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Title page:

**NEUTRALIZATION OF TUMOR NECROSIS FACTOR RELATED APOPTOSIS
INDUCING LIGAND (TRAIL) REDUCES SPINAL CORD INJURY DAMAGE IN MICE**

Running title: Role of TRAIL in spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) is a major cause of disability, its clinical outcome depending mostly upon the extent of damage where proapoptotic cytokines play a crucial role. In particular, the inducers of apoptosis belonging to TNF receptor superfamily and their respective ligands are upregulated after SCI. In this study, the role of the proapoptotic cytokine TRAIL in SCI-induced damage was investigated in the mouse. SCI resulted in severe trauma, characterized by prominent inflammation-related damage and apoptosis. Immunostaining for TRAIL and its receptor DR5, was found in the white and grey matter of the peri-lesional area, as also confirmed by Western blotting experiments. Immunoneutralization of TRAIL resulted in improved functional recovery, reduced apoptotic cell number, modulation of molecules involved in the inflammatory response (FasL, TNF- α , IL-1 β and MPO) and the corresponding signaling (caspase-8 and -3 activation, JNK phosphorylation, Bax and Bcl-2 expression). Since Glucocorticoid-Induced TNF receptor superfamily-related protein (GITR) activated by its ligand (GITRL) contributes to SCI-related inflammation, interactions between TRAIL and GITRL were investigated. SCI was associated with up-regulated GITR and GITRL expression, a phenomenon prevented by anti-TRAIL treatment. Moreover, the expression of both TRAIL and DR5 was reduced in tissues from mice lacking the GITR gene (GITR^{-/-}) in comparison with wild type (WT) mice suggesting that TRAIL and GITRL activated pathways synergise in the development of SCI-related inflammatory damage. Characterization of new targets within such molecular systems may constitute a platform for innovative treatment of SCI.

Keywords: neuronal death; cytokines; GITR/GITRL; neuroinflammation; immunoneutralization; pharmacological treatment

INTRODUCTION

Spinal cord injury (SCI) is a major cause of disability. The functional decline following SCI is contributed by both mechanical injury and mechanism factors set into motion by trauma (Profyris *et al*, 2004). The mechanical forces imparted to the spinal cord cause tissue disruption, with axonal injury, inducing death of neurons that are very unlikely to be regenerated (De Vivo *et al*, 1987; Tator, 1995). Moreover, neuronal death continues for hours after SCI, due to multiple mechanisms including excitotoxicity, vascular abnormalities and inflammation (Liu *et al*, 1999; Tator and Koyanagi, 1997; Hausmann, 2003). The clinical outcome of SCI depends, in part, on the extent of secondary damage, which evolves with contribution of apoptosis (Lu *et al*, 2000).

Cytokines, with special regard to the TNF superfamily, have been suggested to be responsible for increased apoptotic rate in the central nervous system (CNS), involving both neurons (Robertson *et al*, 2001) and glia (Sato JI and Kuroda Y, 2001). In this line, Plunkett *et al* (2001) described upregulation of CD95L and TNF-Related Apoptosis-Inducing Ligand (TRAIL) mRNA following excitotoxic SCI in the rat.

TRAIL is a member of the TNF superfamily which binds to five receptors (Wiley *et al*, 1995; Pitti *et al*, 1996). Among these, DR4 and DR5 are type I membrane proteins (Pan *et al*, 1997a; Pan *et al*, 1997b; Sheridan *et al*, 1997) that contain a cytoplasmic death domain and transduce a death signal (Walczak *et al*, 1997). Both DR4 and DR5 are present in the CNS of mammals (Dörr *et al*, 2002) and mediate the detrimental effects of TRAIL in brain ischemia (Martin-Villalba *et al*, 1999) and in β -amyloid-dependent neurotoxicity (Cantarella *et al*, 2003). Although it has been demonstrated that after SCI, expression of TNF, CD95 and CD95L is increased at the lesion site (Zurita *et al*, 2001; Xu *et al*, 1998; Li *et al*, 2000a), the *in vivo* role of the receptors belonging to TNFR superfamily and the respective ligands in SCI is still controversial. Neutralization of TNF (Lee *et al*, 2000) and CD95L (Demjen *et al*, 2004) reduces the number of apoptotic cells after SCI. Demjen *et al* (2004), showed that neutralization of CD95L, but not of TNF, promotes functional recovery after SCI. Moreover, recent evidence demonstrated a relevant

role of GITR in the regulation of the inflammatory response in SCI (Nocentini *et al*, 2008). GITR is a protein, originally cloned in a glucocorticoid-treated hybridoma T cell line (Nocentini *et al*, 2007). It is expressed in several cells and tissues, including T cells, where it acts as a co-stimulatory molecule (Ronchetti *et al*, 2007; Ronchetti *et al*, 2004; Tone *et al*, 2003) following activation by its ligand (GITRL), mainly expressed on antigen presenting cells and endothelial cells (Krausz *et al*, 2007).

Here we show for the first time the involvement of TRAIL in the pathophysiology of SCI and that neutralization of TRAIL reduces apoptotic cell death and improves the functional and histopathological outcome after SCI. Besides we show the role of GITR in the regulation of TRAIL pro-inflammatory property.

MATERIALS AND METHODS

Animals

Male CD1 adult mice (25-30g, Harlan Nossan) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Spinal Cord Injury

Mice were anesthetized using chloral hydrate (400 mg/kg body weight). We used the clip compression model described by Rivlin and Tator (Rivlin and Tator, 1978) and produced SCI by extradural compression of a section of the SCI exposed via a four-level T5-T8 laminectomy, in which the prominent spinous process of T5 was used as a surgical guide. A six-level laminectomy was chosen to expedite timely harvest and to obtain enough SCI tissue for biochemical examination. With the aneurysm clip applicator oriented in the bilateral direction, an aneurysm clip with a closing force of 24 g was applied extradurally at T5-T8 level. The clip was then rapidly released with the clip applicator, which caused SC compression. In the injured groups, the cord was compressed for 1 min. Following surgery, 1.0 cc of saline was administered subcutaneously in order to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27°C for a survival period of 20 days. Food and water were provided to the mice *ad libitum*. During this time period, the animals' bladders were manually voided twice a day until the mice were able to regain normal bladder function. Sham-injured animals were only subjected to laminectomy.

Experimental Design

Mice were randomized into 4 groups (N= 40 animals/group, randomized 20 (10 to histology and 10 to western and myeloperoxidase assay) at 24 h and 20 for motor score). Sham animals were subjected to the surgical procedure except that the aneurysm clip was not applied and they were

treated intraperitoneally (i.p.) with vehicle (saline) or ANTI-TRAIL (Alexis Biochemicals) (50 µg/kg) 30 min before surgical procedure. The remaining mice were subjected to SCI (as described above) and treated with an i.p. bolus of vehicle or ANTI-TRAIL (50 µg/kg) 30 min before SCI.

Experimental groups study GTR^{-/-} mice

Mice were randomly allocated into the following groups: 1) SCI-GTR^{+/+} group: mice were subjected to SCI; 2) SCI-GTR^{-/-} group: mice were subjected to SCI; 3) control GTR^{+/+} group (sham): GTR^{+/+} mice were subjected to the surgical procedures as the above except that the aneurysm clip was not applied and 4) control GTR^{-/-} group (sham): GTR^{-/-} mice were subjected to the surgical procedures as the above groups except that the aneurysm clip was not applied.

Mice from each group were sacrificed 24 h following SCI in order to collect samples for the evaluation of the parameters as described below.

Grading of motor disturbance

The motor function of mice subjected to compression trauma was assessed in a blinded fashion once a day for 10 days after injury. Recovery from motor disturbance was graded using the modified murine Basso, Beattie, and Bresnahan (BBB) (Basso et al, 1995) hind limb locomotor rating scale (Joshi and Fehlings, 2002a; Joshi and Fehlings, 2002b). The following criteria were considered: 0=No hind limb movement; 1=Slight (<50% range of motion) movement of 1-2 joints; 2=Extensive (>50% range of motion) movement of 1 joint and slight movement of one other joint; 3=Extensive movement of 2 joints; 4=Slight movement in all 3 joints; 5=Slight movement of 2 joints and extensive movement of 1 joint; 6=Extensive movement of 2 joints and slight movement of 1 joint; 7=Extensive movement of all 3 joints; 8=Sweeping without weight support or plantar placement and no weight support; 9=Plantar placement with weight support in stance only or dorsal stepping with weight support; 10=Occasional (0-50% of the time) weight-supported plantar steps and no coordination (Front/hind limb coordination); 11=Frequent (50-94% of the time) to consistent (95-100% of the time) weight-supported plantar steps and no coordination; 12=Frequent to consistent weight-supported plantar steps and occasional coordination; 13=Frequent to consistent

weight-supported plantar steps and frequent coordination; 14=Consistent weight-supported plantar steps, consistent coordination and predominant paw position is rotated during locomotion (lift off and contact) or frequent plantar stepping, consistent coordination and occasional dorsal stepping; 15=Consistent plantar stepping and coordination, no/occasional toe clearance, paw position is parallel at initial contact; 16=Consistent plantar stepping and coordination (Front/hind limb coordination) and frequent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 17=Consistent plantar stepping and coordination and frequent toe clearance and predominant paw position is parallel at initial contact and lift off; 18=Consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 19=Consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and lift off; 20=Consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk instability; 21=Consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk stability.

Immunohistochemistry

Twenty-four hours after SCI, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 µm sections were prepared from paraffin embedded tissues. After deparaffinisation, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked with biotin and avidin (DBA), respectively. Sections were incubated overnight with anti-Fas-L, anti-Bax, anti-Bcl-2, anti-GITR, anti-GITRL, anti-DR5, or anti-TRAIL antibodies (1:100). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). To verify binding specificity some

sections were incubated with only the secondary antibody. Immunohistochemical photographs (n=5 slices from each sample collected from each mouse in each experimental group; N per group= 10) were assessed by densitometry as previously described⁴⁵ by using Optilab Graftek software.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (Apotag, HRP kit DBA, Milano, Italy). Briefly, sections were incubated with 15 µg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H₂O₂ for 5 min at room temperature and then washed with PBS. Sections were dipped into terminal deoxynucleotidyltransferase (TdT) buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL positive cells/high-power field was counted in 5 to 10 fields for each coded slide.

Light microscopy

Spinal cord tissues were taken from all the mice in each experimental groups at 24 h following trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5-µm-thick sections. Tissue sections were deparaffinized with xylene, stained with Haematoxylin/Eosin (H&E) and studied using light microscopy (Dialux 22 Leitz). The segments of each spinal cord were evaluated by an experienced histopathologist. Damaged neurons were counted and the histopathologic changes of the gray matter were scored on a 6-point scale: 0, no lesion observed, 1, gray matter contained 1 to 5 eosinophilic neurons; 2, gray matter contained 5 to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one-third of the gray matter area); 5, moderate infarction; (one-third to one-half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion.

Protein extraction

Spinal cord tissue were homogenized with a Polytron homogenizer in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na₃VO₄, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulfonyl-fluoride, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 10% glycerol and 0.2% Triton X-100. The homogenates were then centrifuged at 14,000 rpm for 10 min at 4 °C. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976).

Western blot analysis

Cellular protein (30 µg) were electrophoresed on 8% and 12% polyacrylamide gel and transferred to a nitrocellulose membranes (Amersham). The membranes were incubated at room temperature overnight with an anti-DR5 antibody (Alexis Biochemicals) (1:1000), or an anti-TRAIL antibody (BD Transductions Laboratories) or anti-Caspase-8 antibody (Cell Signaling Technology), or anti-Caspase-3 antibody (Cell Signaling Technology), or anti-JNK1 antibody (Santa Cruz Biotechnology) or anti-p-JNK1 antibody (Santa Cruz Biotechnology), or anti-GITR antibody (R&D System), or anti-GITRL antibody (Santa Cruz Biotechnology), or anti-β-Tubulin antibody (Santa Cruz Biotechnology). The secondary HRP-conjugated antibody (Amersham) and a chemiluminescence blotting substrate kit assay (Amersham) were used for immunodetection.

All the experiments were repeated at least three times; β-tubulin (Santa Cruz Biotechnology) was used as an internal control to validate the right amount of protein loaded in the gels. One mouse was used for each quantification.

Measurement of TNF- α and IL-1 β

Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as previously described in PBS containing 2 mM of phenyl-methyl sulfonyl fluoride to evaluate TNF- α and IL-1 β tissue levels. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation) according to the manufacturer instructions. All TNF- α and IL-1 β determinations were performed in duplicate serial dilutions.

Myeloperoxidase Activity

MPO activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in the spinal cord tissues as previously described (Mullane, 1989) at 24 h after SCI. The time of 24 h after SCI was chosen in agreement with other studies (Genovese *et al*, 2005). MPO activity was defined as the quantity of enzyme degrading 1 μ mol of peroxide/min at 37°C and was expressed in milliunits per g of wet tissue.

Materials

All compounds were obtained from Sigma-Aldrich Company Ltd. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter).

Statistical evaluation

All values in the figures and text are expressed as mean \pm standard deviation (SD) of N observations. For the *in vivo* studies N represents the number of animals studied. For all the experiments, the respective figures are representative of a single experiment out of three comparable experiments, performed on different days on the sample collected from all the animals of each group. Data, pooled from all the appropriate experiments, were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered significant.

BBB scale data were analyzed by the Mann-Whitney test and considered significant when p-value was < 0.05 .

RESULTS

The expression of TRAIL and its receptors is induced by SCI in the mouse.

We examined the expression of TRAIL and its receptor DR5 in mice 24 h after SCI. Western blot analysis of spinal cord proteins extracts from sham-operated mice groups did not show TRAIL expression and very little DR5, whereas spinal cord sections obtained from mice subjected to SCI exhibited an increased expression of TRAIL and DR5 (Figure 1, blots). Similar information were obtained through immunostaining. No specific immunostaining for either TRAIL (Figure 1a and densitometry graph e) or DR5 (Figure 1c and densitometry graph f) was observed in spinal cords obtained from sham-operated mice and specific immunostaining for TRAIL (Figure 1b and densitometry graph e) and DR5 (Figure 1d and densitometry graph f) were found in both white matter and grey matter of the spinal cord tissues of SCI.

TRAIL neutralizing antibody prevents SCI induced apoptosis, and improves motor function and related tissue scores in the mouse.

Subsequently, animals were treated with a TRAIL neutralizing antibody and perilesional spinal cord tissues underwent TUNEL staining for evaluation of apoptosis. Virtually no apoptotic cells were detectable in the spinal cord tissue from sham-operated mice (Figure 2a). Twentyfour hours after the trauma, tissues obtained from SCI mice demonstrated a marked appearance of dark brown intercellular apoptotic fragments (Figure 2b, see number of TUNEL positive cells panel d) in the grey matter of the spinal cord tissues. In contrast, tissues obtained from TRAIL-neutralizing antibody-treated mice displayed a lesser number of apoptotic fragments (Figure 2c, see number of TUNEL positive cells panel d).

Concomitantly, animals were evaluated for motor activity score by using the modified BBB hind limb locomotor rating scale (Basso *et al*, 1995). Animals undergone SCI showed significant decrease of motor activity. On the other hand, motor activity was significantly improved in animal treated with TRAIL neutralizing antibody (Figure 3). Sham operated animals did not show any change in motor activity score (data not shown).

In addition, the severity of the trauma at the level of the perilesional area, assessed by alteration of the white matter (Figure 4b and densitometry graph d), was evaluated at 24 h after injury. Significant damage to the spinal cord was observed in tissues from SCI mice when compared with sham-operated mice (Figure 4a). Notably, significant protection against spinal cord injury was observed in mice treated with TRAIL neutralizing antibody (Figure 4c and densitometry graph d).

TRAIL neutralizing antibody inhibits apoptosis associated activation of caspases and JNK in mice undergone SCI.

The increased expression of TRAIL and DR5 was paralleled by the activation of caspase-8 in SCI (Figure 5a). Treatment of mice subjected to SCI with TRAIL neutralizing antibody significantly reduced the activation of caspase-8 in the spinal cord extracts. To test whether the effect of the TRAIL neutralizing antibody resulted in inhibition of SCI-induced apoptotic cell death, we examined the expression of active caspase-3, an enzyme that is known to be expressed after SCI and that is critically involved in the execution of the mammalian apoptotic cell death program (Springer et al, 1999; Ekshyyan and Aw, 2004). Indeed, western blot analysis revealed that caspase-3 was cleaved in the spinal cord of SCI 24 h after injury. Treatment of mice subjected to SCI with TRAIL neutralizing antibody significantly prevented such effect (Figure 5b).

24 h after SCI, the expression of phospho-JNK in spinal cord was also investigated. Western blot analysis showed a significant increase of phospho-JNK in spinal cords from mice undergone to SCI, whereas TRAIL neutralizing antibody treatment prevented the SCI-induced (Figure 5c) expression of this kinase. The expression of phospho-JNK was compared with the basal expression of JNK-1 in all groups.

Protein expression of Bax and Bcl-2 correlates with biochemical and morpho-functional data in mice undergone SCI and treated with TRAIL neutralizing antibody.

To confirm morphological and biochemical data on apoptosis, the apoptosis-related family of protein Bcl-2 was studied by means of immunohistochemistry. Spinal cord sections from sham-

operated mice did not show specific immunostaining for Bax (Figure 6a) while SCI operated mice exhibited positive staining for Bax (Figure 6b and densitometry graph 6d). Anti-TRAIL treatment reduced the degree of positive staining for Bax in spinal cord sections from SCI treated mice (Figure 6c and densitometry graph 6d).

In addition, spinal cord from sham-operated mice demonstrated positive staining for Bcl-2 (Figure 7a and densitometry graph 7d). Spinal cord sections obtained from SCI operated mice exhibited significantly less staining for Bcl-2 (Figure 7b and densitometry graph 7d). Anti-TRAIL treatment upregulated protein expression of anti-apoptotic Bcl-2 (Figure 7c and densitometry graph 7d).

TRAIL neutralizing antibody blunts TRAIL-dependent increase of tissue proapoptotic proinflammatory molecules in animals with SCI

Our data show that damage occurring in SCI tissues is depending, at least in part, upon the effect of the proapoptotic cytokine TRAIL. It is reported that tissue damage is unlikely to be determined by a unique factor, but rather by the concerted action of several molecules of the same or related families, eventually recruited by an ignition factor (Fleming *et al*, 2006) and, thus, we investigated whether the functional inactivation of TRAIL modulates the expression of other cytokines, such as FasL, TNF- α , IL-1 β and/or activation of MPO, participating to the inflammation following SCI.

First of all, we studied the expression of the cytokine FasL, which is already known to play a role in SCI in the mouse, 24 h after injury (Demjen *et al*, 2004). Immunohistochemistry data showed that, whereas specific FasL immunoreactivity is absent in sections from the spinal cord of sham operated rats (Figure 8a, and densitometry graph 8d), it is on the other hand well detectable in sections from mice undergone SCI (Figure 8b and densitometry graph 8d). The positive staining was localized in various cells in the grey matter. Treatment of mice with TRAIL neutralizing antibody implied a significant decrease of FasL immunoreactive staining in SCI spinal cord sections (Figure 8c and densitometry graph 8d).

A similar pattern was observed for TNF- α , IL-1 β and MPO, as assessed by colorimetric assay, showing that changes occur in sham-operated animals, in contrast with the dramatic increase

scored in animals undergone SCI. Interestingly, treatment with TRAIL neutralizing antibody produced a significant reduction of these molecules in the tissues from SCI animals (Figure 8 e, f, and g).

Reciprocal regulatory interplay between TRAIL and GITR in mice with SCI.

A recently characterized cytokine, GITRL, appears to play a role in the tissue damage occurring in the SCI model (Nocentini *et al*, 2008). However, in spite of the wide literature reporting relationships between TRAIL and other cytokines, there are no data relating TRAIL and GITRL. For this reason, and with the aim to unravel novel molecular target for therapy, we addressed the question whether the two molecules could have reciprocal interplay within the SCI model.

To do so, the expression of GITR and GITRL in spinal cord was investigated by immunohistochemistry at 24 h after SCI. A significant increase in GITR (Figure 9b, d, and densitometry graph 9e) and GITRL (Figure 10b, c, and densitometry graph 10e) expression was observed in the spinal cords from mice subjected to SCI. On the contrary, anti-TRAIL treatment prevented the SCI-induced GITR (Figure 9c and densitometry graph 9e) and GITRL (Figure 10c and densitometry graph 10e) expression. The specific immunostaining for GITR (Figure 9a and densitometry graph 9e) and GITRL (Figure 10a and densitometry graph 10e) appears dot-shaped in the white matter of sham-operated mice. Western blot analysis (Figure 9f and 10f) confirmed the results obtained with immunohistochemistry.

To verify whether GITR modulates SCI-induced TRAIL-activity, we evaluated both TRAIL and DR5 expression in GITR lacking mice (GITR^{-/-}) undergoing SCI. Levels of TRAIL and DR5 immunoreactivity were significantly attenuated in GITR^{-/-} mice subjected to SCI as compared to SCI-wild type (WT) mice (data not shown).

DISCUSSION

It is known that proapoptotic cytokines play a relevant role as contributors to neuronal damage and functional impairment associated with SCI (Harrington *et al*, 2005; Lee *et al*, 2000). In this line, increased FasL and TNF- α expression have also been reported after SCI, although their neutralization is not always correlated to functional outcome (Demjen *et al*, 2004; Genovese *et al*, 2008; Yu *et al*, 2009). In this paper, we show the prominent role of TRAIL and related molecules in the cell death phenomena related to SCI, as well as how the molecular phenomena underlying can be partially prevented by neutralization of TRAIL, resulting in significant improvement of histopathological and functional outcome.

Indeed, the expression of TRAIL and its DR5 death receptor were significantly increased in mice undergone SCI in mice. These data, in line with those showing lack of expression of TRAIL and relatively scarce expression of DR5 in the spinal cord of intact mice, are well in accordance with a number of literature reports suggesting that TRAIL is not expressed in the normal central nervous system of mammalian species, which rather display weak expression of the DR5 death receptor (Cantarella *et al*, 2003; Aktas *et al*, 2007). On the other hand, expression of TRAIL increases dramatically after various types of nervous tissue injuries, as those occurring after vascular accidents (Martin-Ventura *et al*, 2007), challenge of neurons with toxic stimuli, including amyloid-beta (Cantarella *et al*, 2003). In the same line, increased expression of TRAIL has been detected by immunohistochemistry in the post-mortem brains of Alzheimer's patients, localized in the close vicinity of amyloid plaques (Uberti *et al*, 2004).

Interestingly, post-SCI impairment of motor activity was significantly improved in animals treated with TRAIL neutralizing antibody. It has been reported that, although SCI is associated with significant elevation of various cytokines in the spinal cord tissue, frequently, this does not correspond to amelioration of their functional performances (Profyris *et al*, 2004). These data suggest that, although involved, each single cytokine may have a contribution role, rather than a primary role in maintaining neuroinflammatory parameters associated with SCI and other CNS

injury models. However, it is likely to hypothesize that some molecules, such as, for example, TRAIL, may have a major coordinating role in the cytokine orchestra of neuroinflammation, and thus its neutralization implies improvement of neuronal function and, therefore, of motor performances.

In line with the TRAIL-related signalling, the increased expression of the cytokine and its receptors in the SCI is associated with increased activation of the initiator caspase-8 as well as the effector caspase-3, suggesting that TRAIL is setting into motion the cell death machinery (Ashkenazy and Dixit, 1998; Almasan, 2003). Moreover, systemic administration of an TRAIL neutralizing antibody resulted in reduced expression of TRAIL in SCI spinal cord lysates, as well as in reduced activation of caspase-8 and caspase 3. The relevant result that the perilesional edema and alteration of the white matter present in SCI mice was significantly reduced strongly suggests that TRAIL is directly involved in SCI-related tissue damage and, thus, its neutralization can bring about relevant improvement of pathophysiological parameters altered in SCI. In fact, this is in line with observations made for other proapoptotic cytokines the expression of which is increased in the brain in course of neuronal damage (Martin-Villalba *et al*, 1999). Moreover, SCI is not the only model of nervous system damage in which neutralization of TRAIL results in improvement of neuronal function. For example, it has been reported that TRAIL antagonism results in decreased amyloid-beta-induced toxicity in human neuronal cells in vitro (Cantarella *et al*, 2003; Uberti *et al*, 2007).

To confirm the specificity of detrimental effects of TRAIL in SCI, we found that the expression of the cell death-related phosphorylated form of kinase JNK was increased 24 h after SCI in spinal cords. Increased phosphorylation of JNK has been reported after treatment with TRAIL in other in vivo and also in vitro models (Corazza *et al*, 2006; Jurewicz *et al*, 2006; Cantarella *et al*, 2007) and represents a step of the canonical transduction pathway set into motion after binding of TRAIL to its DR5 death receptor (Jaganathan *et al*, 2002). Thus, it appeared

obvious that prevention of TRAIL detrimental effects in SCI by means of TRAIL neutralizing antibody was associated with decrease activation of the death related kinase JNK.

Evidence showed that proinflammatory and proapoptotic cytokines, including TNF- α , IL-1 β , FasL regulate the cellular events after SCI (Streit *et al*, 1998, Martin-Villalba *et al*, 1999). In the present study, we have demonstrated by immunohistochemistry a significant increase in positive staining for TNF- α in SCI mice group compared with sham-operated animal groups as well as FasL. Besides, Taoka *et al* (1997) showed that activated neutrophils are involved in SCI induced-trauma in rats. We demonstrated that the administration of TRAIL neutralizing antibody in SCI reduced the expression of both TNF- α and FasL as well as the inflammatory cell infiltration as assessed by the specific granulocyte enzyme MPO after SCI. The reduced neutrophil recruitment represents an important additional mechanism for the protective effects of the anti TRAIL treatment. Neutrophils recruited into the tissue can, in fact, contribute to tissue destruction by the production of reactive oxygen metabolites (Carlson *et al*, 1998; Tator, 1995), granule enzymes, and cytokines that further amplify the inflammatory response by their effects on macrophages and lymphocytes (Chatham *et al*, 1993).

It has been recently demonstrated that the TNFR superfamily member GITR plays a co-stimulatory role in T cells and a pro-inflammatory role in other cells of the immune system (Nocentini *et al*, 1997; Nocentini *et al*, 2007; Nocentini and Riccardi, 2009). Activation of GITR by its ligand appears to play a role also in the tissue damage occurring in the SCI model, as demonstrated by the higher motor score and lower inflammatory mediators levels in GITR^{-/-}, compared to wild type mice (Nocentini *et al*, 2008). As a counterproof, it has been demonstrated that GITR-Fc fusion protein inhibits GITR triggering and protects from the inflammatory response after spinal cord injury (Nocentini *et al*, 2008).

In the same line, Razmara *et al.*, (2009) showed that Fn14-TRAIL, a chimeric intercellular signal exchanger, attenuates experimental autoimmune encephalomyelitis inflammatory hallmarks. In this study, it is shown that neutralization of TRAIL by in vivo treatment with TRAIL neutralizing

antibody results in diminished expression of GITR and its ligand. Moreover, in GITR^{-/-} mice the expression of either TRAIL and its receptor DR5 is reduced following SCI, supporting the hypothesis that the interplay between the two systems is synergic and suggest that TRAIL and GITRL may play a leading role in the mechanisms of inflammation-driven neurodegeneration .

In summary, we have shown that the cytokine TRAIL plays a primary role in the SCI model. TRAIL induces apoptotic neuronal death, tissue damage and functional impairment in mice undergone SCI. Neutralization of TRAIL implies a dramatic improvement in all these parameters. The effects of TRAIL appear mediated by activated caspases and involved the expression of apoptosis-related genes, such as Bcl-2 and Bax. In a similar line, neutralization of TRAIL abrogated the expression of various inflammatory molecules, including GITRL and its receptor GITR.

In conclusion, neutralization of TRAIL could be envisioned as a novel potential pharmacological tool contributing to prevention of spinal cord injury-related damage.

Disclosure/Conflict of interest

No conflict of interest thereof subsists for any of the authors related to the subject of the report.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Expression of TRAIL and its DR5 receptor in mice underwent SCI. Blots: expression of DR5 (upper) or TRAIL (middle) in either sham operated or SCI mice. Lower blot: constitutive β -tubulin expression. Representative images from immunohistochemical analysis of TRAIL (photographs a and b) or DR5 (photographs c and d) performed on section from the spinal cord or sham operated (a, c) or SCI (b, d) mice. Histograms: densitometric analysis of the expression of TRAIL (e) and its DR5 (f) receptor in sham operated or SCI mice, was assessed. Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. Data are means \pm SD of 10 mice for each group. * p <0.01 vs. Sham. Inserts: higher magnification.

Figure 2. TRAIL neutralizing antibody injection prevents SCI induced apoptosis in the grey matter. TUNEL staining for evaluation of apoptosis in sham operated (photograph a), SCI (photograph b), or SCI mice treated with a TRAIL neutralizing antibody (photograph c). The number of apoptotic nuclei in the three groups is compared in the histogram in d). Figure is representative of at least 3 experiments performed on different experimental days. Inserts: higher magnification. Data are means \pm SD.

Figure 3. TRAIL neutralizing antibody injection improves hind limb motor disturbance after spinal cord injury in the mouse. BBB motor score (expressed as arbitrary units) in mice underwent SCI after treatment with a TRAIL neutralizing monoclonal antibody. The degree of motor disturbance was assessed every day until 10 days after SCI by Basso, Beattie, and Bresnahan (BBB) criteria. Treatment with anti-TRAIL reduces the motor disturbance after SCI. Values shown are mean \pm SD of 10 mice for each group. * p <0.01 vs. SCI+anti-TRAIL.

Figure 4. Histological alteration score in the SCI in the mouse after treatment with TRAIL neutralizing antibody. Edema and alterations of the white matter in tissues from sham operated (panel a), untreated (panel b) and TRAIL-neutralizing antibody treated (panel c) SCI mice. Panel D:

histological score (arbitrary units) for tissue damage in sham operated (SHAM), TRAIL-neutralizing antibody (ANTI-TRAIL, empty bars), and untreated (vehicle, filled bars) SCI mice. wm: White matter; gm: gray matter. This figure is representative of at least 3 experiments performed on different experimental days. Values shown are mean \pm SD of 10 mice for each group. * p <0.01 vs. SCI. Inserts: higher magnification.

Figure 5. Expression of caspase-8 and -3, and of the stress kinase JNK-1 in the tissues from SCI mice treated with a TRAIL neutralizing antibody. *Blots A:* Western blot analyses of caspase-8; *Blot B:* caspase-3 and the house keeping β -tubulin, in the tissues from sham operated (SHAM), SCI (SCI) and SCI pretreated with a TRAIL neutralizing antibody (SCI antiTRAIL). *Blots C:* Western blot analyses of the stress kinase JNK-1 (upper right blot), and its phosphorylated form (p-JNK-1, lower right blot), in the tissues from sham operated (SHAM), SCI (SCI) and SCI pretreated with a TRAIL neutralizing antibody (SCI anti-TRAIL). Densitometric analysis of the expression of Caspase 8 (A1) and caspase 3 (B1) and p-JNK-1 (C1) Data are expressed as percentage of relative intensity versus control.

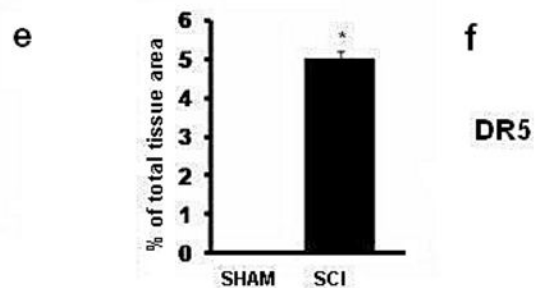
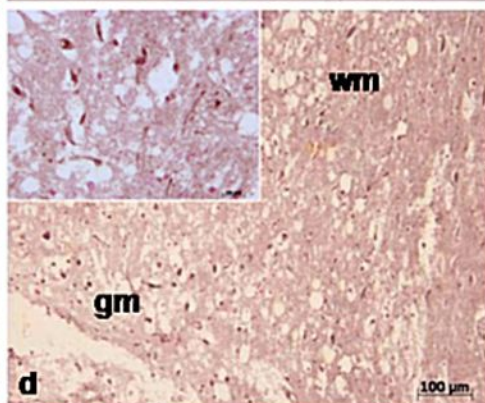
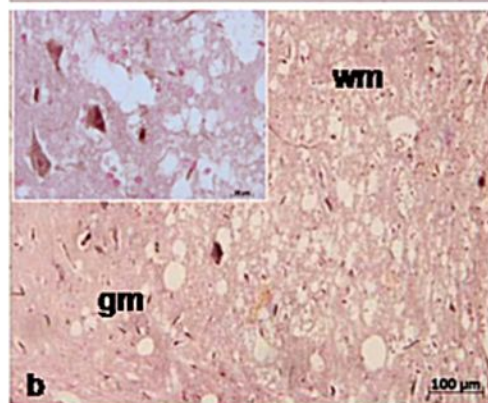
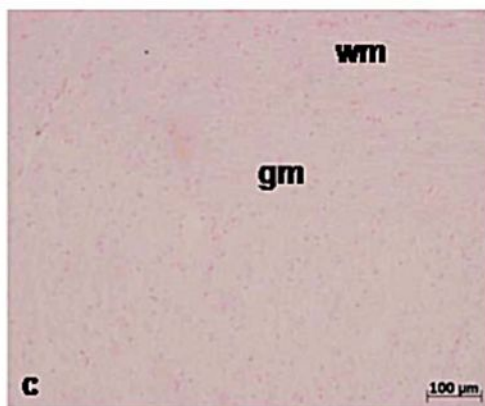
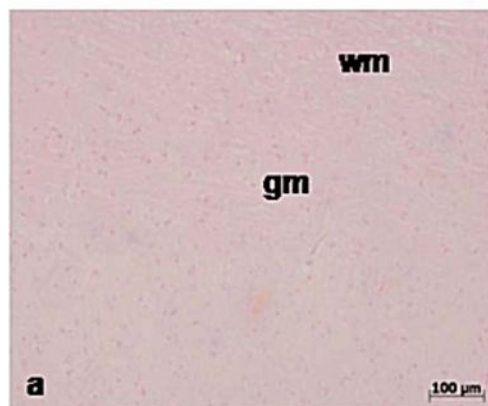
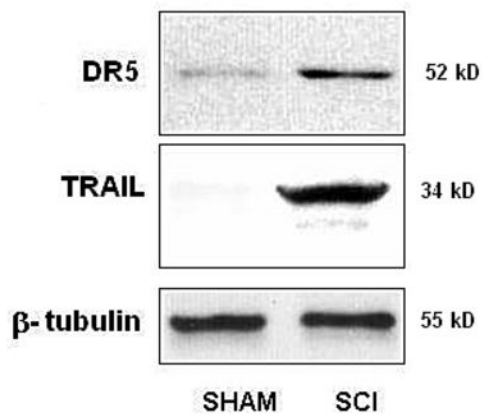
Figure 6. Bax expression in tissue section from sham-operated mice (a) and SCI (24 h),(b) and anti-TRAIL treated mice (c). Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for Bax (d) from spinal cord tissues was assessed. This figure is representative of at least 3 experiments performed on different experimental days. * p <0.01 vs. Sham. ° p <0.01 vs SC I+ vehicle. Data are means \pm SD. Insert: higher magnification.

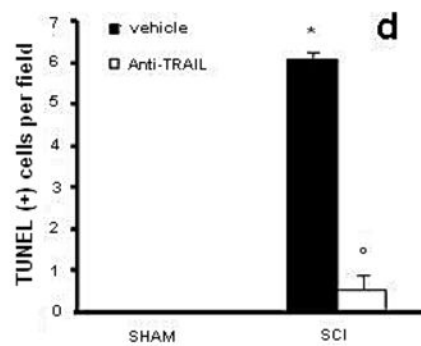
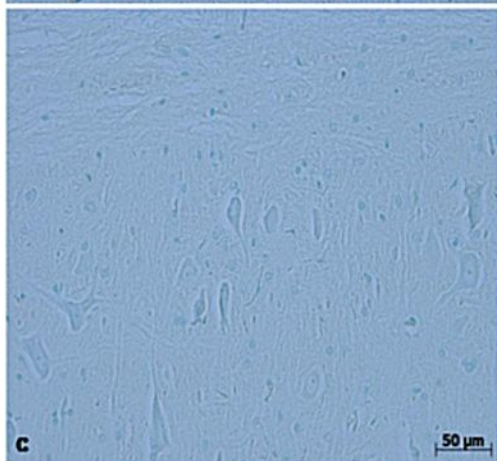
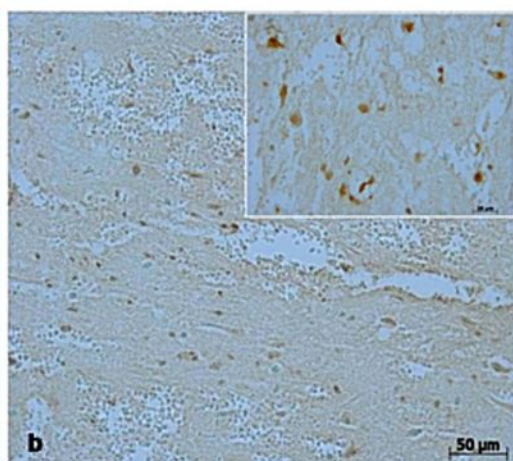
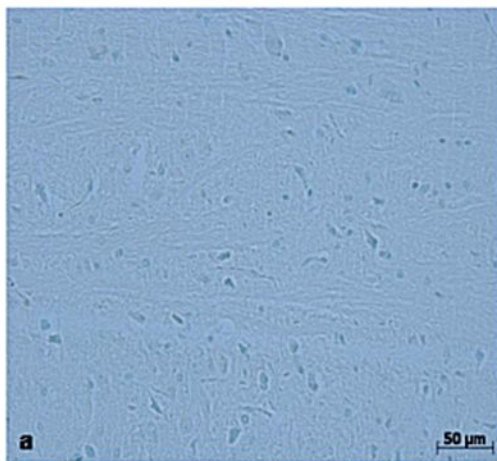
Figure 7. Immunohistochemical expression of Bcl-2 in tissue section from sham-operated mice (a) and SCI (24 h),(b) and anti-TRAIL treated mice (c). Densitometry analysis of immunocytochemistry photographs for Bcl-2 (d) from spinal cord tissues was assessed. Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. * p <0.01 vs. Sham. ° p <0.01 vs SC I+ vehicle. Data are means \pm SD. Inserts: higher magnification.

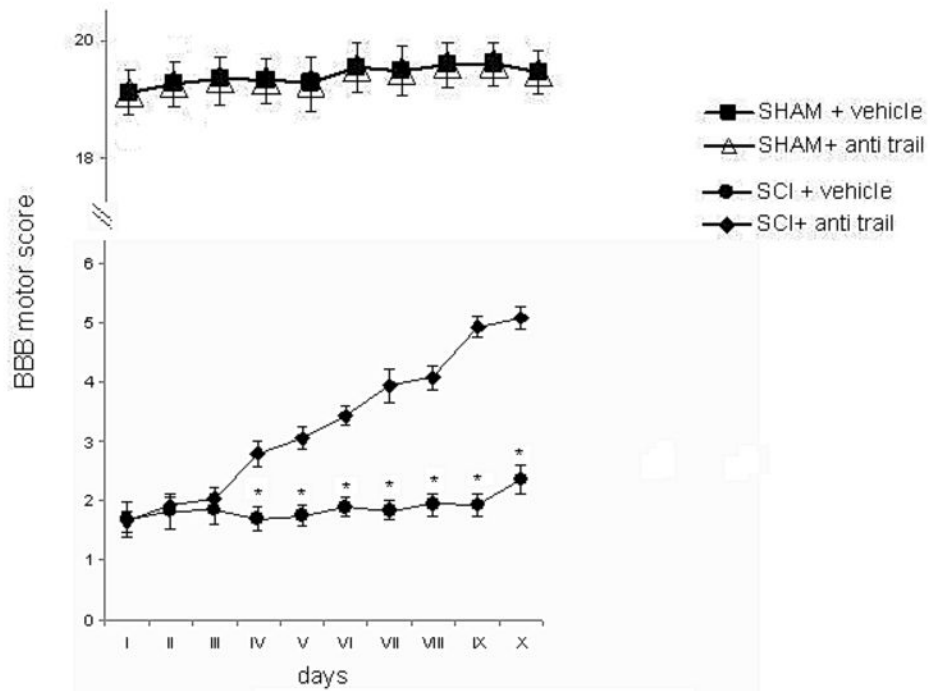
Figure 8. Immunohistochemical and immunoenzymatic evidence of different cytokines in the spinal cord of SCI mice. Specific immunostaining for Fas-ligand (panel a) in spinal cord sections from sham-operated, SCI (24 h), (panel b) and anti-TRAIL treated mice (panel c). Densitometry analysis of immunocytochemistry photographs for Fas-ligand (d) from spinal cord tissues was assessed. Data are expressed as % of total tissue area. Tissue levels of TNF- α (panel e) and IL-1 β (panel f) in spinal cords from SCI (24 h) mice. Panel g: MPO activity in spinal cord from SCI mice. This figure is representative of at least 3 experiments performed on different experimental days. * $p < 0.01$ vs. Sham. $^{\circ}p < 0.01$ vs SCI+vehicle. Data are means \pm SD. Insert: higher magnification.

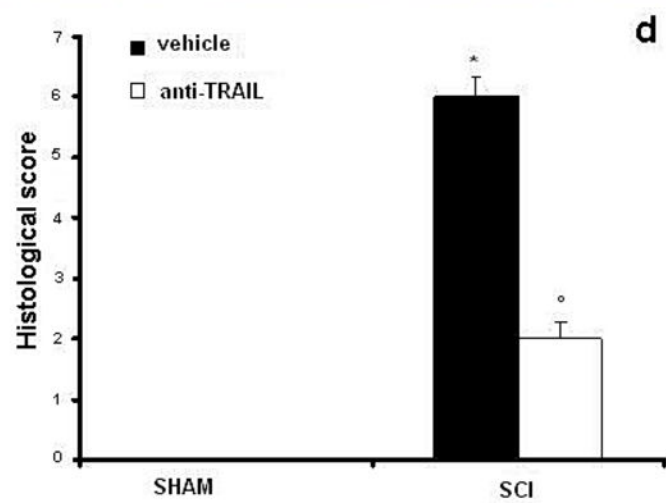
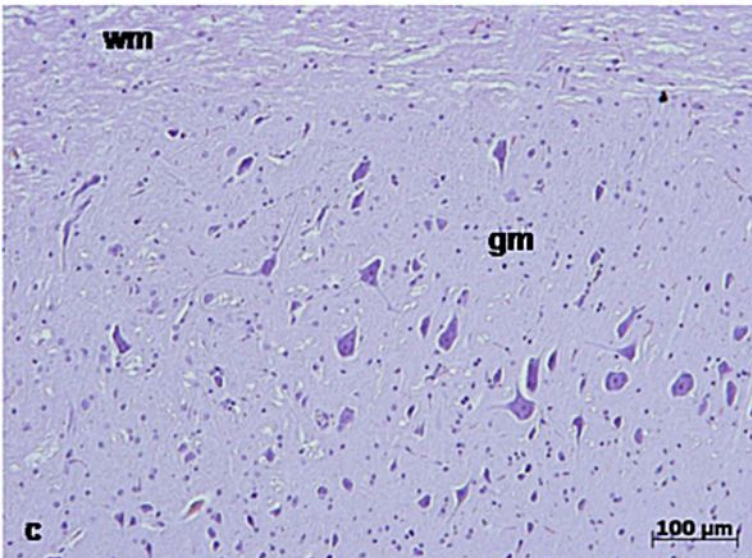
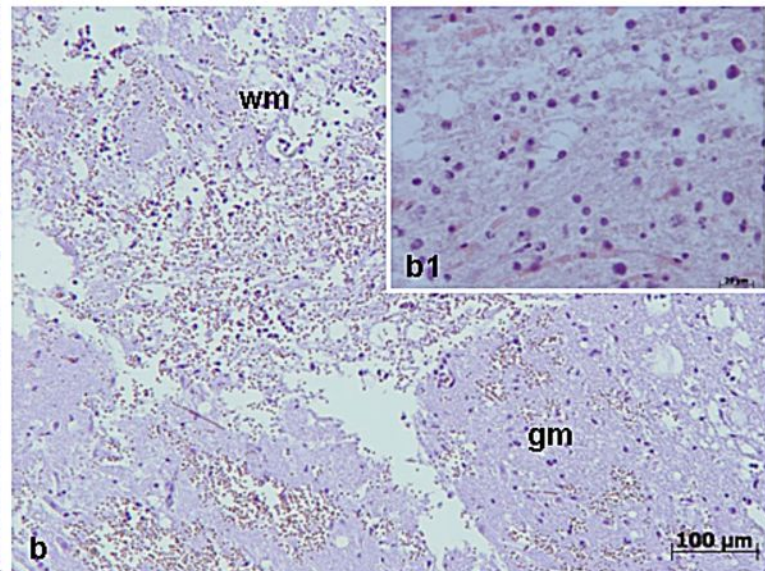
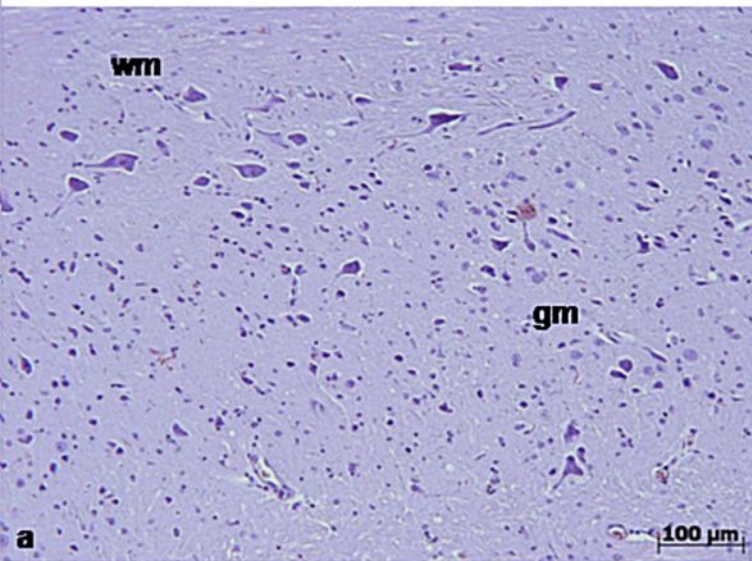
Figure 9. Immunoreactivity for GITR in tissue sections from sham-operated (panel a), SCI (Panel b), anti-TRAIL treated (panel c;) SCI mice. Panel e: Densitometry analysis of immunocytochemistry photographs for GITR from spinal cord tissues. Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. * $p < 0.01$ vs. Sham. $^{\circ}p < 0.01$ vs SCI+vehicle. Data are means \pm SD. Panel d: Western blot analysis of GITR protein in spinal cords from sham-operated (SHAM), SCI, or anti-TRAIL treated SCI mice (SCI + anti-TRAIL). Insert: higher magnification.

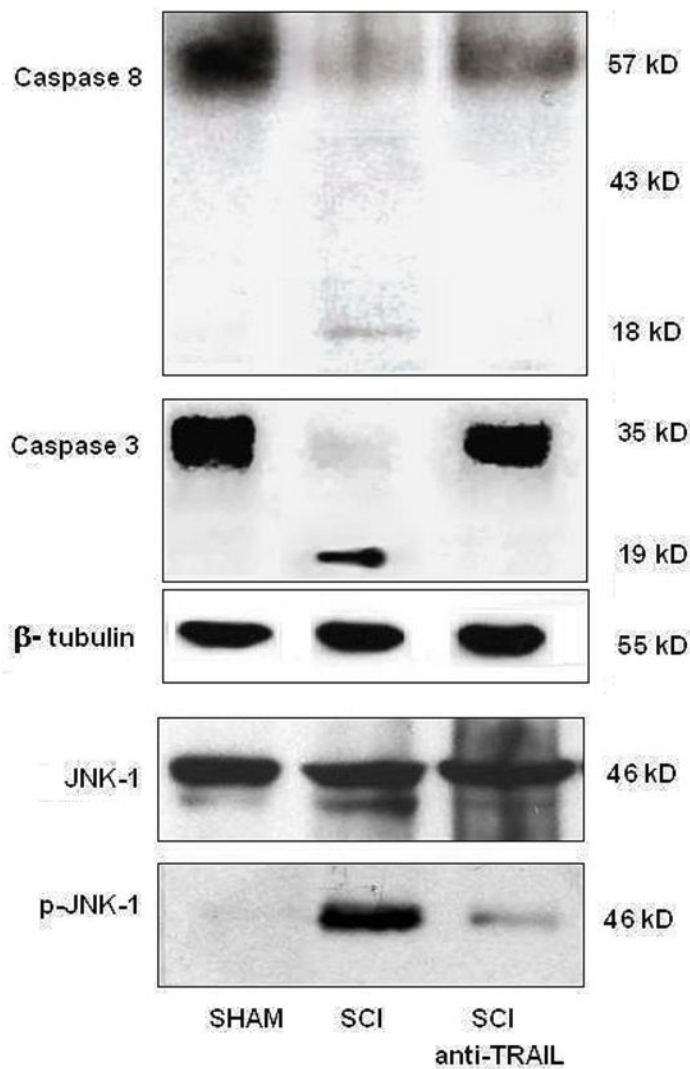
Figure 10. Immunoreactivity for GITRL in tissue sections from sham-operated (panel a), SCI (Panel b), anti-TRAIL treated (panel c;) SCI mice. Panel d: Densitometry analysis of immunocytochemistry photographs for GITRL from spinal cord tissues. Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. * $p < 0.01$ vs. Sham. $^{\circ}p < 0.01$ vs SCI+vehicle. Data are means \pm SD. Panel e: Western blot analysis of GITRL protein in spinal cords from sham-operated (SHAM), SCI, or anti-TRAIL treated SCI mice (SCI + anti-TRAIL). Insert: higher magnification.



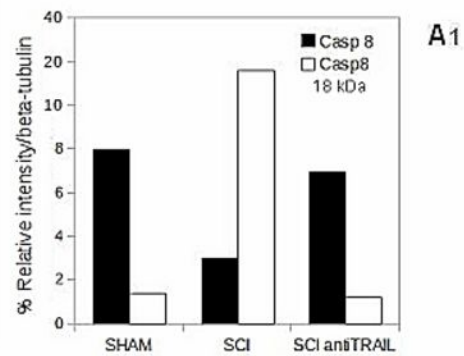




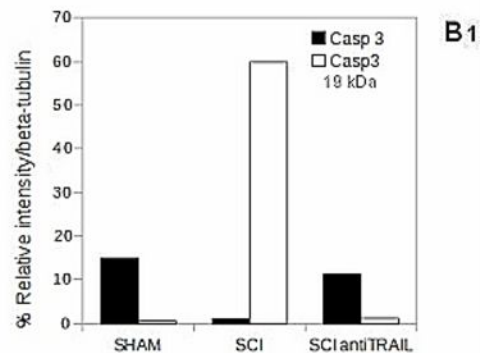




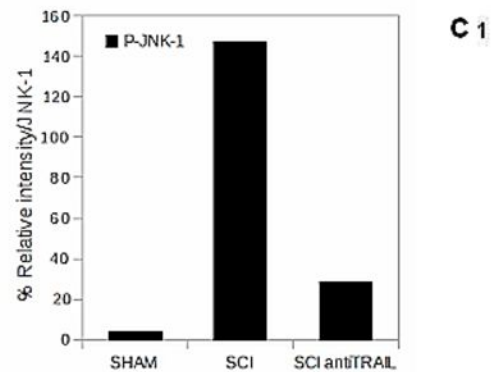
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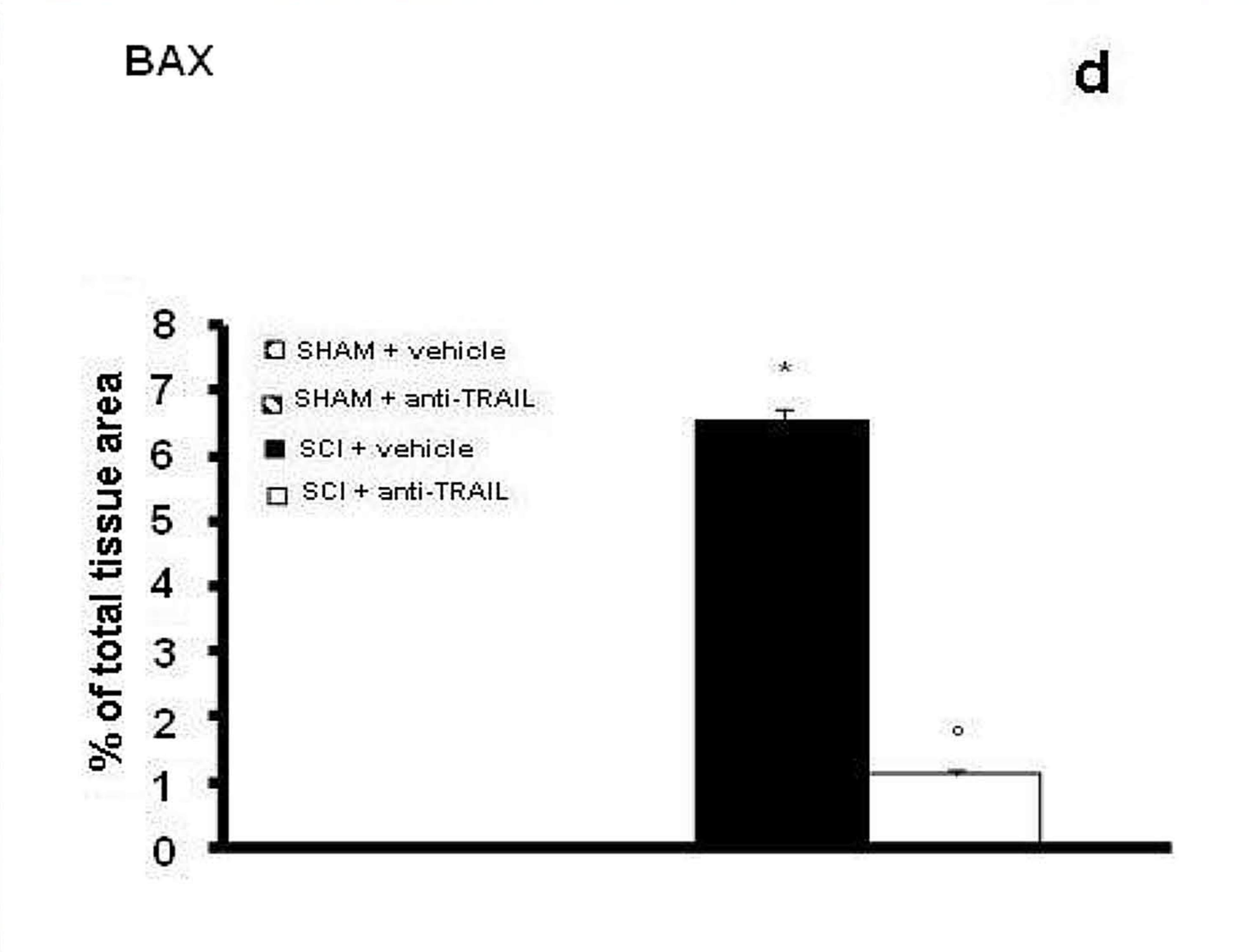
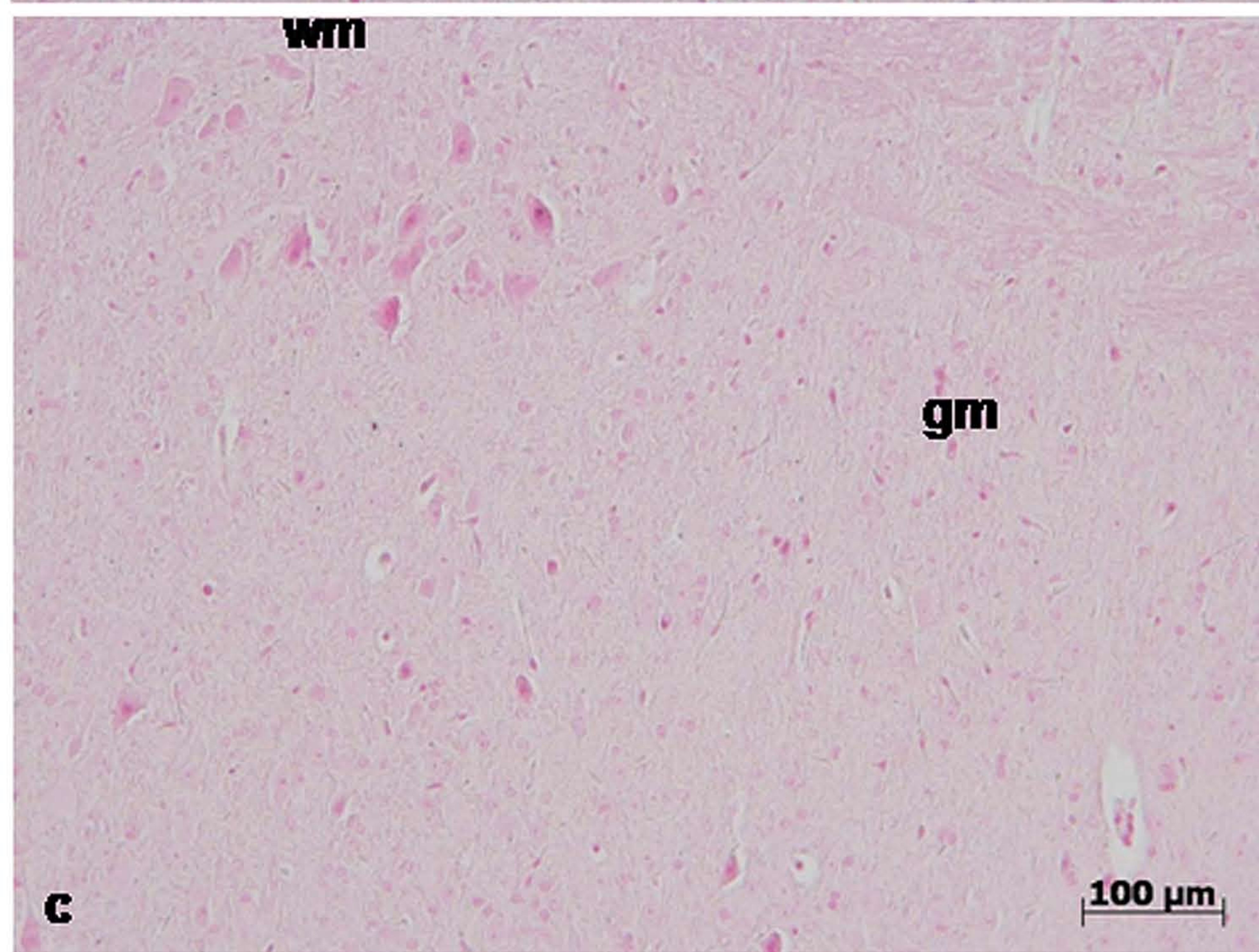
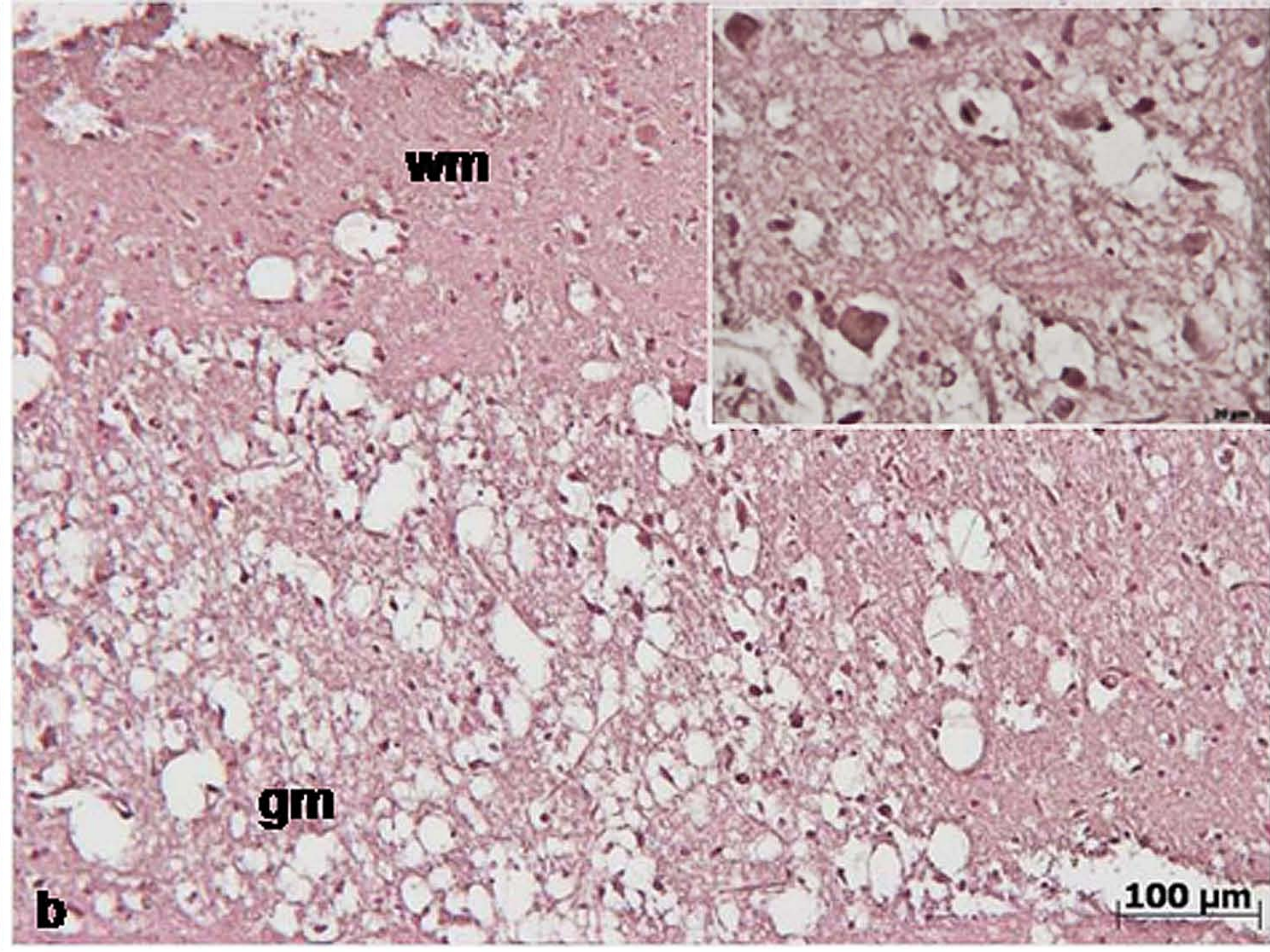
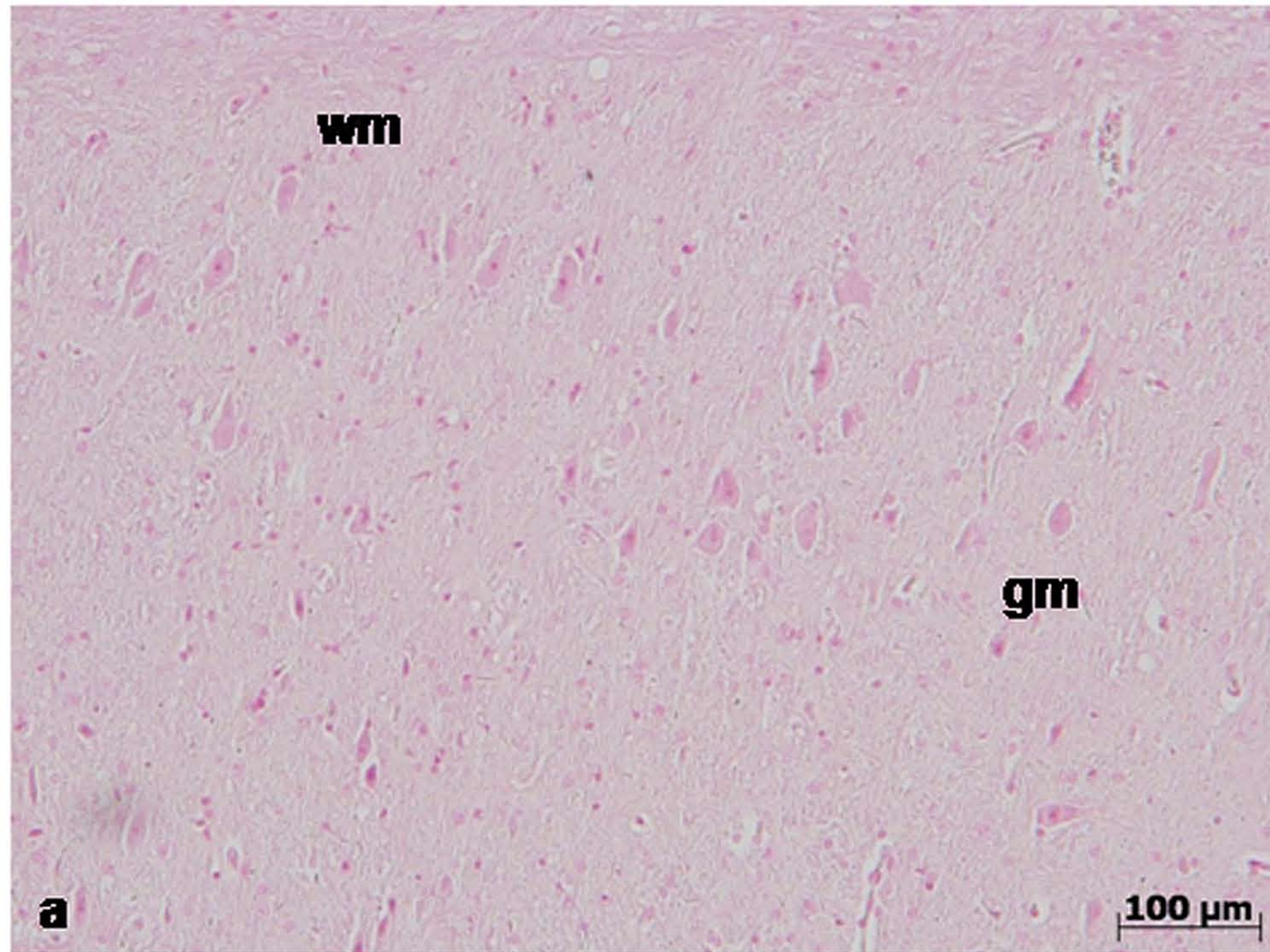


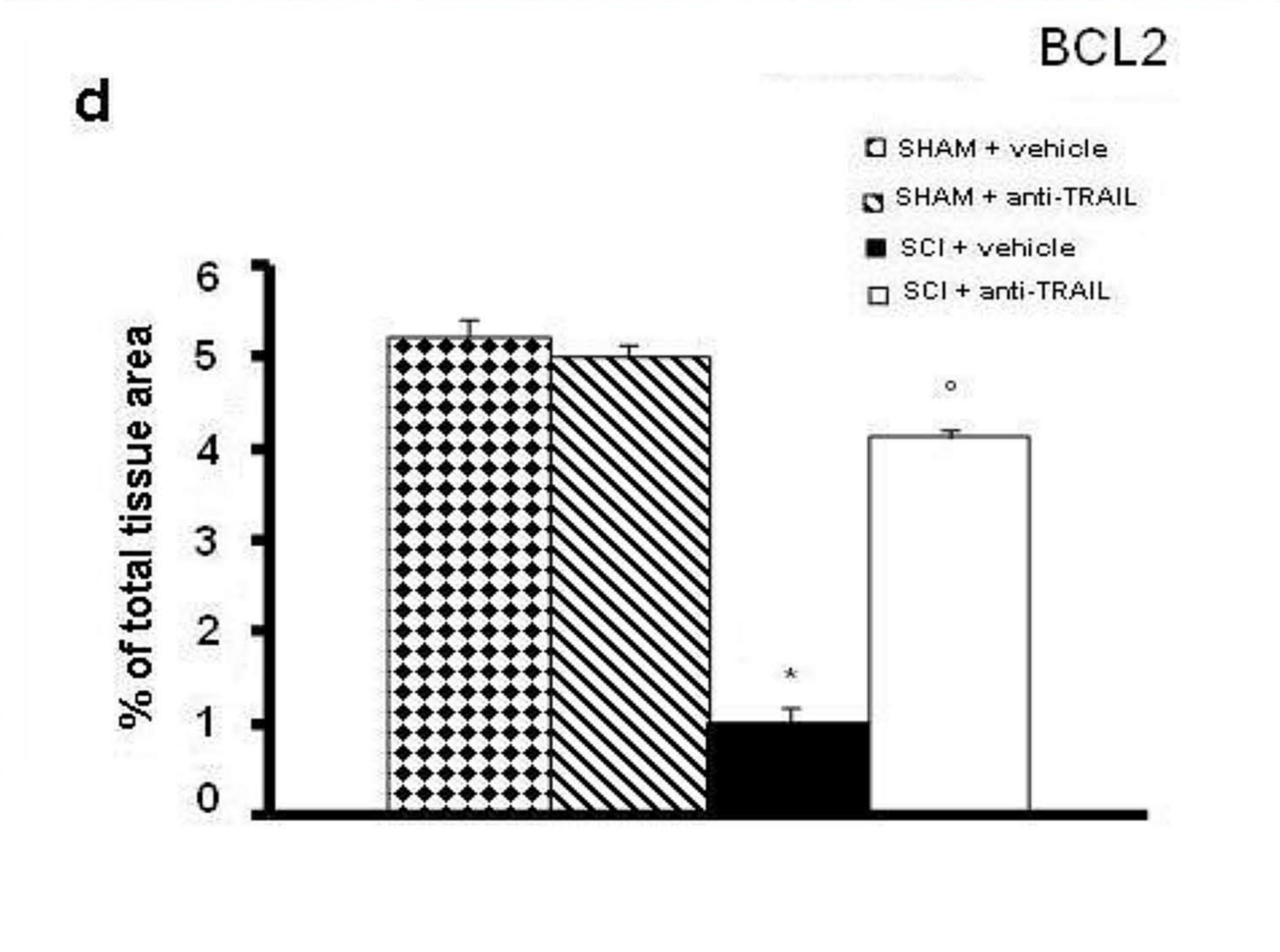
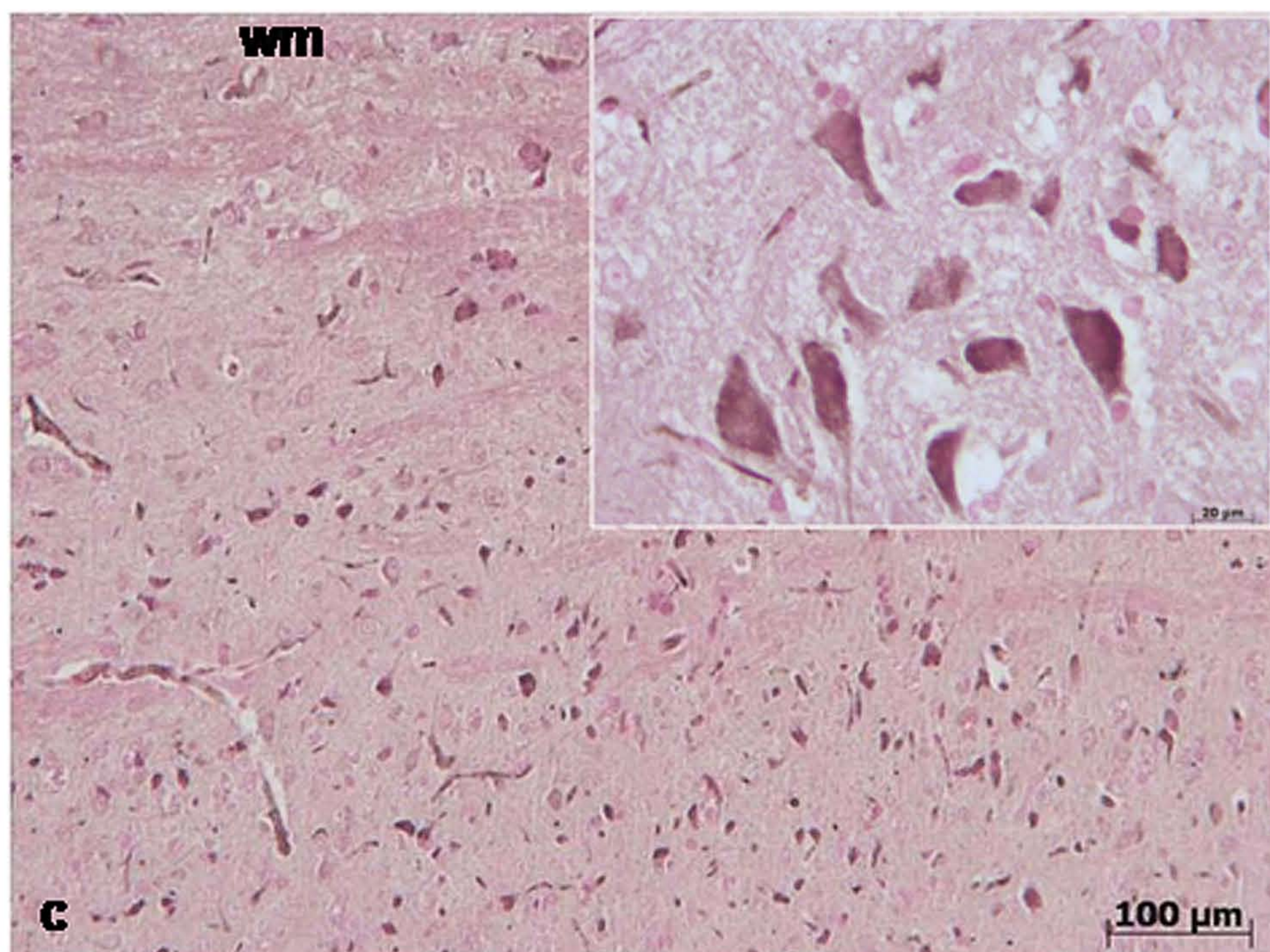
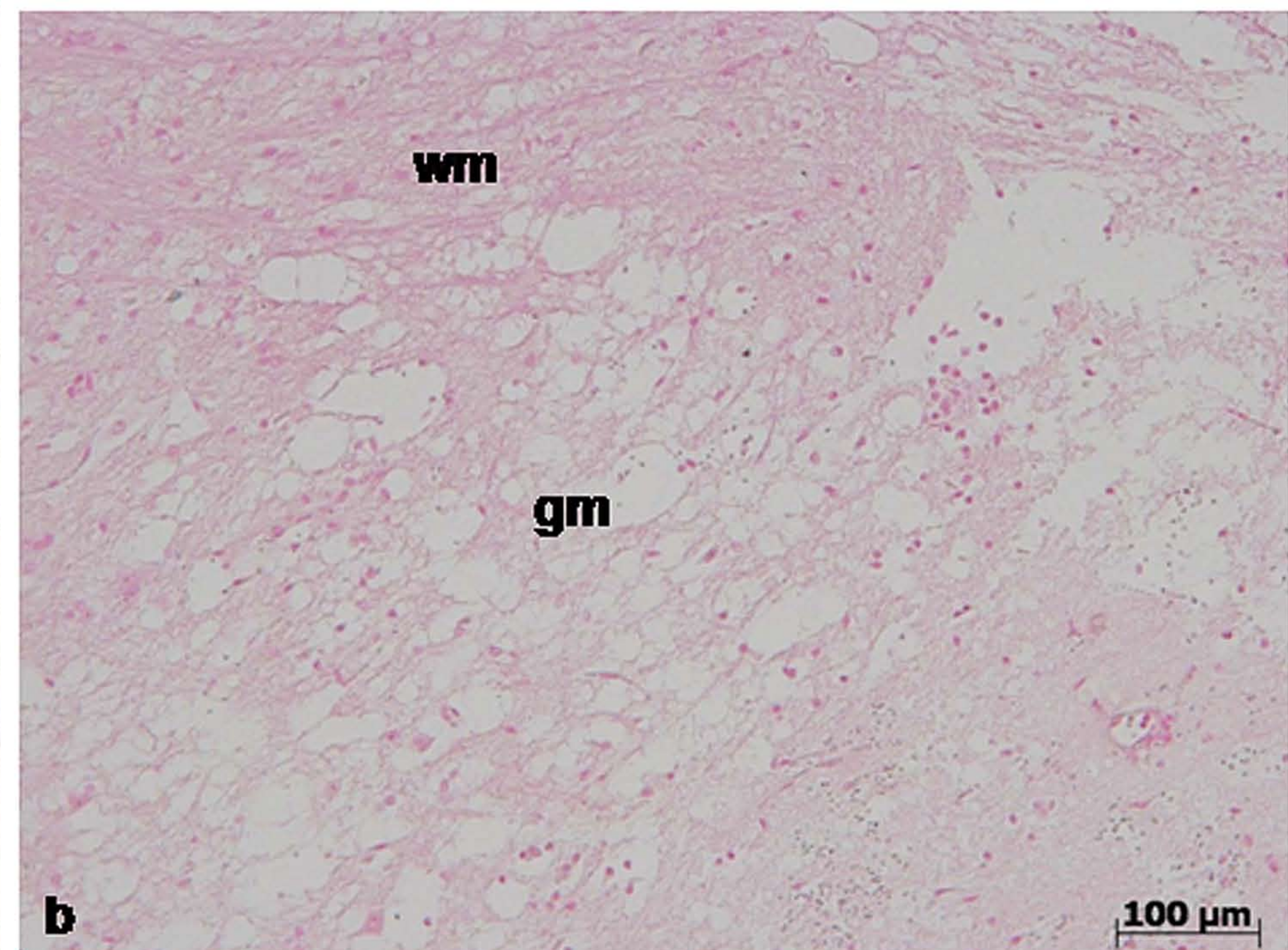
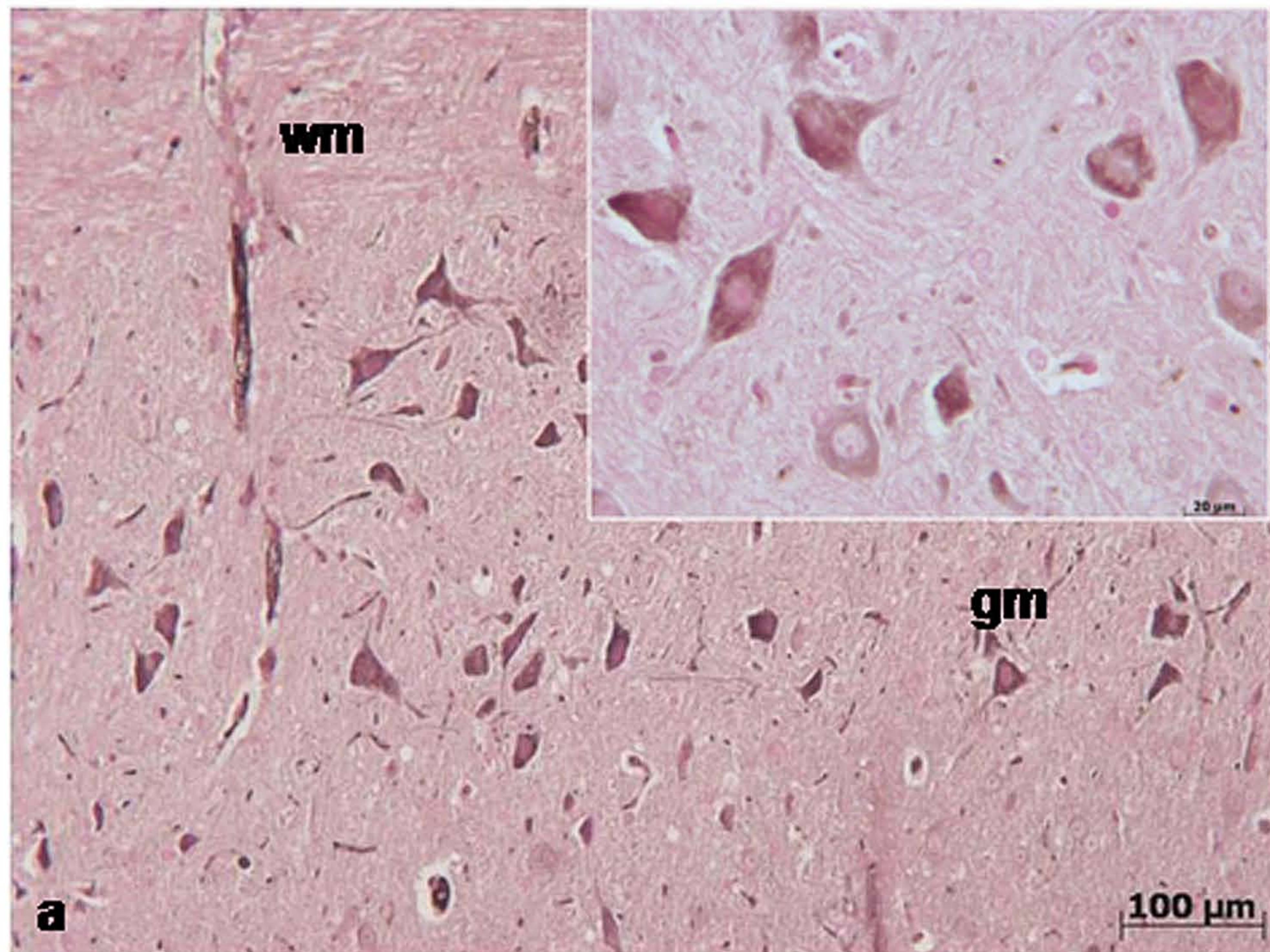
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C







Fas ligand

