked with 4% normal goat serum for 30 minutes. The sections were incubated with indicated primary antibodies overnight at

4 °C, including anti-BLyS, (Santa Cruz Biotechnology, Santa Cruz, CA), anti-NgR (a gift from Dr. Peihua Lu, Shanghai Jiaotong University) (Yu et al. 2006), anti-Myc polyclonal (MBL, Naka-ku Nagoya, Japan), anti-glial fibrillary acidic protein (GFAP; an astrocyte marker) (Millipore, Beijing, China) and anti-CD11b (OX42; a macrophage/microglial cell marker) (AbD Serotec, UK), respectively.

To control the specificity of anti-NgR antibody (Yu et al. 2006), 6-8 week old wild-type and NgR knock-out C57BL/6 mice were used (supplemental Fig.8). The mouse brain coronal sections were similarly prepared as described for rat tissues described above. The sections were then incubated with indicated primary antibodies overnight at 4 °C, including anti-NgR (1:200 dilution) (Yu et al. 2006), anti-Myc polyclonal (1:200 dilution), and anti-Tuj 1 (a neuron specific marker) (Millipore, Beijing, China). After PBS washes, Alexa Flour 488-conjugated goat anti-rabbit or Alexa Flour 594-conjugated goat anti-mouse secondary antibodies (Invitrogen, Eugene, OR) were applied for 2 hours in the dark at room temperature. After PBS wash, the slides were mounted in mounting medium supplemented with 1 µg/ml DAPI (Vector Laboratories, Inc. Burlingame, CA). Images were collected using a Zeiss-Axioplan 2 epifluorescent microscope (Carl Zeiss, Germany).

Reference:

- Scheff SW, Rabchevsky AG, Fugaccia I, Main JA, Lump PP, Jr. (2003) Experimental modeling of spinal cord injury: Characterization of a force-defined injury device. *Journal of neurotrauma* 20:179-193.
- Yu PP, Zhu HQ, Lu PH, Huang LD. (2006) Preparation and identification of rabbit antibody against LRR of Nogo-66 receptor (NgR). *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi*.22:633-5. in Chinese.

Table 1. Primers used in RT-PCR

Name	primer sequence (5'-3')	product size (bp)
<i>Actin</i>	U: GTTCGGCTTCTGGCGTGTGA	293
	D: ACAATACCGTGTTCAATGGGGTAC	
<i>NgR</i>	U : CTCTCAGCTCCCATGATGGC	295
	D: GTGGAAGGTTCAATAGGCTCTA	
<i>NgRH1</i>	U: CTCACCATCCTCTACCTGTTC	296
	D: TCGCCGTCATCCTCATCTTTG	
<i>NgRH2</i>	U: AAAGCCCGTTCGCTCTGGAAT	302
	D: CTGGTGCAGTTCTTGGCAGG	
<i>BCMA</i>	U: CCAGCAGTCTCCTAATCTTCG	314
	D: CACGTCACCCTTGGCATCAC	
<i>TACI</i>	U: CAGTCTGTAACAGGAGAGCTC	292
	D: CTTCCGCAAGTGTGTCCATC	

Supplemental figure legends

Supplemental figure 1. Nogo Receptors and BLYS receptors are selectively expressed in early chicken neurons. Total RNAs of chicken RGC or DRG at different developmental stages are isolated. RNA expressions of *NgR*, *NgRH1*, *NgRH2*, *BCMA* and *TACI* are detected by RT-PCR.

Supplemental figure 2. BLYS inhibits rat cortical neuron growth. E17 rat cortical neurons are dissociated and cultured on the substrates containing PBS or recombinant Nogo-66 or BLYS. a. Cortical neurons are fixed and stained with monoclonal antibody Tuj 1 against class III β -tubulin. Scale bar, 100 μ m. b. Quantification of E17 rat cortical neuron neurite length inhibition shown in c with Student's Two tailed t test (** $p < 0.0001$). The number of neurites that are counted (means $\pm$ SEM) for each treatment is indicated in parentheses.

Supplemental figure 3. NgR-Fc can block the interaction between AP-BLYS and NgR. The same amount of AP, AP-Nogo-66, or AP-BLYS proteins are incubated with COS-7 expressed NgR. After extensive washes, incubated COS-7 cells are lysed and quantified by measuring AP activity. Two tailed t test * $p < 0.04$. Error bars represent SEM. AP, alkaline phosphatase; AP-Nogo-66, alkaline phosphatase and Nogo-66 fusion protein; AP-BLYS, alkaline phosphatase and extracellular BLYS fusion protein; FLAG-BLYS, FLAG peptide tagged BLYS protein; NgR-Fc, extracellular NgR and Fc

fusion protein.

Supplemental figure 4. Inhibition of neurite outgrowth by BLyS is dependent on RhoA activation. **a.** E12 chicken embryo DRGs are cultured on the inhibitory substrates. 15 μ M Y27632 or PBS is added to the culture medium. DRGs are fixed and stained using monoclonal antibody Tuj 1 against class III β -tubulin. Scale bar: 100 μ m. **b.** Quantification of DRG neurite length on inhibitory substrates with the addition of Y27632 or PBS as indicated in **a**. Neurite length analysis is carried out double-blindly. The number of neurites that were counted (means $\pm$ SEM) for each treatment is indicated in parentheses.

Supplemental figure 5. BLyS cannot activate NF kappa B pathway through NgR complex in COS-7 cells. COS-7 cells are co-transfected with an *Elam-luciferase* reporter plasmid and various receptor plasmids. Addition of BLyS or Nogo-66 does not activate NF kappa B pathway through NgR. Error bars represent SEM.

Supplemental figure 6. BLyS expression is associated with astrocytes and macrophages/microglial cells at spinal cord injury sites. Frozen rat spinal cord sections are stained using an indirect immunohistological method. **a-d**, BLyS co-localizes with astrocytes at spinal cord injury sites. **a**, anti-BLyS staining (green). **b**, anti-GFAP staining labels astrocytes (red). **c**, DAPI (blue). **d**, merged image of a-c.

a'-d', BLyS expression is expressed at a low level in normal spinal cords. **a'**, anti-BLyS staining (green). **b'**, anti-GFAP staining (red) **c'**, DAPI (blue). **d'**, merged image of **a'-c'**. BLyS co-localizes with macrophages/microglial cells at spinal cord injury sites (**e-f**) but not in normal spinal cord (**e'-f'**). **e** and **e'**, anti- BLyS staining (green). **f** and **f'**, anti-cd11b (OX42) staining (red). **g** and **g'**, DAPI (blue). **h** and (**h'**) are merged images of **e-g** and **e'-g'**, respectively. Scale bar, 10µm.

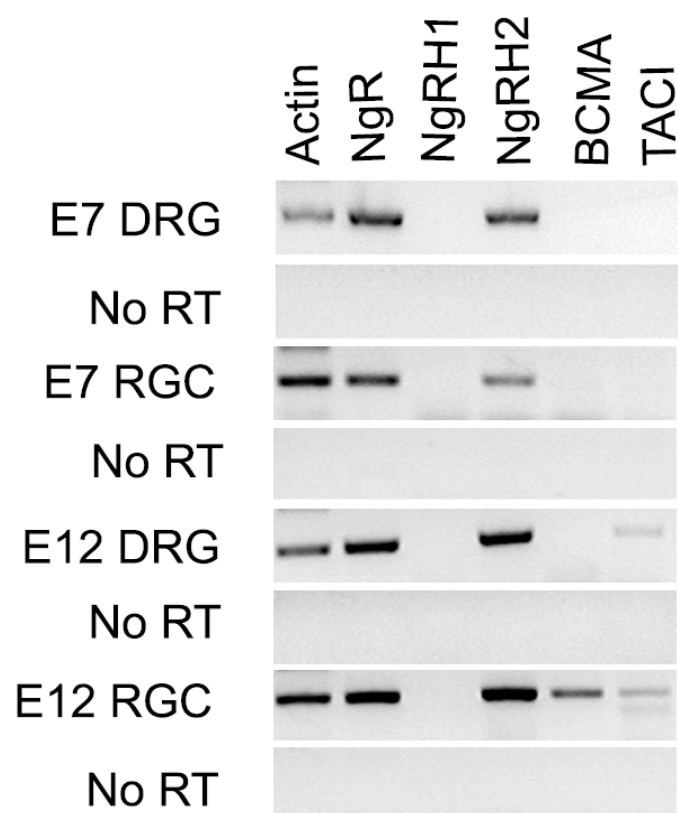
Supplemental figure 7. NgR expression is associated with astrocytes and macrophages/microglial cells at spinal cord injury sites. Frozen rat spinal cord sections are stained using an indirect immunohistological method. **a-d**. NgR co-localizes with astrocytes at spinal cord injury sites. NgR expression was detected on 7th day after spinal cord injury. **a**, anti-NgR staining (green). **b**, anti-GFAP staining shows astrocytes (red). **c**, DAPI (blue). **d**, merged image of **a-c**. **a'-d'**, NgR expression is associated with astrocytes while its expression is lower in normal spinal cords. **a'**, anti-NgR staining (green), **b'**, anti-GFAP staining (red), **c'**. DAPI (blue). **d'**, merged image of **a-c**. **e-h** and **e'-h'**, SCI (**e-h**) and normal (**e'-h'**) spinal cord sections are stained with a rabbit anti-Myc antibody as a negative control for the anti-NgR antibody. **e** and **e'**, anti-Myc staining (green). **f** and **f'**, anti-GFAP staining (red). **g** and **g'**, DAPI (blue). **h** and **h'** are merged images of **e-g** and **e'-g'**, respectively. **i-l**, NgR co-localizes with macrophages/microglia. **i**, anti-NgR staining (green). **j**, anti-cd11b (OX42) staining (red). **k**, DAPI (blue). **l**, a merged image of **i-k**. **i'-l'**. NgR expression is associated with macrophages/microglia and its expression is

at a lower level in normal spinal cords. **i'**. anti-NgR staining (green). **j'**. Anti-cd11b (OX42) (red). **k'**. DAPI (blue). **l'**. merged image of **i'**-**k'**. Scale bar, 20µm.

Supplemental figure 8. NgR expression in wild type and NgR deficient mice.

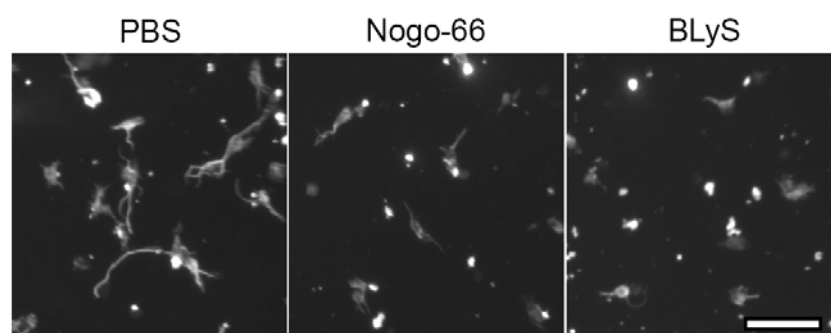
Coronal brain sections from wild- type (NgR WT) and NgR knock-out (NgR KO) C57BL/6 mice were stained using an indirect immunohistological method. **a-d**, NgR expression in the lateral precentral area and sensorimotor cortex I of wild- type mouse brain tissue (NgR WT). **a**, anti-Tuj 1 (red). **b**, anti-NgR (green). **c**, DAPI (blue). **d**, a merged image of **a-c**; **e-h**, Lack of NgR expression in the lateral precentral area and sensorimotor cortex I of knock-out mouse brain tissue (NgR KO). **e**, anti-Tuj 1 (red). **f**, anti-NgR (green). **g**, DAPI (blue). **h**, a merged image of **e-h**; **i-l**, wild- type mice (NgR WT) brain sections were stained with the anti-Myc antibody as a negative control. **i**, anti-Tuj 1 (red). **j**, anti-Myc (green). **k**, DAPI (blue). **l**, a merged image of **i-l**. Scale bar, 40µm

Supplemental figure 1

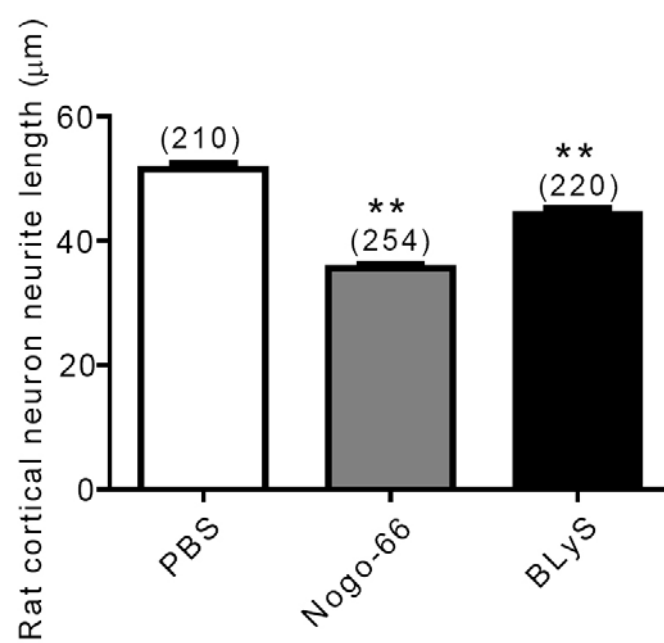


Supplemental figure 2

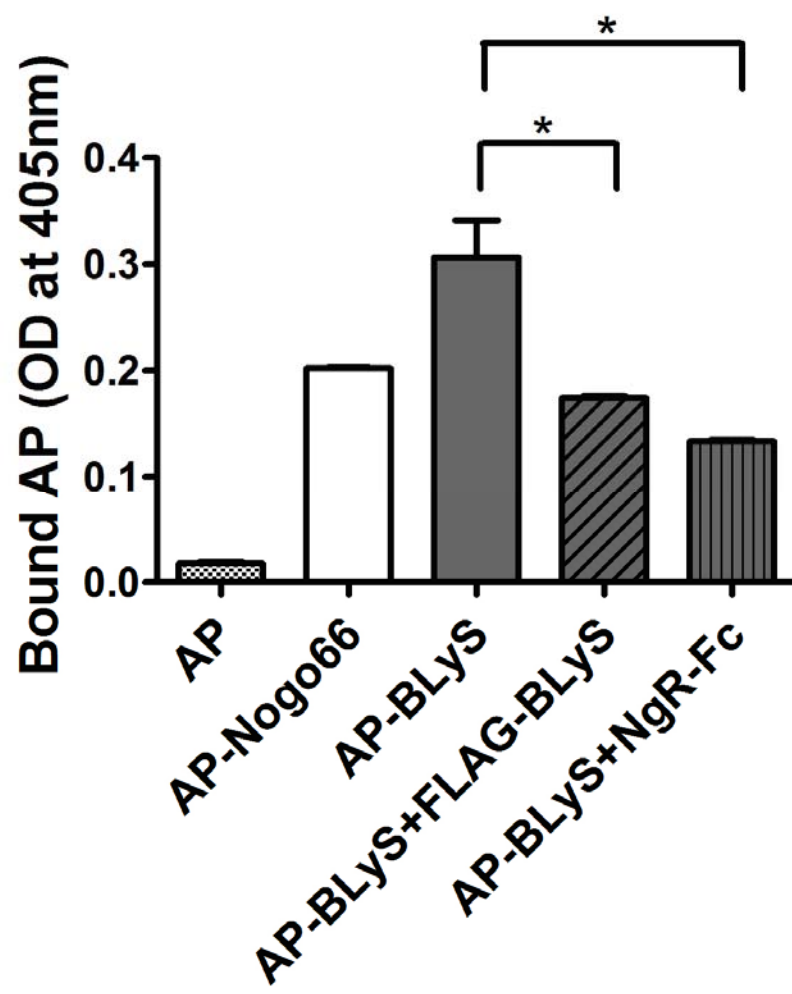
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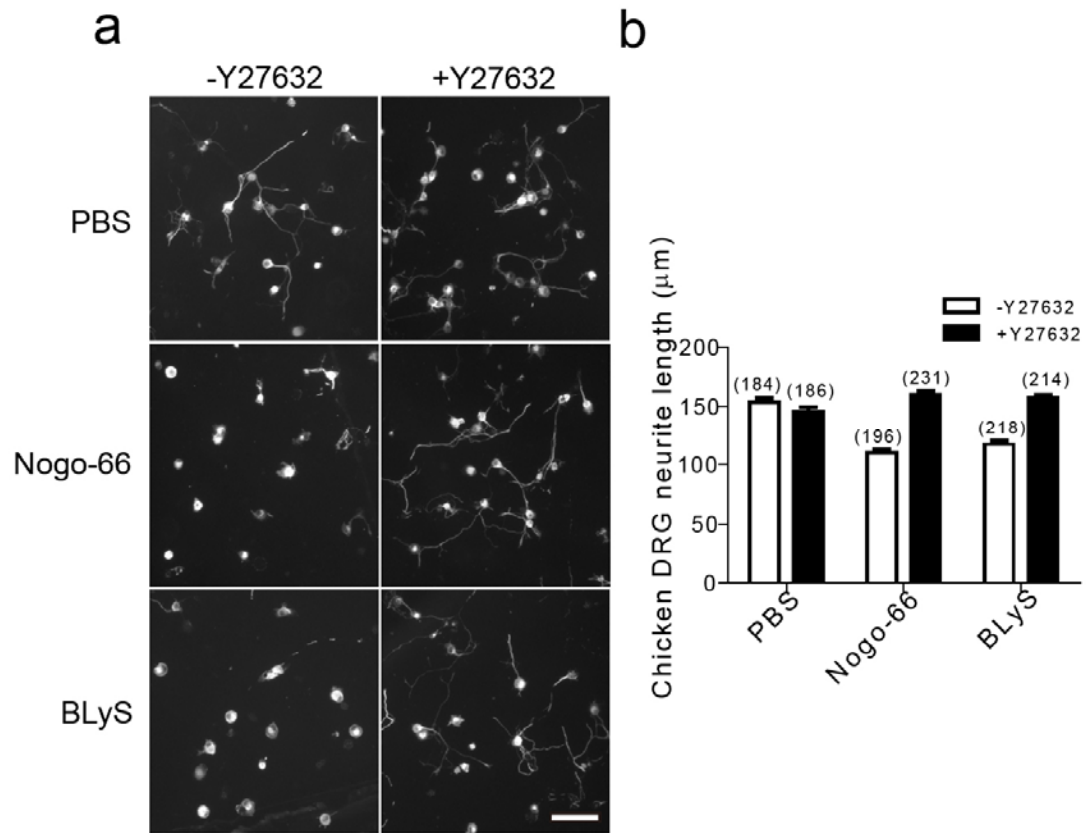
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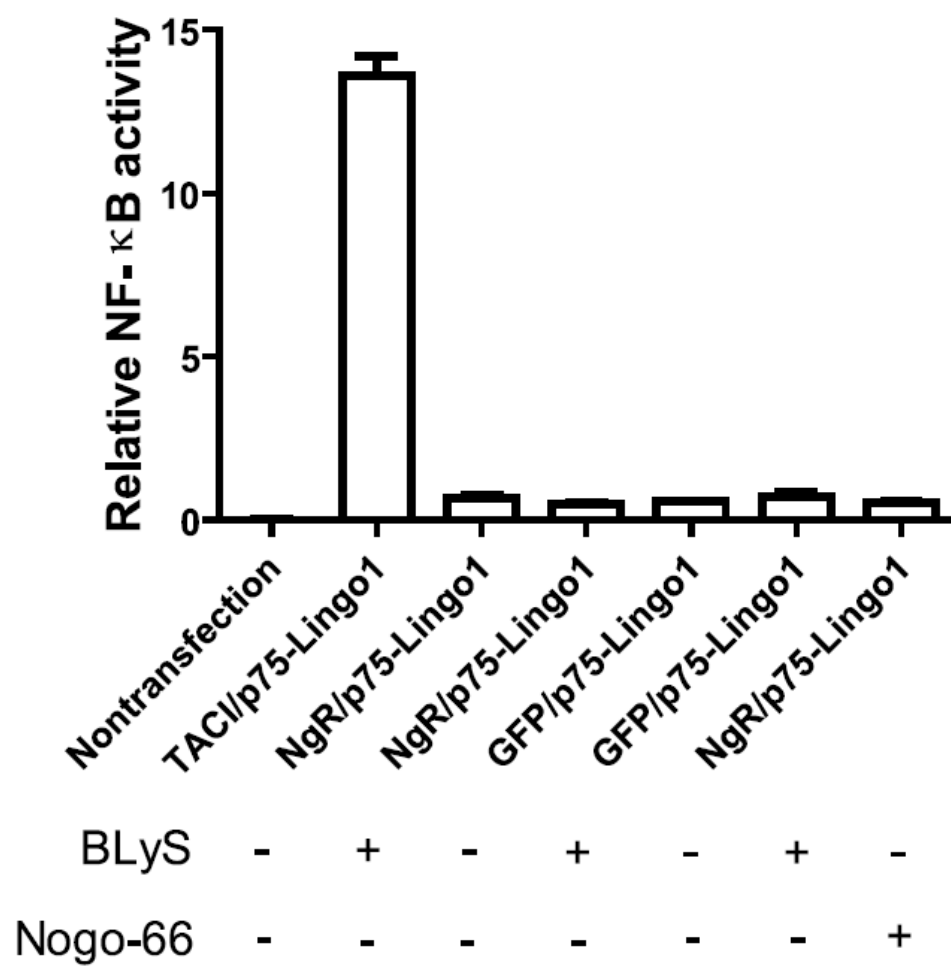
Supplemental figure 3



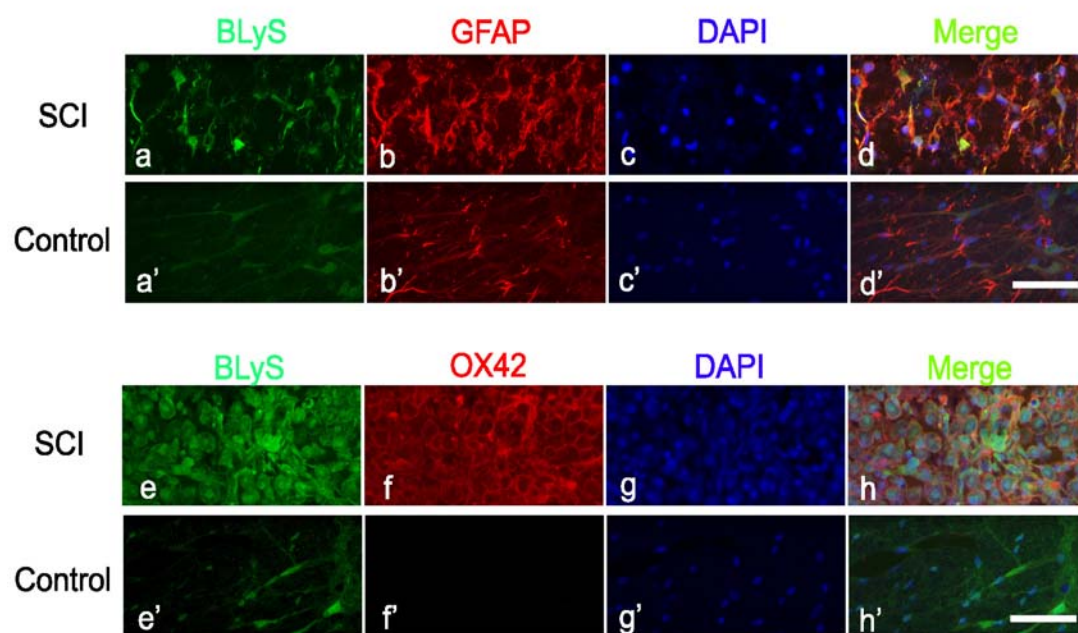
Supplemental figure 4



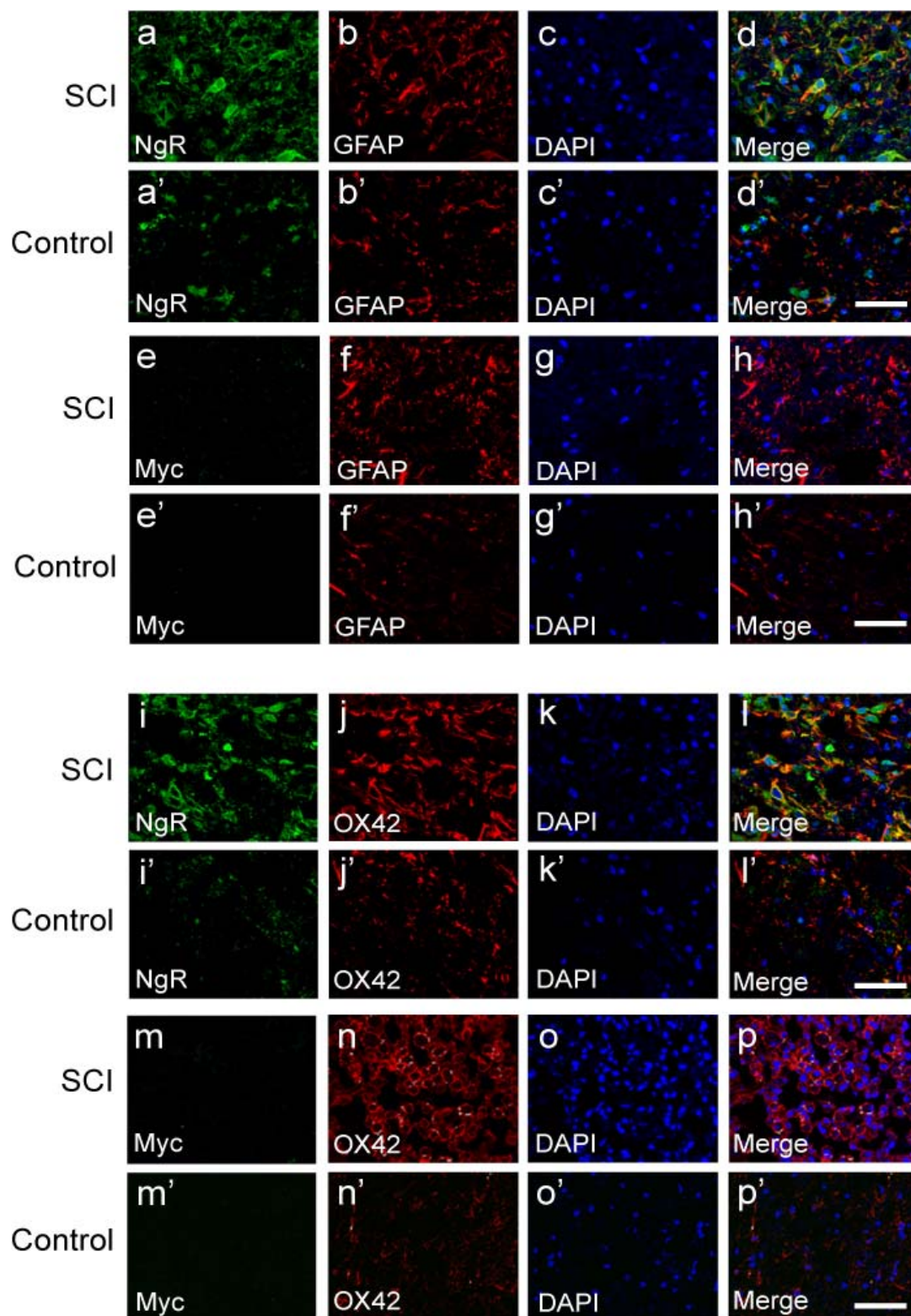
Supplemental figure 5



Supplemental figure 6



Supplemental figure 7



Supplemental figure 8

