

# Immunoexpression of gemins 2 and 4 in the rat spinal cord. Is the SMN complex a new target in investigations of sporadic amyotrophic lateral sclerosis pathogenesis?

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## Abstract

*Sporadic amyotrophic lateral sclerosis (sALS) is a neurodegenerative disease leading to degeneration and loss of motoneurons in different structures of the nervous system. Although aetiology of the disease is unknown, it is hypothesized that the survival motor neuron (SMN) protein which protects motoneurons in spinal muscular atrophy, may play a similar role in ALS. Relatively little is known about normal expression and functions of the SMN complex compounds, i.e. SMN protein and the related gemins. Therefore, we have decided to examine the physiological expression of SMN and gemins 2 and 4 in spinal cords of healthy Wistar rats at different age using immunofluorescence and immunohistochemical methods. Our study revealed that (1) in rat spinal cord neurons, the immunoexpression of SMN and gemins 2 and 4 is present through the whole animal lifespan although the reactive cells reveal different intensity of the immunolabeling, (2) both SMN and gemin 2, and SMN and gemin 4 are present in the same motoneurons, (3) immunoexpression of gemin 2 and 4 decreases slightly with aging.*

**Key words:** SMN, gemin, ALS, rat.

## Introduction

Mutation of the telomeric form of the *SMN* gene (*SMN-1*) causes spinal muscular atrophy (SMA). As a result of the mutation (deletion of the exons 7,8 of *SMN-1* gene) delayed development of skeletal muscles and degeneration of motoneurons in the spinal cord and brain stem are observed. Clinically, there are three forms of SMA of different severity. In the early infantile form (form I, Werdig-Hoffman disease), clinical symptomatology is the most severe and quickly leads

to patient disability and death. In forms II and III, the course and symptomatology of the disease are more benign. Encoded by the *SMN* gene, the survival motor neuron (SMN) protein together with other proteins forms the SMN complex of a key importance for the motoneuron function. The SMN complex closely cooperates with many other multifunctional proteins, including small nuclear ribonucleoproteins (snRNAs), and – acting frequently with uridine – plays a role in the *SMN* gene splicing process [1,8,9,11]. Except functions in the assembly of smRNAs it probably also par-

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ticipates in the assembly, metabolism, and transport of other classes of ribonucleoproteins.

Apart from the SMN protein, the SMN complex contains several other proteins including gemin 2 and gemin 4. Gemin 2 is present in the cell cytoplasm, processes [23], and in gems – small structures localized in the nucleus. Gems are similar in size and number to Cajal bodies and play a role in gradual maturation of RNA. In the SMN complex, gemin 2 binds directly to the SMN protein. A small complex composed only of SMN and gemin 2 represents its inactive form whereas the high molecular form containing SMN, gemin 2, 3 and 4 is an active form that can bind substrates [17]. Gemin 2 plays a role in stabilization of the SMN complex [16], in export of smRNAs from the nucleus, and in their assembly in the cytoplasm [12].

Gemin 4, similarly to gemin 2, is localized in the cytoplasm and nucleus. Besides location in nucleolar gems, the protein is also present in nucleoli. Such localization of gemin 4 suggests that the SMN complex may also function in preribosomal RNA processing or ribosome assembly. Unlike gemin 2, gemin 4 does not bind with the SMN protein directly but interacts with it via gemin 3 [2].

Formerly published few studies on various experimental models [6,10] have not answered how long the SMN complex is active in individual life. Our previous investigations on healthy rats showed preserved immunoexpression of SMN and gemin 3 during the whole animal lifespan [19]. In the double staining immunofluorescent method we also demonstrated that both SMN and gemin 3 were expressed in the same motoneurons. The aim of the present study was to attempt to answer whether: (1) SMN and gemin 2, and SMN and gemin 4 – similarly to SMN and gemin 3 – are expressed in the same motoneuron; (2) immunoexpression of gemins 2 and 4 remain unchanged during the rat lifespan; and (3) whether aging influences immunoreactivity for gemins 2 and 4.

## Material and methods

The experimental material consisted of spinal cords obtained from 27 adult male Wistar rats. Rats were divided into 9 groups composed of 3 rats at age of 1, 10, 20, 30, 60, 150, 200, 250 and 350 days. All animal using procedures were accepted by the 4<sup>th</sup> Local Animal Experimentation Ethics Committee and were in agreement with the European Union rules on the care and use of laboratory animals.

Animals were anaesthetized with Nembutal (20 mg/kg b.w., *i.p.*) and perfused through the ascending aorta with 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer pH 7.4 and then with 4% formaldehyde in 0.1 M phosphate buffer pH 7.4. Next, the laminectomy was performed and spinal cords were removed. The material was soaked in 4% formaldehyde for 2 h, embedded in paraffin, and cut on the 40 µm-thick sections.

On glass-mounted sections of spinal cords, immunohistochemistry for light microscopy was executed according to avidin-biotin-peroxidase method. Material was dehydrated in alcohol, pretreated 3 × 10 min in microwave in 10 mM citrate buffer pH 6.0 for antigen retrieval, and labelled with primary antibodies (Ab) against SMN (Santa Cruz Biotechnology, 1 : 250), gemin 2 (Santa Cruz Biotechnology, 1 : 500), and gemin 4 (Santa Cruz Biotechnology, 1 : 1000) using Goat F(ab)2 Fragment anti-mouse IgG-biotin (Beckman Counter, 1 : 1500) and Goat F(ab)2 Fragment anti-rabbit IgG-biotin (Beckman Counter, 1 : 1500), respectively. The diaminobenzidine (DAB) was used as a chromogen.

For fluorescent microscopy, double immunofluorescent reactions for SMN and gemin 2, and for SMN and gemin 4 were performed. The first step of the procedures was the same as for single immunohistochemistry but then simultaneous incubation with polyclonal rabbit anti-SMN Ab and one of two primary antibodies: monoclonal mouse anti-gemin 2 Ab or monoclonal mouse anti-gemin 4 Ab was performed. Following the incubation, the sections were washed and incubated for 1 h at 37°C with secondary antibodies: goat anti-mouse Alexa Fluor 594 (Invitrogen – Molecular Probes, 1 : 100) and goat anti-rabbit Alexa Fluor 488 (Invitrogen – Molecular Probes, 1 : 100).

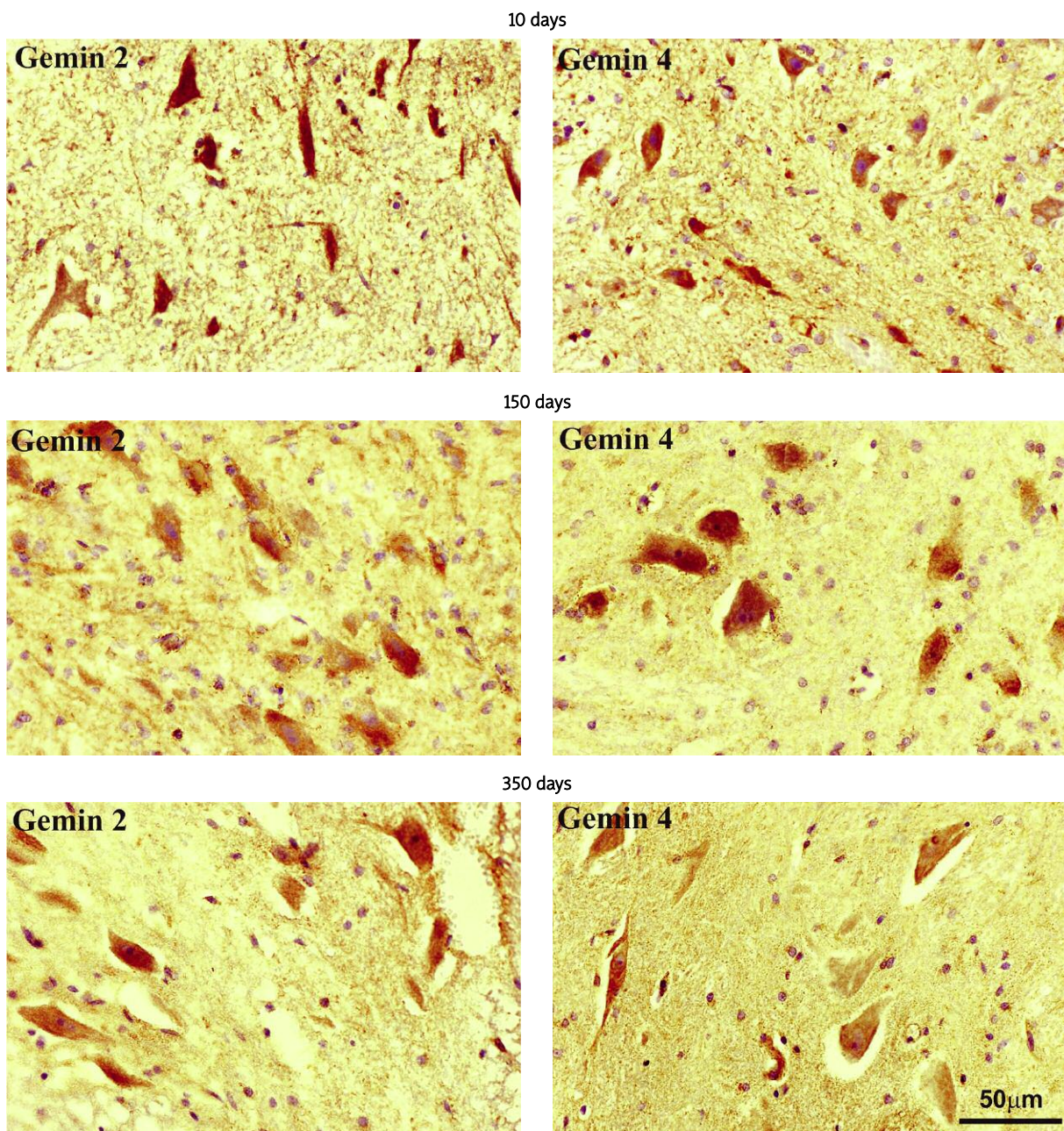
Specificity of the immunostainings was verified by the execution of the “negative control” staining, in which the procedure with the primary antibodies was omitted in the incubation mixture.

The labelled material was analyzed and captured with an Optiphot-2 Nikon microscope (Japan) equipped with the appropriate filters and a DS-L1 Nikon camera (Japan). For the analysis of immunostaining, image processing software IPP+ (Image Pro Plus) was used. Surface plot profiles were performed from selected sections representing each investigated time point. Blue colour of hematoxylin counterstaining was removed electronically from the analysis. At the plots, the colour of each pixel indicates the level of intensity of the immune reaction.

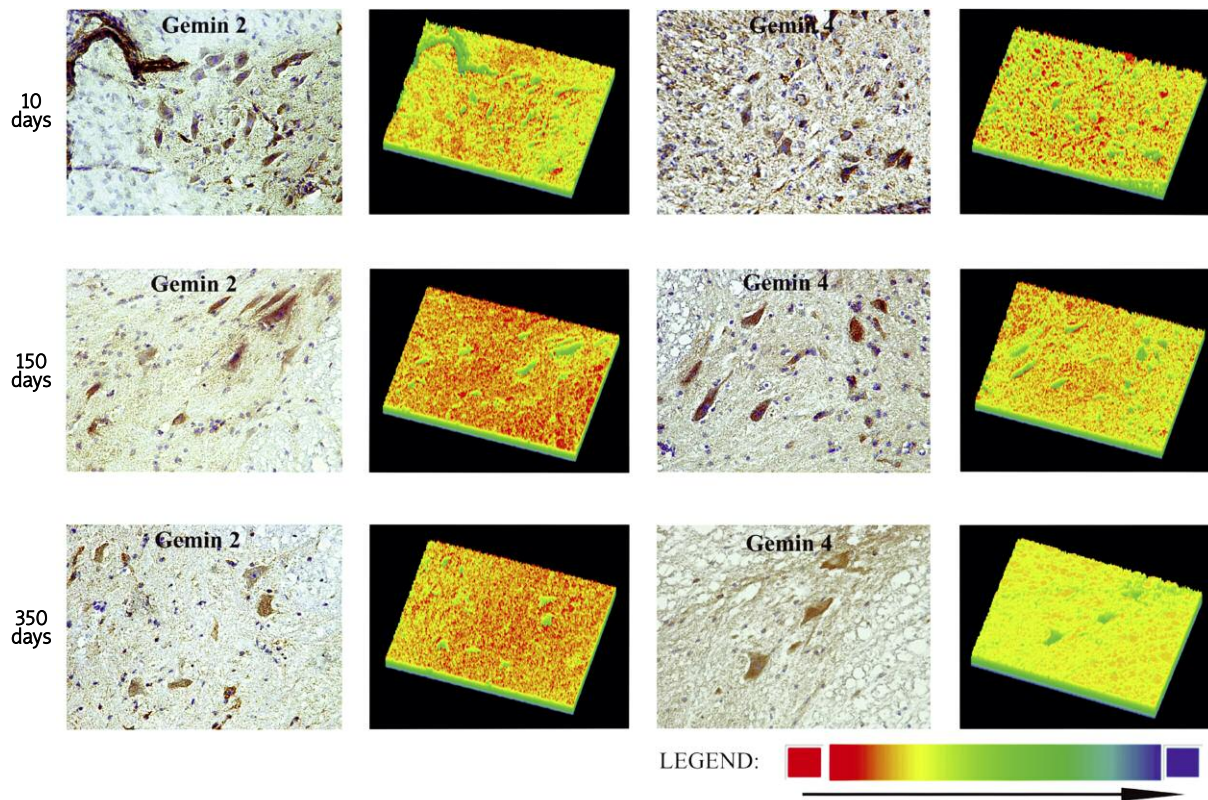
## Results

Results of our study indicate that both gemin 2 and gemin 4 are expressed during the whole lifespan of a rat but in different intensity. In an early postnatal period (up to 10 days of life) spinal cord neurons were small, and only weak immunoreactivity for the analyzed gemins was observed (Figs. 1 and 2). The level of the im-

munosignal for gemin 2 was generally weaker than that observed for gemin 4. In that period, immunoreactivity for the investigated proteins was also seen in astroglia. In rats at the age from 20 to 150 days, more intense reaction for gemins 2 and 4 in the motoneurons was seen but the signal was unequal within the individual cells and varied from heavy to weak (Figs. 1 and 2). At 350 day of rat lifespan the level of immunoexpression



**Fig. 1.** Immunoreactivity for gemins 2 and 4 in healthy rats at different time points of the animal lifespan. Note the different level of the immunostainings in individual neurons.



**Fig. 2.** Surface plot profiles of the selected sections analyzed in pseudocolours. Visible different intensity of the immunosignals in DAB staining.

of both gemins seems to be weaker than in the earlier time points. Similarly to previous observations, the stained motoneurons showed various intensity of the immunosignal, and some of them even demonstrated a negative reaction for the labelling to gemin 2 and 4 (Figs. 1 and 2).

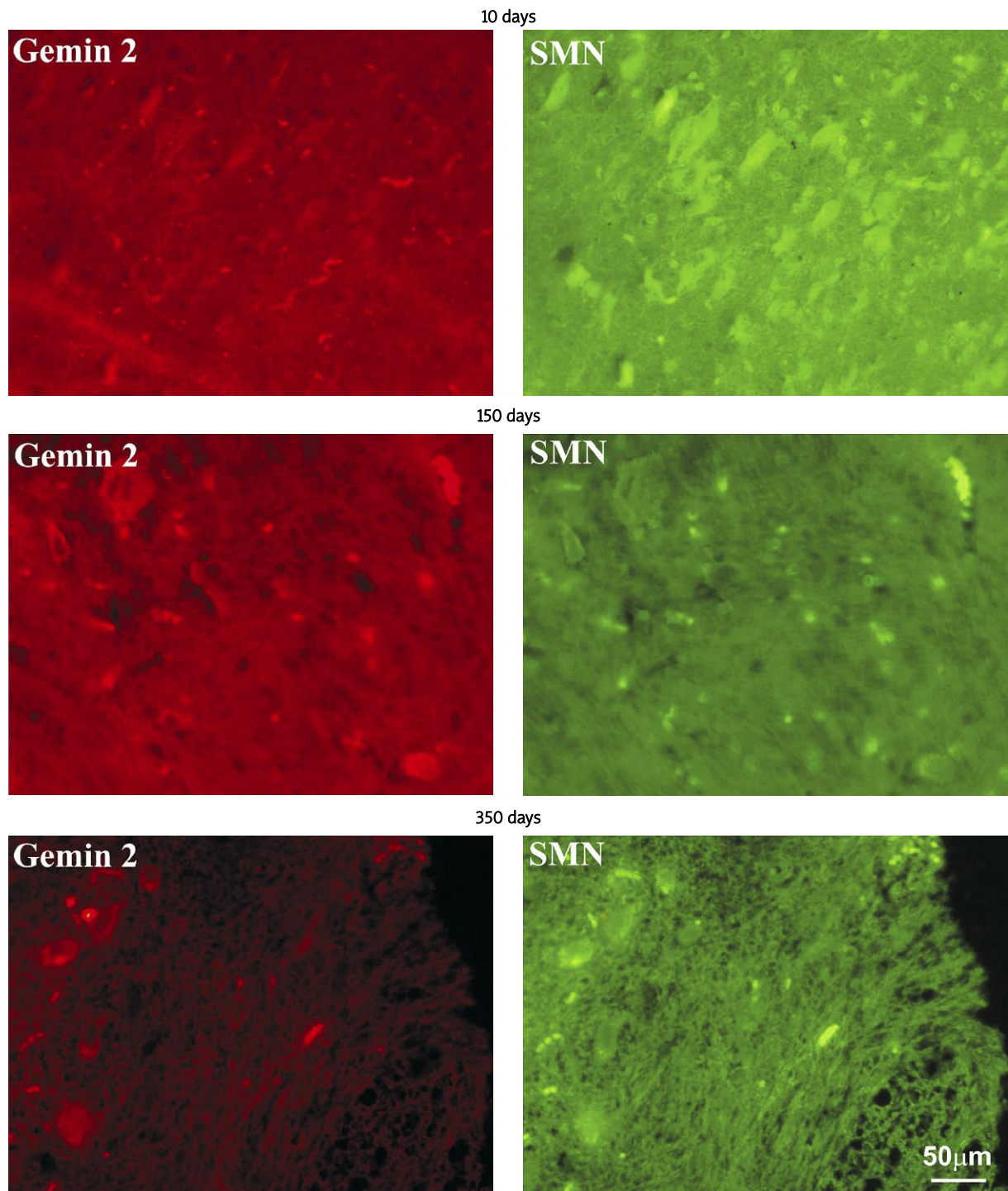
A double staining technique for immunofluorescence revealed the colocalization of SMN protein with gemin 2 and gemin 4 within individual spinal cord neurons (Figs. 3 and 4).

## Discussion

Our experimental study on rats at different age revealed that – similarly to gemin 3 [19] – gemins 2 and 4 are expressed in the same motoneurons as SMN protein. Since the immunoreactivity for the investigated proteins was observed in all examined animals it suggests that in healthy rats the SMN complex is active from the animal birth to its death. Although the immunolabeling for gemins 2 and 4 was various in different neurons and even some of them were immu-

nonegative, general results of the immune reactions in young and old animals showed slightly decreased immunoreactivity for the investigated gemins in old rats. Therefore, our investigations imply that aging probably has only a little influence on the expression of the investigated proteins.

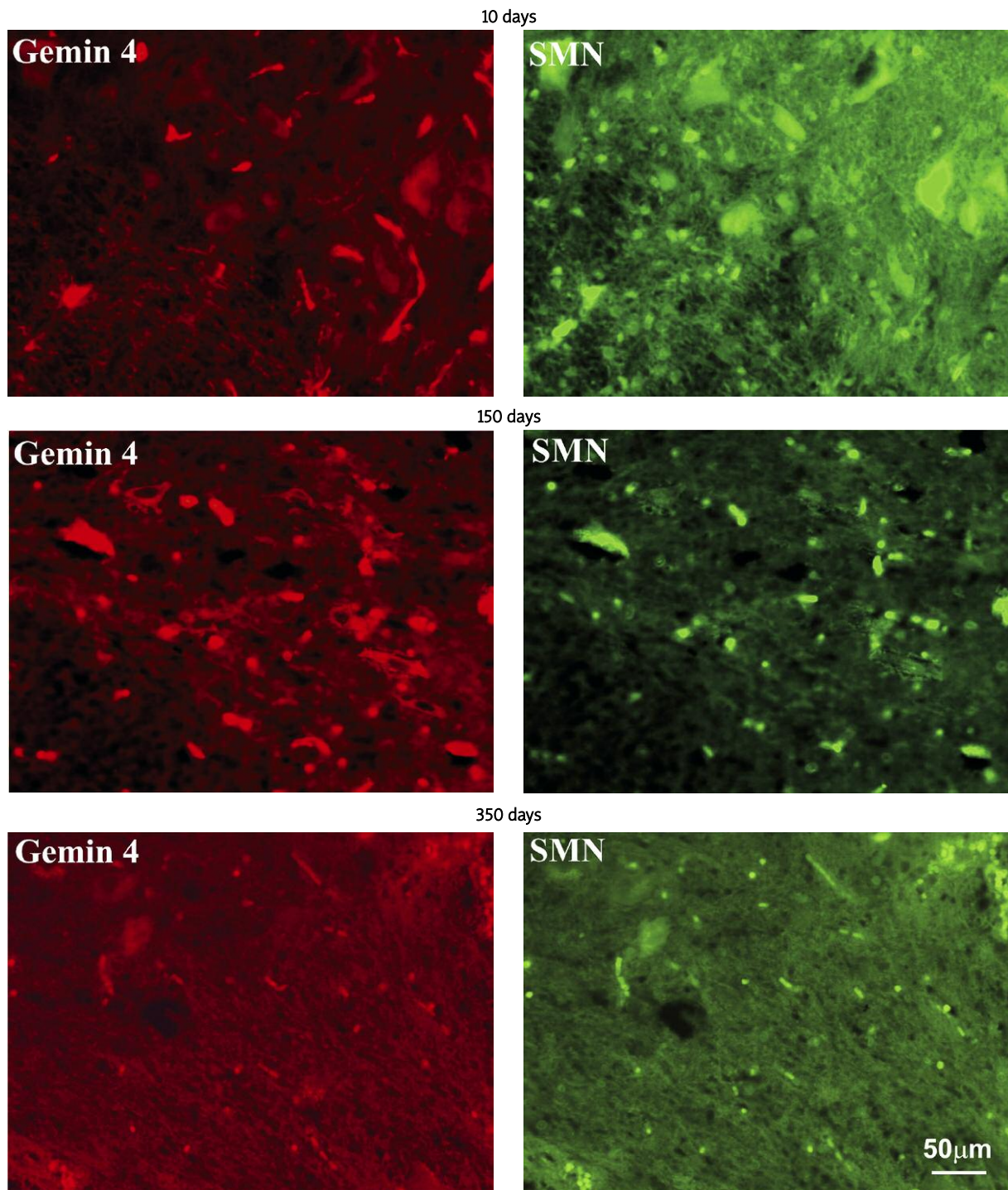
Gemin 2 and 4 as compounds of the SMN complex may play a role in sALS pathogenesis. Their participation seems to be reasonable because aberrant RNA processing is regarded as one of the molecular mechanisms of the disease, similarly to axonal transport defects, glutamate excitotoxicity, mitochondrial dysfunction or deficient protein quality control. But the relationship between the SMN complex and sALS has not been established yet. Investigations revealed that an increased number of *SMN* gene copies can be a risk factor for sALS [3]. On the other hand, results of the experimental studies on SMA and fALS indicate that presence of the SMN protein makes a course of these diseases more benign [13,24]. A wild allele of *SMN* genes plays a role in genetics of sALS [4]. In a mouse model of ALS, deficiency of the SMN gene



**Fig. 3.** Double immunofluorescent reaction for gemin 2 and SMN reveals co-expression of the proteins in the same motoneurons.

enhances the disease progression [20]. Deficiency of the SMN protein also causes severity of sporadic ALS [21]. In human sALS the level of the SMN protein, which

was investigated in anterior horn cells was reduced [18]. Therefore, it seems possible that non mutated *SMN* gene plays a protective role for motoneurons. But why



**Fig. 4.** Double immunofluorescent staining for gemin 4 and SMN shows colocalization of the proteins.

does a normal *SMN* gene not protect motoneurons in sALS? Perhaps not *SMN* alone but other compounds of the *SMN* complex like gemins prevent motoneurons from degenerating.

Although the role of the *SMN* gene and *SMN* protein in sALS is intensively investigated there is scarce data in the literature concerning functions of particular gemins in the *SMN* complex and sALS. Even in

recently published handbooks [7], reviews [14,22], experimental [5,15,20] and human [3,18] investigations, there are no references concerning that issue. Therefore, we hope that our experimental study on immunoexpression of the selected gemins in healthy rats will initiate a series of investigations on new factors which could participate in very complex pathogenesis of ALS. Such investigations are important because they can offer novel opportunities to understand this dramatic disease.

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