Research article

Regulation of stearoyl-CoA desaturase-1 after central and peripheral nerve lesions

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Abstract

Background: Interruption of mature axons activates a cascade of events in neuronal cell bodies which leads to various outcomes from functional regeneration in the PNS to the failure of any significant regeneration in the CNS. One factor which seems to play an important role in the molecular programs after axotomy is the stearoyl Coenzyme A-desaturase-1 (SCD-1). This enzyme is needed for the conversion of stearate into oleate. Beside its role in membrane synthesis, oleate could act as a neurotrophic factor, involved in signal transduction pathways via activation of protein kinases C.

Results: *In situ* hybridization and immunohistochemistry demonstrated a strong up-regulation of SCD at mRNA and protein level in regenerating neurons of the rat facial nucleus whereas non-regenerating Clarke's and Red nucleus neurons did not show an induction of this gene.

Conclusion: This differential expression points to a functionally significant role for the SCD-1 in the process of regeneration.

Background

It is well known that damaged nerve fibers in the peripheral nervous system (PNS) have the capacity for complete regeneration followed by the restoration of appropriate synaptic connectivity and full functional recovery. However, damaged nerve fibers in the central nervous system (CNS) only demonstrate an abortive sprouting response without any functional regeneration. Up to now, little is known about the molecular programs leading to these fundamental differences. To gain insight into the molecular differences, we have compared the differentially regulated genes of axotomized PNS neurons to those of axotomized CNS neurons by differential display polymerase chain reaction [1]. In the following study, we concentrate on one clone which corresponds to the 3' end of the rat stearoyl-Coenzyme A desaturase-1 (SCD-1).

The first mammalian cDNA of this enzyme was isolated in 1986 from rat liver [2]. Subsequently, two different SCD isoforms (SCD-1 and SCD-2) were identified in mouse 3T3-L1 adipocytes [3,4]. Zheng et al. identified a third SCD isoform (SCD-3) which is exclusively present in skin [5] and most recently Miyazaki et al. identified a novel heart-specific isoform (SCD4)[6].

Stearoyl-CoA desaturase is responsible for the rate limiting step in the ?^{9}-cis desaturation of a spectrum of methylene-interrupted fatty acyl-CoA substrates [7]. One of the preferred substrates, stearoyl-CoA, is desaturated, resulting in oleoyl-CoA which is further converted into its corresponding fatty acid oleate. Beside its function as an energy store in the form of triacylglycerides and its presence in biological cell membranes, several studies may show an involvement of oleate in second messenger cascades. Biochemical studies demonstrated the activation of protein kinase C (PKC) by fatty acids and the subsequent phosphorylation of regeneration-associated genes such as the 43 kilodalton growth associated protein (GAP-43) [8,9]. Recently, *in vitro* studies showed that oleate could promote axonal outgrowth and neuronal clustering [10]. This induction of neuronal outgrowth was coupled with the activation and accumulation of GAP-43 by a PKC dependent mechanism [11].

Here, we describe the up-regulation of SCD-1 on the mRNA and protein level in the regenerating facial and hypoglossal nucleus. In contrast, SCD-1 could neither be detected in axotomized Clarke's nucleus nor in Red nucleus neurons.

Results
PNS injury
Radioactive ISH demonstrated a strong induction of SCD-1 mRNA in neurons of the operated facial nucleus starting at 3 days post operation (dpo), the earliest survival time investigated. Figure 1 shows dark field (Fig. 1A) and bright field (Fig. 1B) microscopy of the facial nucleus 3 dpo. Numerous strongly labeled cells (white arrows) were visible which could be identified as motor neurons (black arrows) by bright field microscopy (Fig. 1B). High power magnification of axotomized facial nucleus neurons, as shown in Figure 1C and 1D (7 dpo) verified the cell-specific SCD-1 expression.

In contrast, no significant SCD-1 mRNA could be detected in non-axotomized neurons (black arrow) of the control side 3 days p.o. (Fig. 1E,1F). Dark field analysis of these cells (Fig. 1E) displayed a homogenous distribution of the silver grains.

No specific signal could be obtained using the sense probe at this or any other time point (Fig. 2A and 2B). Sham operated and control animals displayed no induction of SCD-1 mRNA in the facial nucleus (Fig. 2C and 2D).

To quantify the up-regulation of SCD-1 mRNA in axotomized facial nucleus neurons, we measured the signal intensity of operated and non-operated neurons. To avoid experiment-related differences in the intensity (e.g. due to different exposure times), we analyzed all slides separately. Table 1 shows the intensity of *in situ* hybridization signal of the operated and non-operated side per survival time. Two different distributions between the two groups can be observed. First, repeated measure analysis yielded significant differences with respect to the measured intensity between operated and non-operated side (p < 0.0001) with a clear up-regulation on the operated side. Second, univariate analysis of operated and non-operated side shows significant differences on the operated side with respect to the measured intensity between the different survival times (p = 0.0025) while no significant time-effect could be proven on the non-operated side (p = 0.2234). This explains the existing significant interaction between both sides, operated as well as non-operated, and the different survival times (p = 0.0003), i.e. the measured intensity changes over time in dependence on the respective side.

To illustrate the quantification data, means of intensity were plotted against the survival time (Fig. 3). Up-regulation of SCD-1 mRNA was first observed at the first time point investigated (3 dpo) and was evident up to 42 days, with a peak at 7 dpo. At 84 days after lesion, values had returned to basal levels.

To prove that the up-regulation of SCD-1 mRNA is a general response to axotomy of cranial motoneurons, we used the hypoglossal nerve transection model as a second PNS axotomy paradigm. In accordance with the results in the facial nucleus model, SCD-1 mRNA was only detected in axotomized hypoglossal neurons (Fig. 4A and 4B, right side was transected). Again, no specific signal was visible using the sense probes (data not shown).

To extend the results obtained by ISH to the protein level, immunohistochemistry was performed using a polyclonal antibody against the SCD isoforms. At 7 and 14 dpo a strong up-regulation of the SCD protein in axotomized facial nucleus neurons could be observed. Figure 5 illustrates this up-regulation within the operated facial nucleus 7 dpo (Fig. 5A). High power magnification (Fig. 5C) demonstrated the cytoplasmic staining in every visible neuron (white arrows). In contrast, non-operated facial nucleus neurons expressed only basal levels of SCD protein (Fig. 5B and 5D). No signal could be detected by omitting the primary antibody (Fig. 2E).
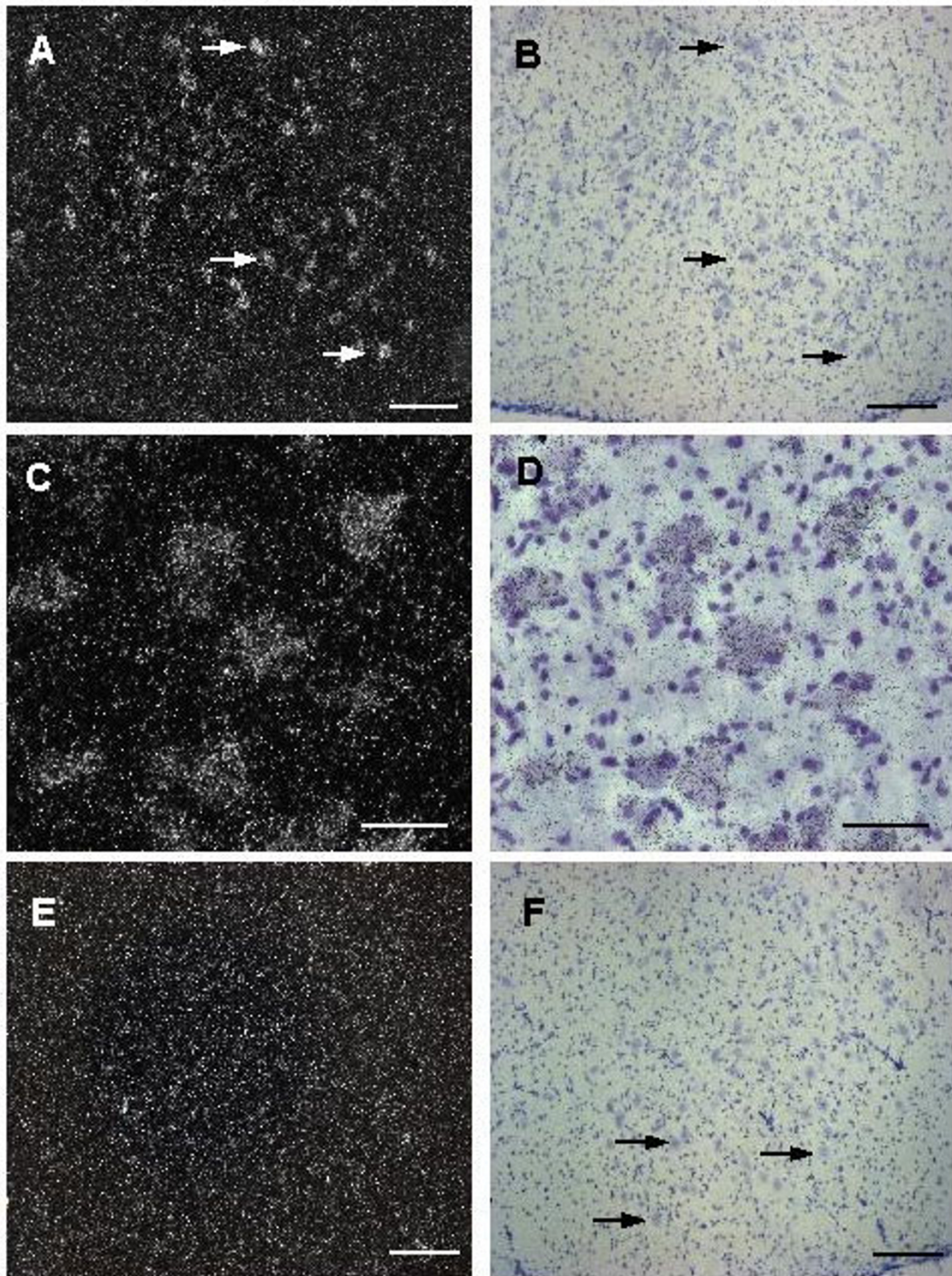
Figure 1
SCD-1 specific radioactive in situ hybridization of facial nucleus neurons 3 and 7 dpo. A and B show the operated side 3 dpo; numerous strongly labeled cells (arrows) are visible in dark filed microscopy (A) which were identified as neurons by bright field microscopy of the same area (B, arrows). High power magnification of axotomized facial nucleus neurons 7 dpo (C and D) verifies the neuron-specific SCD-1 expression due to their large diameter and characteristic thionine staining. No SCD-1 mRNA induction could be identified in non-axotomized neurons (arrows) of the control side (E and F). Scale bars represent: 200 μm (A, B, E and F), 50 μm (C and D).
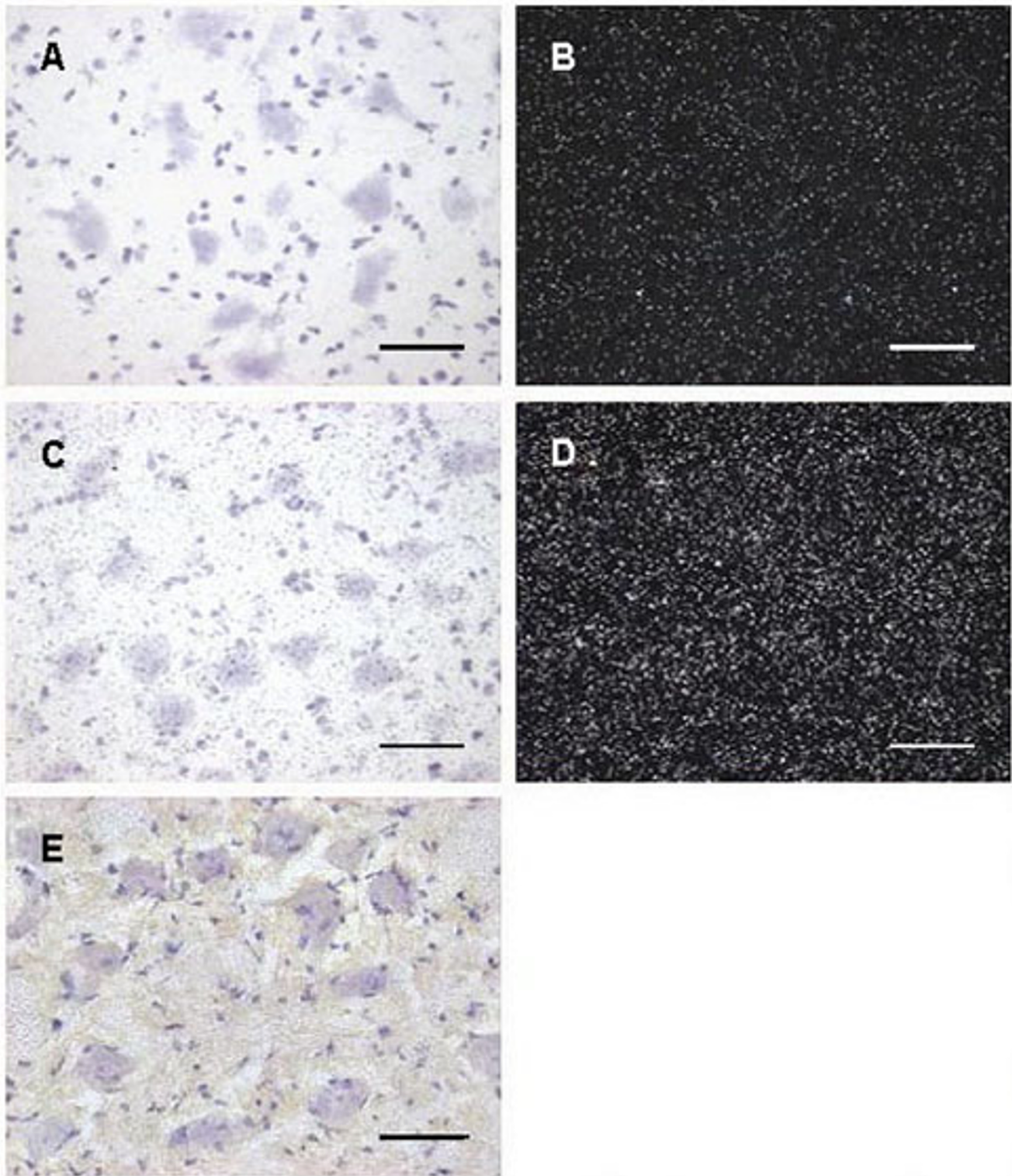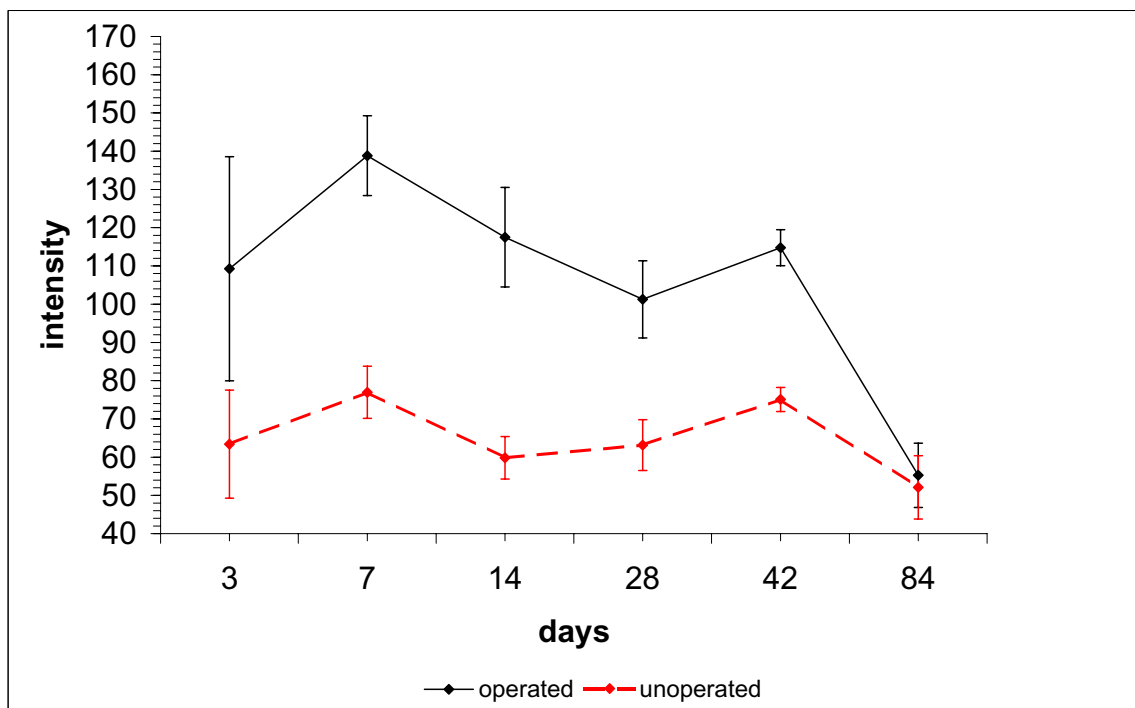
Figure 2
A and B show the operated facial nucleus 3 dpo. Neither in bright field (A) nor in dark field (B) a specific *in situ* hybridization signal could be observed with the sense probe. C and D: In sham operated animals no SCD-1 up-regulation was observed using antisense probes. E shows the negative control of the immunohistochemistry, no signal could be detected by omitting the primary antibody. Scale bars represent: 50 μm.

Table 1: Characteristics of operated and non-operated facial nucleus neurons per days post operation (dpo)

| dpo	Side	N	Mean	SEM	Minimum	Maximum
3	operated	3	109.25	29.31	70.28	167.87
	unoperated	3	63.41	14.13	47.19	91.56
7	operated	3	138.83	10.44	117.99	150.40
	unoperated	3	76.96	6.82	69.36	90.57
14	operated	3	117.50	13.02	94.62	139.71
	unoperated	3	59.83	5.56	49.51	68.56
28	operated	3	101.26	10.11	83.99	119.02
	unoperated	3	63.15	6.64	50.18	72.13
42	operated	3	114.77	4.69	105.89	121.81
	unoperated	3	75.08	3.17	69.45	80.44
84	operated	3	55.25	8.42	44.87	71.93
	unoperated	3	52.11	8.29	40.25	68.07

Mean, SEM (standard error of the mean), Minimum and Maximum corresponds to the intensity of the *in situ* hybridization signal.

Figure 3
Quantification of the radioactive in situ hybridization signal shows the means of intensity ± SEM for the operated and non-operated side. The operated side (continuos line) shows a slight drop at 28 days compared to 42 days, which is due to a shorter exposure time of these slides indicated by a lower constitutive expression of the non-operated side (dotted line).
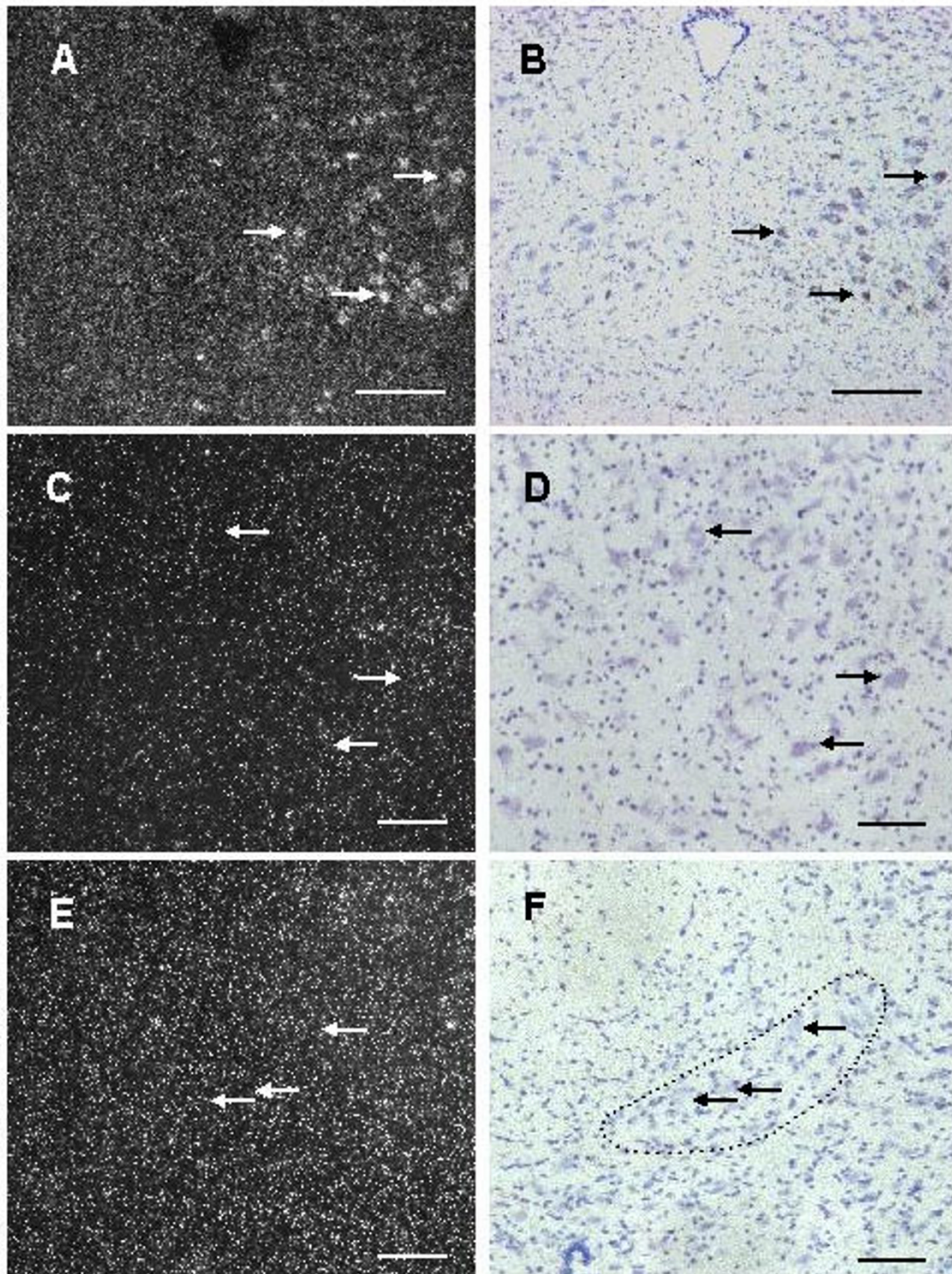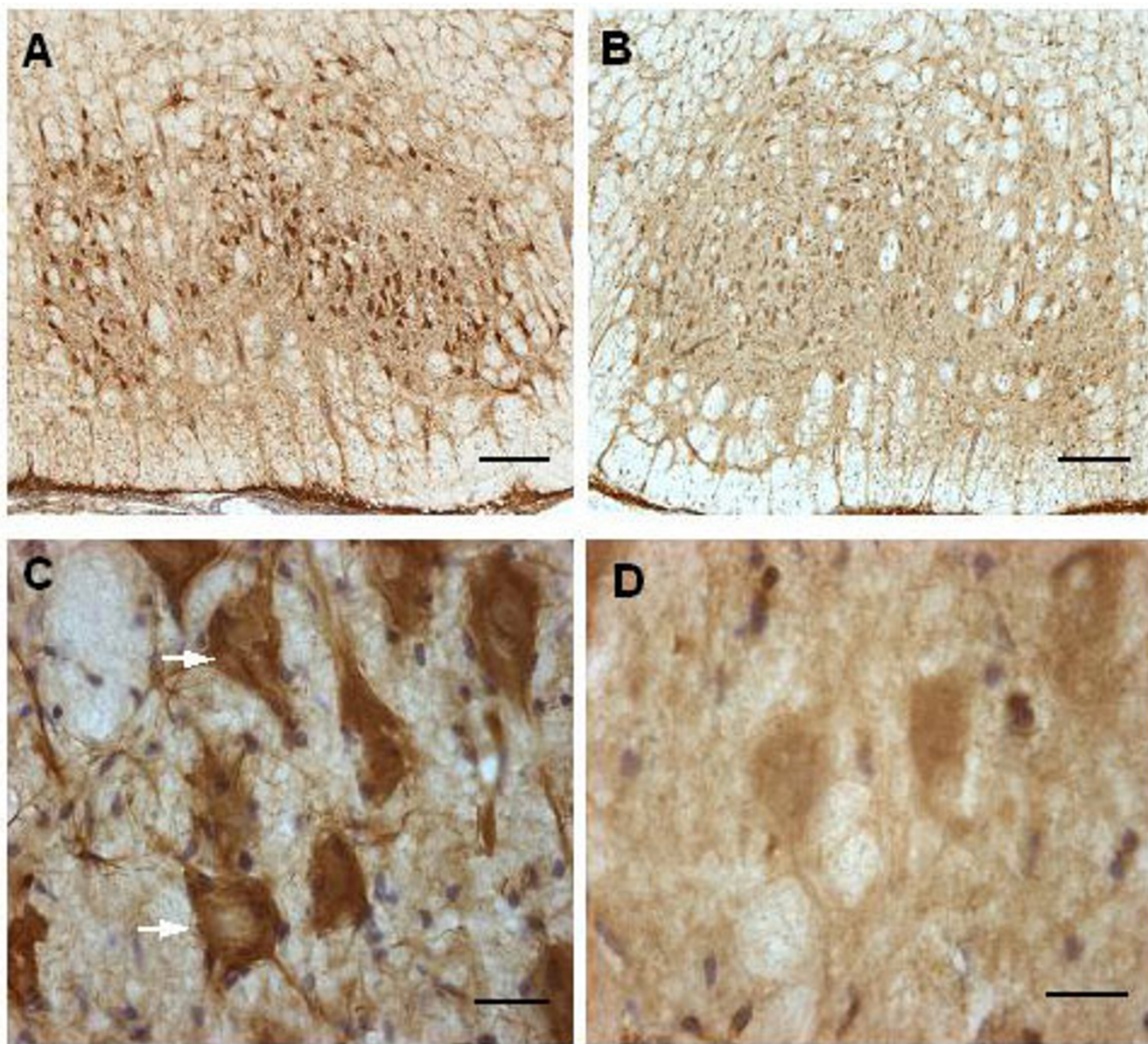
Figure 4
A and B show the hypoglossus model. SCD-1 mRNA could be only detected in axotomized neurons (arrows). C and D show axotomized Red nucleus neurons. No up-regulation of SCD-1 mRNA could be observed in dark (C) or in bright (D) field analysis. E and F show the Clarke's nucleus (dotted line); no up-regulation could be observed in the axotomized neurons. Arrows indicate the position of neurons in the corresponding bright field microscopy. Scale bars represent 200 μm (A, B) and 50 μm (C-F).

Figure 5
Immunohistochemistry against the SCD. A shows operated facial nucleus. Numerous immunoreactive cells are visible. In high power magnification (C), these cells could be identified as neurons (white arrows) due to their typical phenotype. B and D show the non-operated facial nucleus. No up-regulation of SCD could be identified. Scale bars represent 200 µm (A, B), 20 µm (C, D).

CNS injury
By using ISH, SCD-1 mRNA could not be detected at any time point (3, 7, 14 and 28 dpo) in axotomized as well as in non-axotomized Clarke's and Red nucleus neurons (Figs. 4C,4D,4E,4F).

Figure 4E and Fig. 4F show axotomized Clarke's nucleus neurons 7 dpo. A specific hybridization signal could neither be detected in dark field (Fig. 4E) nor in bright field (Fig. 4F) analysis. No specific signal was detected using the sense probe (data not shown).

Discussion

One major area of interest in spinal cord research is the identification of regeneration-associated genes which are responsible for the success or failure of functional neuronal regeneration. Here, we describe the differential expression pattern of SCD-1 in response to axotomy of PNS and CNS neurons. SCD-1 mRNA and protein was highly expressed in axotomized neurons of the regenerating facial and hypoglossal nucleus. This up-regulation was already detectable at the earliest survival time studied (3 dpo), peaked at 7 to 14 dpo and was hardly visible at 84 dpo. In contrast, mRNA was not induced in non-regenerating axotomized Clarke's nucleus and Red nucleus neurons, nor in non-axotomized control neurons.

This induction of SCD-1 is in line with other reports which indicate that the terminal component of the fatty acyl desaturase system (i.e. the stearoyl-CoA desaturase) and not the preceding components (NADH cytochrome b_5 reductase and cytochrome b_5) appears to be under regulatory control, as seen in developmental, hormonal or metabolic processes [12,13].

Regarding the functional role of the up-regulation of SCD-1 in regenerating motoneurons, two possible functions can be discussed: 1. the production of phospholipids as cell membrane components, 2. an involvement in signal transduction pathways.

In the context of the first hypothesis, one can assume that the induction of SCD-1 is necessary for the process of regeneration of peripheral neurons as neuronal outgrowth is always coupled with the new synthesis of cell membranes. This hypothesis is supported by the fact that oleate, which is the major product of SCD-1, is a major structural fatty acid in adult cell membranes, accounting for up to 30% of the fatty acid content of the membrane. Furthermore, it is one of the most abundant fatty acids in developing neuronal growth cones and synaptosomes [14,15]. Recently, in vitro studies could show that astrocyte-derived oleate was incorporated into newly formed neuronal phospholipids in outgrowing neuronal cell cultures [16].

Regarding the second hypothesis, experimental data point to an involvement of the SCD-1 in signal transduction pathways. Biochemical studies demonstrated the activation of PKC by free fatty acids [8,17]. In particular, oleate and arachidonic acid were shown to activate the a, ß and ? subtypes of PKC with a subsequent phosphorylation of the regeneration-associated protein GAP-43 [9]. *In vivo* studies already demonstrated the involvement of GAP-43 in the process of facial nerve regeneration [18]. Recently, *in vitro* studies could also show the activation of PKC by oleate in neuronal cell cultures [16]. This activation led to the accumulation and activation of GAP-43 in outgrowing neurons, indicating that oleate could act as a neurotrophic factor [11,19]. Additionally, Schaechtner and Benowitz investigated the phosphorylation GAP-43 via PKC activation in nerve terminal membranes. This activation was stimulated by arachidonic acid as well as oleate [20]. These studies suggest a route whereby SCD-1 and its product oleate can directly affect the process of regeneration. The oleate could be produced by the neurons themselves as shown in our model or derive from glial cells mainly astrocytes as shown in previous investigations [10].

Conclusion

Taken together, we demonstrate for the first time an involvement of the SCD-1 in the context of neuronal regeneration. Beside an involvement in the production of cell membrane components, the enzyme also play an important role in signal transduction pathways via the activation of PKC and subsequent phosphorylation of downstream proteins which are known to be involved in neuronal outgrowth.

Methods
Surgery
Adult Sprague Dawley rats were used for all experiments. CNS lesions were performed by a lateral funiculotomy of the spinal cord at spinal vertebra C2 or Th9 respectively. For lesions of the PNS, the right facial nerve was transected at the foramen stylomastoideum or the right hypoglossal nerve was transected below the tendon of the digastric muscle. Sham operated animals were used as controls. Three rats were used for each survival time (3, 7, 14, 28, 42, and 84 days) and three unoperated animals were used as further controls. All lesions were performed as described earlier [1].

In situ hybridization and immunohistochemistry
The cDNA fragment of 350 bp containing the 3'end of the SCD-1 mRNA (Ass. nr.: J02585) was subcloned into the p-zero cloning vector (Invitrogen) according to the manufacturer's protocol. No significant homology to other genes, including SCD2, SCD3 or SCD 4 was found. Transcription and labeling reaction was carried out according to the manufacturer's protocol (Roche Diagnostik) using 30 µCi of ^{35}s-a SUTP (Amersham Pharmacia). *In situ* hybridization (ISH) and immunohistochemistry were performed as described earlier [1]. The polyclonal antiserum against the C terminus of the SCD [21] was used at a dilution of 1:1000.

Statistical analysis
For quantification of the *in situ* hybridization signal, the quantification software KS300 (Zeiss; Göttingen) was used. A minimal number of 3 sections per animal and at least 3 animals per survival time were analyzed. Facial

nucleus neurons were identified in bright field microscopy by their size and characteristic morphological profile. The mean intensity of the signal caused by the presence of the silver grains (belonging to the previously identified neurons) was subsequently analyzed in dark field analysis. This procedure allowed for the cell specific measurement of the radioactive *in situ* hybridization signal.

A mean intensity value for each animal was calculated for the operated and non-operated side separately because of different numbers of sections per animal. A generalized linear model (GLM) with respect to repeated measurements was then fitted to the data to investigate side to side effects. The comparison of sides, operated and non-operated, was carried out using the F-test; p-values of less than or equal to 0.05 were regarded as statistically significant. All statistical analyses were performed using SAS V8.0 Software (SAS Institute Inc., Cary, NC, USA).

Authors' contributions

SB: corresponding author, conceived and coordinated the experiments; KP: performed the immunohistochemistry and the animal experiments; AB: assisted (substantial) in writing the manuscript and the statistical analysis; CS: performed the radioactive in situ hybridization; JO: generated the antibody against the SCD; NH: performed the statistical analysis; EH: generated the ddPCR experiments by which the SCD clone was identified; JN: most of the work was done in his lab, substantial contribution to the experiments and in the discussion of the results; FWS: performed radioactive in situ hybridization (together with CS), assisted by the ddPCR; AS: assisted in coordination of the experiments, writing the manuscript and funding the study

All authors read and approved the final manuscript.

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