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Involvement of brain-derived neurotrophic factor and sonic hedgehog in the spinal cord plasticity after neurotoxic partial removal of lumbar motoneurons

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ABSTRACT

Adult mammals could spontaneously achieve a partial sensory-motor recovery after spinal cord injury, by mechanisms including synaptic plasticity. We previously showed that this recovery is associated to the expression of synapsin-I, and that sonic hedgehog and Notch-1 could be also involved in plasticity. The role of brain-derived neurotrophic factor and glutamate receptors in regulating synaptic efficacy has been explored in the last decade but, although these mechanisms are now well-defined in the brain, the molecular mechanisms underlying the so called "spinal learning" are still less clear.

Here, we measured the expression levels of choline acetyltransferase, synapsin-I, sonic hedgehog, Notch-1, glutamate receptor subunits (GluR1, GluR2, GluR4, NMDAR1) and brain-derived neurotrophic factor, in a motoneuron-depleted mouse spinal lesion model obtained by intramuscular injection of cholera toxin-B saporin. The lesion caused the down-regulation of the majority of analysed proteins. Moreover, we found that in lesioned but not in control spinal tissue, synapsin-I expression is associated to that of both brain-derived neurotrophic factor and sonic hedgehog, whereas GluR2 expression is linked to that of Shh. These results suggest that brain-derived neurotrophic factor and sonic hedgehog could collaborate in modulating synaptic plasticity after the removal of motoneurons, by a mechanism involving both pre- and post-synaptic processes. Interestingly, the involvement of sonic hedgehog showed here is novel, and offers new routes to address spinal cord plasticity and repair.

1. Introduction

It has been widely demonstrated that adult mammals could achieve a partial sensory-motor recovery after spinal cord injury (SCI) by means of various forms of neuroplasticity consisting in the reorganization of spared pathways by mechanisms involving activity-dependent synaptic plasticity. However, part of this recovery could be achieved also spontaneously in sedentary conditions (de Leon et al., 1998; Wolpaw and Tennissen, 2001; Edgerton et al., 2004; Gulino et al., 2007b, 2010b). The molecular feature of synaptic plasticity has been extensively studied in the hippocampus, as it represents the principal mechanism underlying learning and memory. In fact, it is known that long-term modifications of synaptic efficacy are regulated presynaptically by the expression and phosphorylation of various synaptic vesicle proteins including synapsin-I (Greengard et al., 1993; Hilfiker et al., 1999; Gulino et al., 2007b, 2010b) and postsynaptically by changes in the expression and trafficking of glutamate receptors (Lisman, 2003; Mellor, 2006; Rao and Finkbeiner, 2007). In particular, both N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors are fundamental for cortical and hippocampal synaptic plasticity, although with distinct roles (Rao and Finkbeiner, 2007; Kessels and Malinow, 2009; Keifer and Zheng, 2010; Rebola et al., 2010). A fundamental role in modulating both pre- and post-synaptic changes is exerted by brain-derived neurotrophic factor (BDNF) (McAllister et al., 1999; Huang and Reichardt, 2001; Kohara et al., 2001; Vaynman et al., 2003). In fact, synapsin-I is considered as a downstream effector of BDNF (Jovanovic et al., 2000; Gómez-Pinilla et al., 2001a; Vaynman et al., 2003). Moreover, it seems clear that the activity-dependent release of BDNF could regulate the synthesis and synaptic delivery of glutamate receptors in different brain areas (Narisawa-Saito et al., 1999; Madara and Levine, 2008; Li and Keifer, 2009) and, conversely, the glutamate receptor activity could modulate BDNF release (Jourdi et al., 2009; Clarkson et al., 2011).

More recently, other molecules traditionally considered as morphogenetic factors, such as sonic hedgehog (Shh) and Notch-1

Abbreviations: BSA, bovine serum albumin; BDNF, brain-derived neurotrophic factor; CTB-SAP, cholera toxin-B saporin; NMDAR1, N-methyl-D-aspartate receptor 1; PBS, phosphate buffered saline; SC, spinal cord; SCI, spinal cord injury; SDS, sodium dodecyl sulphate; Shh, sonic hedgehog.

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(Traiffort et al., 1999; Androutsellis-Theotokis et al., 2006; Fuccillo et al., 2006; Breunig et al., 2007), have also been implicated in synaptic plasticity (Wang et al., 2004; Costa et al., 2005; Gulino et al., 2010b).

The mechanisms underlying spinal cord (SC) plasticity could be similar to those described above (Gómez-Pinilla et al., 2001b, 2002, 2004; Gulino et al., 2004, 2007b, 2010b; Chen et al., 2005; Ying et al., 2005). However, many experimental evidence are still necessary to better clarify the molecular mechanisms underlying the so called "spinal learning", with particular attention to the glutamate receptors, whose role in the SC plasticity is still unclear, and to understand the possible relationship between the above described plasticity-related factors.

Here, we have used a murine model of selective lumbar motoneuron depletion obtained by injection of the retrogradely transported, ribosome inactivating toxin, cholera toxin-B saporin (CTB-SAP) into the gastrocnemius muscle (Llewellyn-Smith et al., 2000; Wiley and Kline, 2000; Fargo and Sengelaub, 2004; Gulino et al., 2010b). After the histological characterization of the lesion, the expression levels of synapsin-I and glutamate receptors have been evaluated in relation to those of BDNF, Shh and Notch-1.

2. Materials and methods

2.1. Neurotoxic lesion and experimental groups

Young adult male mice ($n = 44$) (Charles River, Strain 129, 5 weeks aged, weight: 20–25 g) were used. Animal care and handling were carried out in accordance with the EU Directive 2010/63/EU, and have been approved by the Ethical Committee at the University of Catania (IACUC). The animals were housed in groups of three or four per cage, under standard conditions of temperature, light and humidity, with ad libitum access to water and food. They were randomly allocated into four experimental groups as described below. Surgical procedures were performed under aseptic conditions, with the animals under deep anaesthesia by ketamine/xylazine (10 mg ketamine/2 mg xylazine per 100 g body weight, i.p.). All efforts were made to minimize the number of animals used and their discomfort.

Anaesthetized mice received CTB-SAP (Advanced Targeting Systems, San Diego, CA, USA) unilaterally or bilaterally in the medial and lateral gastrocnemius muscles at a dose of 3.0 μg/2.0 μl PBS per muscle as described previously (Gulino et al., 2010b). CTB-SAP is a retrogradely transported, ribosome-inactivating toxin (Llewellyn-Smith et al., 2000; Wiley and Kline, 2000), and its efficiency in producing a selective motoneuron depletion after injection in the target muscle has been proven (Fargo and Sengelaub, 2004; Gulino et al., 2010b), thus representing an effective model of primary neurodegeneration.

In the present experiment, a first group of mice received a bilateral injection of CTB-SAP (total dose: 12.0 μg) and were sacrificed by decapitation one week later (LES, $n = 18$). Similarly, a second group of mice received a bilateral injection of an equal volume of PBS only and were sacrificed one week later (SHAM; $n = 12$). A third group of animals were left untreated and used as normal controls for Western blot analyses (NC; $n = 10$). For histological analyses, other animals received a unilateral injection of CTB-SAP (total dose: 6.0 μg) into the right medial and lateral gastrocnemius (LES-right, $n = 4$). These animals were injected unilaterally in order to have an internal control for the effect of the neurotoxin. They were sacrificed one week after the lesion.

2.2. Western blotting

All animals with bilateral injection as well as NC were sacrificed by decapitation, under deep anaesthesia. Lumbar SCs were rapidly dissected out and frozen in isopentane cooled in liquid nitrogen, and then stored at −80 °C until analysis.

Tissue samples were homogenized in 10 volumes of cold RIPA lysis buffer (50 mM Tris pH 8.0; 150 mM NaCl; 2 mM EDTA; 1% NP-40; 0.5% sodium deoxycholate; 1% sodium dodecyl sulphate (SDS); 5 μg/ml aprotinin; 1 μg/ml pepstatin; 5 μg/ml leupeptin; 88 μg/ml PMSF) using a glass homogenizer (Potter-Elvehjem, USA) and centrifuged at 10,000 rpm for 20 min at 4 °C. Then, supernatants were collected, aliquoted and stored at −80 °C until analysis. Total protein concentrations were estimated by colorimetric assay (Bradford method) using bovine serum albumin (BSA) as standard. Prior to gel loading, samples were diluted 1:1 with loading buffer 2× (125 mM Tris pH 6.8; 10% glycerol; 4% SDS; 10% β-mercaptoethanol; 0.005% bromophenol blue) and denatured at 100 °C for 5 min.

For Western blot quantification, 20 μg of protein was separated on a 4–20% polyacrylamide gel (Bio-Rad Laboratories, Milano, Italy; Cat. No. 456-1093) in Tris–glycine running buffer (25 mM Tris; 250 mM glycine; 0.1% SDS; pH 8.3) and electrotransferred to a nitrocellulose membrane (Schleicher and Schuell BioScience GmbH, Germany) for 2 h in Tris–glycine transfer buffer (48 mM Tris; 39 mM glycine; 0.02% SDS, 20% ethanol; pH 9.2).

Membranes were blocked overnight at 4 °C with 5% BSA and incubated for 1 h with the primary antibodies, as described in Table 1. Then, membranes were rinsed six times in wash buffer (10 mM Tris pH 7.5; 100 mM NaCl; 0.1% Tween-20) for 5 min and incubated for 1 h with the appropriate peroxidase-conjugate antibodies (Table 1). Peroxidase activity was developed by enhanced chemiluminescent substrate (Pierce Biotechnology Inc., Thermo Scientific Group; Cat. No. 34075) and visualized on a film (Kodak). Then, membranes were blocked again with BSA, and the protocol was repeated for quantification of actin, using a mouse anti-actin primary antibody (Chemicon International, Millipore Group; Cat. No. MAB1501; dilution 1:700) followed by a goat anti-mouse secondary antibody (Pierce Biotechnology Inc., Thermo Scientific Group; Cat. No. 1858413; dilution 1:5000).

The films were digitally scanned and the relative 300 dpi greyscale images were used for optical density (OD) measurement by using Scion Image software (Scion Corporation, USA). The background level was measured on each film, as a mean value measured in several areas of the film, away from the protein bands, and subtracted from the density values of each protein band. Density values relative to all proteins were normalized to actin levels measured in the same membrane. All assays were performed in triplicate.

2.3. Histology and immunohistochemistry

After completion of the survival period, the unilaterally injected animals were deeply anaesthetized and perfused transcardially with 40 ml of room temperature saline followed by 100 ml of ice-cold phosphate-buffered 4% paraformaldehyde (pH 7.4). The lumbar SC was dissected out, postfixed for 1 h and then soaked for 24 h into a phosphate-buffered 20% sucrose solution at 4 °C. Then, the tissue was cut horizontally at 20 μm thickness on a freezing microtome, and the sections were collected into 4 series. One series (i.e. every fourth section) was used for cresyl violet staining, while the other series were used for double-labelling immunofluorescence by using the primary antibodies as described in Table 2.

In brief, sections were mounted on gelatine-coated slides, incubated for 30 min in 5% normal donkey serum and 0.4% Triton X100 in PBS and then overnight at room temperature with the primary antibody solution containing 0.3% Triton X100 and 2% normal donkey serum. Then, sections were washed in PBS and incubated for 1 h with the appropriate Alexa Fluor 488 or 568 donkey anti-rabbit, anti-mouse or anti-goat secondary antibodies (Invitrogen Ltd., UK, dilution 1:500), in PBS plus 2% normal donkey serum and 0.3% Triton X100. After washing in PBS, sections were counterstained for

Table 1
Primary antibodies and the relative secondary antibodies used in Western blotting.

Primary antibody	Distributor and Cat. No.	Dilution	Secondary antibody	Distributor and Cat. No.	Dilution
Mouse anti-ChAT	Immunological Sciences, Roma, Italy; Cat. No. MAB10838	1:400	Peroxidase-conjugate goat anti-mouse	Pierce Biotechnology Inc., Thermo Scientific Group; Cat. No. 1858413	1:6000
Mouse anti-GluR1	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-13152	1:300			
Rabbit anti-synapsin-I	Abcam plc, Cambridge, UK; Cat. No. AB18814	1:500	Peroxidase-conjugate goat anti-rabbit	Pierce Biotechnology Inc., Thermo Scientific Group; Cat. No. 1858413	1:6000
Rabbit anti-NMDAR1	Cell Signaling Technology, Inc., MA, USA; Cat. No. 4204	1:1000			
Rabbit anti-Notch-1 extracellular domain	Upstate Biotechnology, Millipore Group; Cat. No. 07-218	1:700			
Goat anti-BDNF	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-33905	1:200	Peroxidase-conjugate rabbit anti-goat	Chemicon International, Millipore Group; Cat. No. AP106P	1:10,000
Goat anti-Shh precursor	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-1194	1:300			
Goat anti-GluR2	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-7610	1:300			
Goat anti-GluR4	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-13152	1:300			

Table 2
Primary antibodies used in double-labelling immunofluorescence.

1st primary antibody	Distributor and Cat. No.	Dilution	2nd primary antibody	Distributor and Cat. No.	Dilution
Mouse anti-ChAT	Immunological Sciences, Roma, Italy; Cat. No. MAB10838	1:400	Rabbit anti-BDNF	Chemicon International, Millipore Group; Cat. No. AB1534	1:500
Mouse anti-ChAT	Immunological Sciences, Roma, Italy; Cat. No. MAB10838	1:400	Goat anti-Shh precursor	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-1194	1:100
Rabbit anti-synapsin-I	Abcam plc, Cambridge, UK; Cat. No. AB18814	1:100	Goat anti-TrkB	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-20542	1:100
Goat anti-Shh precursor	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-1194	1:100	Rabbit anti-synapsin-I	Abcam plc, Cambridge, UK; Cat. No. AB18814	1:100
Goat anti-Shh precursor	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-1194	1:100	Rabbit anti-GluR2	Abcam plc, Cambridge, UK; Cat. No. AB20673	1:300
Rabbit anti-BDNF	Chemicon International, Millipore Group; Cat. No. AB1534	1:500	Goat anti-Shh precursor	Santa Cruz Biotechnology Inc., Heidelberg, Germany; Cat. No. sc-1194	1:100

5 min with DAPI 1:20,000 (Invitrogen Ltd., UK) in PBS. Slides were coverslipped with Permafluor (Thermo) and stored at 4 °C pending analysis.

2.4. Microscopy and staining quantification

The observation of cresyl violet stained or immunostained SC sections and profile counts were carried out by a Zeiss light and fluorescence microscope coupled with a Zeiss Axiocam camera (Carl Zeiss S.p.A., Arese, Italy). Co-localization studies as well as OD measurements were carried out by using a laser confocal microscope coupled with the appropriate analysis software (Leica Microsystems S.p.A., Milano, Italy).

For all histological measurements, four alternate horizontal SC sections from each animal were analysed at similar rostro-caudal (L3–L5) and dorso-ventral (lamina IX) level. Relative profile numbers on each side were expressed as the average number/section. Only unambiguous immunopositive profiles characterized by an evident nucleus and well defined motoneuronal features were considered. Then, the number of profiles in the ipsilateral (lesioned) side was expressed as percent of the number of profiles counted in the contralateral side.

In order to determine the extent of motoneuronal depletion induced by the neurotoxin, cresyl violet-labelled, as well as ChAT-positive motoneuronal profiles were counted bilaterally in the lesioned compared to the contralateral lumbar SC side. In order to focus on the levels of protein expression in motoneurons, and to also validate histologically the data obtained by Western blot analysis, we have measured the relative OD of ChAT, synapsin-I, GluR2, BDNF and Shh at the cellular level. The average OD was measured from motoneuron profiles outlined manually by the experimenter in high magnification images (400×). The OD measured in the grey matter of a control sections obtained by omitting the primary antibody was used as background staining and subtracted from each measurement. The immunostaining of inter-perikaryal grey matter was evaluated by measuring the average OD of three circular areas (3 cm diameter at 400× magnification) randomly selected within each SC side, at L4–L5 level. The relative OD was expressed as the average OD value calculated between all cells considered in each SC side after background subtraction. Then, the average group values were calculated between all unilaterally lesioned animals ($n = 4$).

2.5. Statistical analysis

Data were analysed either as raw data or as mean ± s.e.m., as appropriate. Differences between lesioned and control groups in Western blot data were evaluated by using Student's *t*-test. Differences between lesioned and untreated SC side in the number of stained cell profiles, or in the relative OD, were analysed by paired Student's *t*-test.

In order to assess whether the expression of synaptic proteins directly involved in synaptic plasticity (synapsin-I, NMDAR1, GluR1, GluR2 and GluR4) could depend from the expression levels of the other analysed proteins, we used the following multivariate regression model:

$$\begin{aligned} \mathrm{P[Protein]} = {} & \beta_0 + \beta_1[\mathrm{BDNF}] + \beta_2[\mathrm{BDNF}]^2 + \beta_3[\mathrm{Shh}] + \beta_4[\mathrm{Shh}]^2 \\ & + \beta_5[\text{Notch-1}] + \beta_6[\text{Notch-1}]^2 + \beta_7[\mathrm{ChAT}] \\ & + \beta_8[\mathrm{ChAT}]^2 + \varepsilon \end{aligned} \quad (1)$$

where P[Protein] is the predicted expression level of one of the five analysed synaptic proteins (synapsin-I, NMDAR1, GluR1, GluR2 and GluR4); the other terms in square brackets are the mean OD values of the relative proteins probably involved in the regulation of synaptic plasticity, as calculated between Western blot replicas for each SC sample and normalized to actin levels; β_0–β_8 represent the regression coefficients and ε is the residual error. As shown in the equation, quadratic terms have been included in the model to account for any non-linearities. ChAT expression levels have also been included in the model to account for any protein variation due to motoneuron depletion.

From this general regression model, we eliminated the non-significant terms using an iterative procedure known as backward stepwise regression. This procedure starts with the complete model and removes iteratively the least significant predictors until only significant variables remain. We used a restrictive α value ($\alpha < 0.05$) for which a given variable was allowed into the model and selected only final models that explained at least 20% of the dataset variance ($R^2 > 0.20$) with a *P*-value of the regression ANOVA less than 0.01. For each model we also examined the relation between the residual errors and the predicted values of protein expression for any signs of systematic trends in the residual variance. Multivariate regression models are the most appropriate statistical method when a given dependent variable could depend from several independent variables, because it provides the best fitting mathematical model, by including only the highly significant predictors into the model, and removing the least significant ones (Gulino et al., 2007b; Valle et al., 2007). All analyses were performed by means of Systat package version 11 (Systat Software Inc., USA).

3. Results

All animals survived surgery and did not show any significant sign of illness, except an animal belonging to the LES group and another belonging to the SHAM group, which died at three and five days after surgery, respectively. Starting at about two days after toxin injection, all mice showed an evident weakness of the injected hindlimb, although they were still able to walk on a horizontal plane. The analysis of cresyl violet stained SC sections from unilaterally lesioned animals demonstrated a statistically significant ($P = 0.02$) 35 ± 9% decrease of the average number of motoneuron profiles in the right (injected), compared to the contralateral side (Fig. 1). This reduction is similar to the decrease of ChAT-positive motoneurons counted in the immunostained sections (38 ± 9%, $P = 0.03$) and also similar to that observed in our previous study (Gulino et al., 2010b). The effects of toxin treatment on cell death, motor function and general health conditions of mice were described in more detail in our previous study (Gulino et al., 2010b).

3.1. Effect of CTB-SAP lesion on protein expression levels

Protein expression in the lumbar SC was measured by Western blotting and the average values of relative OD for each protein

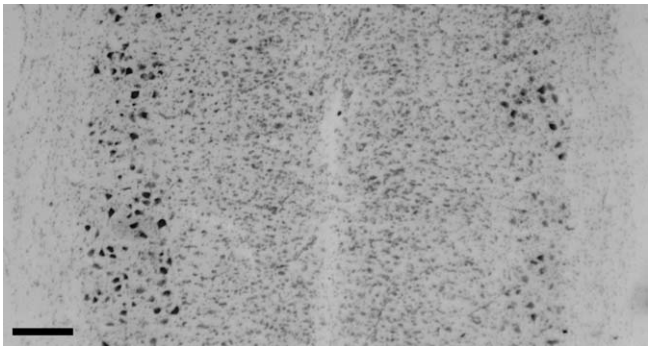

Fig. 1. Light microscope image showing an example of lumbar spinal cord section from a unilaterally lesioned animal, stained with cresyl violet. The effect of CTB-SAP on the number of surviving motoneurons is evident in the lesioned (right), as compared to the contralateral side. The original picture has been converted in greyscale image and adjusted in brightness and contrast. Scale bar: 250 μm.

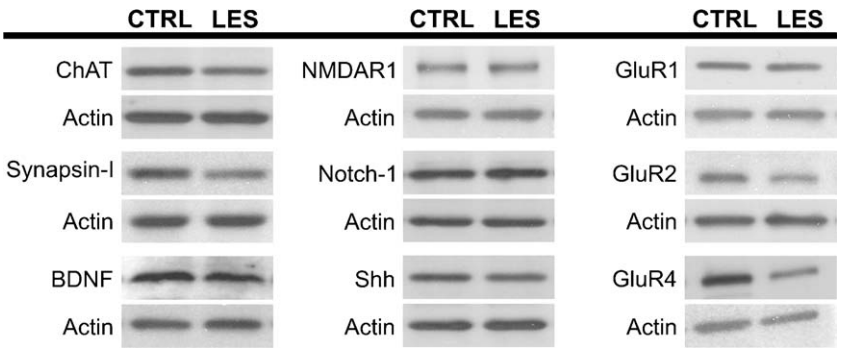

Fig. 2. Examples of Western blots showing immunoreactive bands relative to ChAT, synapsin-I, BDNF, MMDAR1, Notch-1, Shh, and the AMPA receptor subunits GluR1, GluR2 and GluR4, in relation to their corresponding actin signals in control and lesioned mice.

in every group were compared and analysed by Student's *t*-test. Sham lesioned and normal groups did not differ from each other in terms of Western blot data. Therefore, these groups were pooled together in a single control group (CTRL). Data analysis revealed a significant ($P = 0.003$) $23 \pm 5\%$ decrease of the average ChAT expression one week after the lesion in the lumbar SC of bilaterally CTB-SAP lesioned mice, compared to the expression in control animals (Figs. 2 and 3A). Similar results were found for synapsin-I (Figs. 2 and 3B), GluR2 (Figs. 2 and 3H) and GluR4 (Figs. 2 and 3I), which appeared reduced in lesioned animal by $26 \pm 3\%$ ($P = 0.000$), $12 \pm 4\%$ ($P = 0.043$) and $20 \pm 4\%$ ($P = 0.007$), respectively. Conversely, the average expression levels of the other proteins appeared not affected by the lesion, though an evident but not significant reduction of BDNF expression, by $18 \pm 8\%$ ($P = 0.098$) has also been observed.

In order to verify the hypothesis that synaptic plasticity within the SC could be modulated by BDNF and the fate determinants Shh and Notch-1, we used multivariate regression models to correlate the expression levels of synapsin-I or glutamate receptor subunits, which are considered as markers of synaptic plasticity, with those of BDNF, Shh and Notch-1. Moreover, ChAT expression levels were included in the model to account for any relationship with motoneuron depletion itself. After the application of backward stepwise regression to the starting models shown in Eq. (1), we obtained the final equations reported in Fig. 4, which represents the regression models better fitting with the actual values. The application of this method to the CTRL group did not provide any significant correlation (not shown). The results showed that in the lesioned group, the expression of synapsin-I was linked to that of both BDNF and Shh ($R_{17} = 0.814$; $P = 0.004$; Fig. 4A), whereas the expression of GluR2 appeared dependent from that of Shh alone ($R_{17} = 0.748$; $P = 0.003$; Fig. 4D). Conversely, the expression levels of NMDAR1, GluR1 and GluR4 appeared dependent from ChAT expression only (Fig. 4B, C, and E).

3.2. Distribution of protein expression within the lesioned SC

The specific localization of BDNF and its receptor TrkB, synapsin-I, Shh and AMPA receptor subunit GluR2 in the lesioned SC, has been studied by immunohistochemistry and confocal microscopy. The results showed that ChAT-immunopositive neurons are also positive for BDNF (Fig. 5A) and Shh (Fig. 5B). Moreover, we found an evident co-localization of BDNF and Shh in large spinal cells resembling motoneurons (Fig. 5F). Furthermore, Fig. 5C and D shows synapsin-I-positive synaptic terminals distributed around TrkB-positive and Shh-positive large motoneurons, respectively, whereas Fig. 5E shows GluR2-positive large neurons also expressing Shh.

In order to quantify the protein expression levels into the motoneuronal compartment after CTB-SAP lesion, we have measured the relative OD of ChAT, synapsin-I, GluR2, BDNF and Shh into the motoneuronal cytoplasm and cell surface in the lesioned SC side, compared to the contralateral unlesioned side. The results show that the motoneuronal expression of all proteins did not change after lesion (Fig. 6). Notably, ChAT and GluR2 immunoreactivity were increased, although not significantly, by $14 \pm 7\%$ ($P = 0.18$) and $18 \pm 8\%$ ($P = 0.15$), respectively (Fig. 6).

4. Discussion

In our previous study (Gulino et al., 2010b), a neurotoxic SC lesion model was developed in order to study compensatory changes in the SC circuitry after partial depletion of the motoneuronal population. A significant recovery of locomotion has been shown after the post-lesion impairment, and it was probably due to plastic changes occurring within spared motoneurons and their surrounding spinal circuits (Gulino et al., 2010b). This model is useful to evaluate plasticity in the presence of only neurodegenerative processes, without tissue damage or scar formation, and with only a moderate activation of inflammatory mechanisms, known to affect various cell responses such as neurogenesis, fibre sprouting and glial activation (Ekdahl et al., 2003; Kerschensteiner et al., 2004; Klussmann and Martin-Villalba, 2005).

In the present work, the same SCI model was used in order to deepen the investigation of the mechanisms of SC plasticity. Since the linkage between functional recovery and molecular markers of synaptic plasticity has been observed at one week, when recovery was ongoing, but not at one month after the lesion, when functional outcome reached near-normal levels (Gulino et al., 2010b), the present work focused on the events occurring at one week after the lesion. The injection of CTB-SAP into the gastrocnemius muscle determined a partial depletion of the lumbar motoneuron population accompanied by an evident impairment of the hindlimb function. The motoneuron loss was paralleled by a down-regulation of ChAT expression within lumbar SC tissue at one week after the lesion. Given that the majority of acetylcholine release (about 80%) within the SC originates from local motoneuronal activity, whereas the remaining cholinergic activity could be linked to other intraspinal neurons (Phelps et al., 1984; Borges and Iversen, 1986; Gulino et al., 2007a), it is likely that the observed down-regulation of ChAT after the lesion should be caused in large part by the motoneuron loss, and in part by the consequent disruption of spinal circuitry. Thus, it is reasonable that the motoneuron depletion could result in a reduction of the spinal neural activity, which in turn could be responsible for the functional impairment. For a better histological and functional characterization of this and other similar neurotoxic lesion models, see our previous works (Gulino et al., 2004, 2007a, 2010a,b). The decrease of ChAT expression was paralleled by a similar down-regulation of synapsin-I and glutamate receptor subunits GluR2 and GluR4, but not GluR1 and NMDAR1.

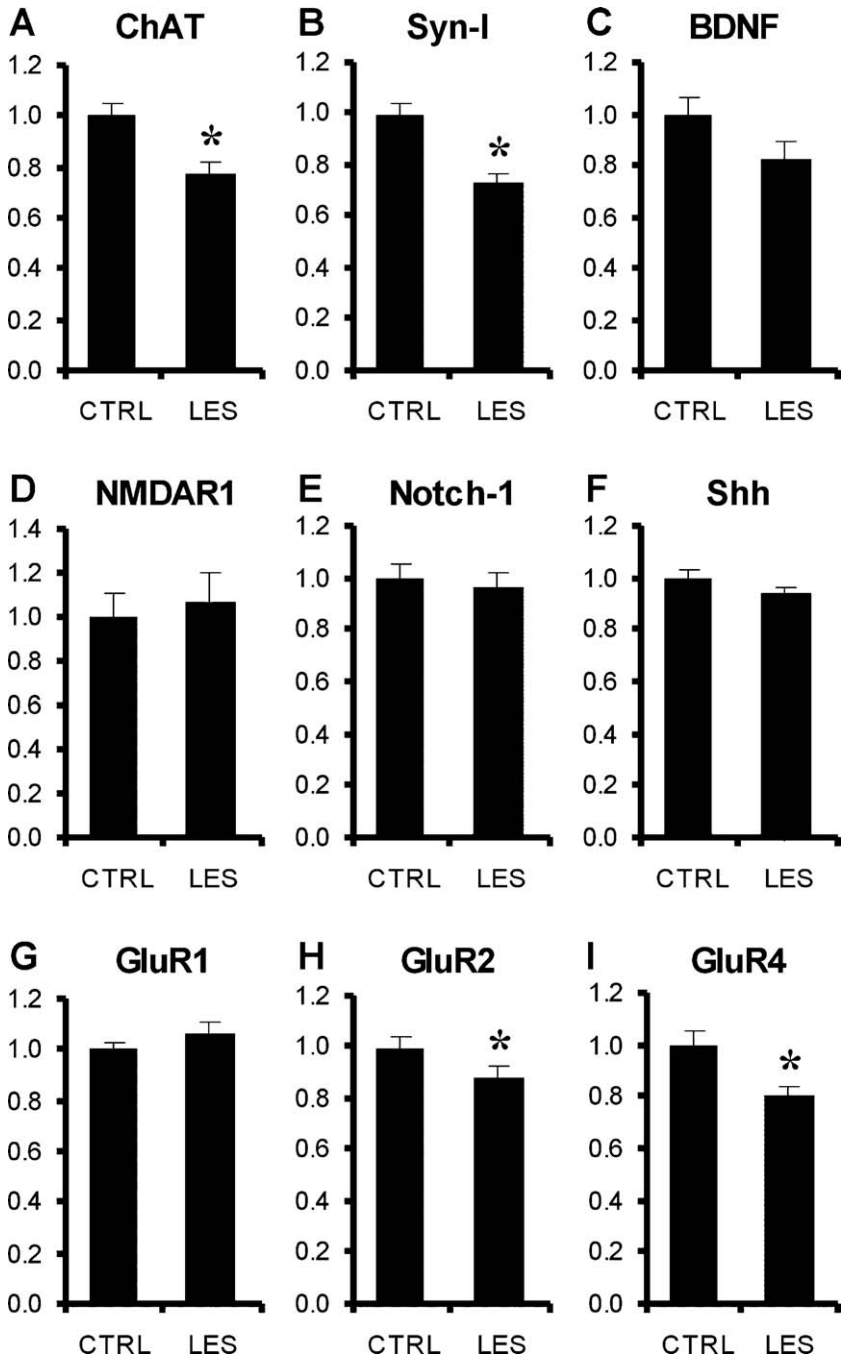

Fig. 3. Western blot data relative to the average expression levels of choline acetyltransferase (ChAT) (A), synapsin-I (B), BDNF (C), NMDAR1 (D), Notch-1 (E), sonic hedgehog precursor (Shh) (F), GluR1 (G), GluR2 (H), and GluR4 (I) in SC homogenates from control and CTB-SAP-lesioned animals. Values are mean ± s.e.m.; they are expressed as arbitrary units and normalized to control levels. Asterisks (*) indicate significant difference from control levels ($P < 0.05$).

Synapsin-I is a phosphoprotein that modulates synaptic plasticity by regulating the trafficking of synaptic vesicles. Therefore, a down-regulation of synapsin-I, also previously shown in this and other lesion models (Gulino et al., 2007b, 2010b) could reflect a decreased number and/or a reduced trafficking of synaptic vesicles (Greengard et al., 1993; Hilfiker et al., 1999). This reduction could be either due to a lower number of synapses, as a consequence of cell death, and/or by a lower activity of the spared synapses linked to the overall reduction of SC activity.

The observed changes in the expression of AMPA receptor subunits is also likely due to the overall reduction of the number and activity of lumbar SC synapses or, otherwise, but not necessarily in contrast, it could depend from changes in the stoichiometry of AMPA receptors, that ultimately can result in events of synaptic plasticity (Cull-Candy et al., 2006; Isaac et al., 2007; Bassani et al., 2009). In fact, we can argue that the reduction of GluR2 could determine an increased number of Ca^{2+}- permeable GluR2-lacking AMPA receptors, which are considered mediators of synaptic plasticity (Cull-Candy et al., 2006). It is also worth to note that an increased number of Ca^{2+}-permeable AMPA receptors could increase glutamate toxicity, thus causing excitotoxic cell death (Nagano et al., 2003; Cull-Candy et al., 2006). Moreover, since the GluR2 subunit is crucial for AMPA receptor trafficking, its down-regulation could also affect the number and function of receptors at the synapses,

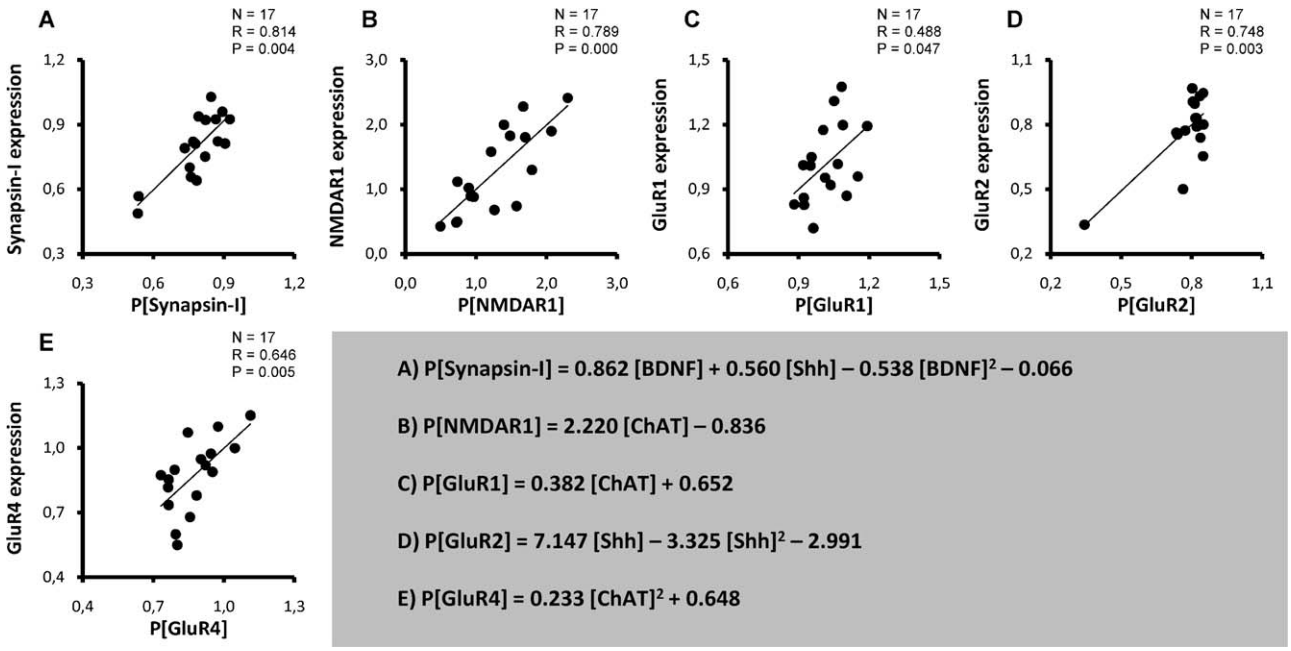

Fig. 4. Significant correlations between the actual expression levels of synapsin-I (A), NMDAR1 (B), GluR1 (C), GluR2 (D) and GluR4 (E), with those predicted by the multivariate regression models, indicated by P[synapsin-I], P[NMDAR1], etc., after the application of backward stepwise regression. The final regression models represented in the graphs are reported in the grey window. As shown by the equations, synapsin-I expression is significantly predicted by both BDNF and Shh, whereas GluR2 is significantly predicted by Shh expression only. On the other hand, the expression levels of NMDAR1, GluR1 and GluR4 are correlated with ChAT expression only.

thus inducing the modification of synaptic efficacy (Bassani et al., 2009; Kessels and Malinow, 2009).

In order to investigate regulatory mechanisms controlling synaptic plasticity by modulating the expression of synapsin-I and glutamate receptors, we have also measured the expression levels of BDNF, already known to be involved in plastic changes (McAllister et al., 1999; Gómez-Pinilla et al., 2002), as well as Shh and Notch-1, whose role in synaptic plasticity is still less clear and are acquiring increasing interest (Costa et al., 2003; Presente et al., 2004; Wang et al., 2004; Costa et al., 2005; Gulino et al., 2010b). The results showed a small and not significant down-regulation of these proteins after the lesion, more evident for BDNF but, interestingly, multivariate regression analysis have shown that synapsin-I expression is associated with those of both BDNF and Shh, but not

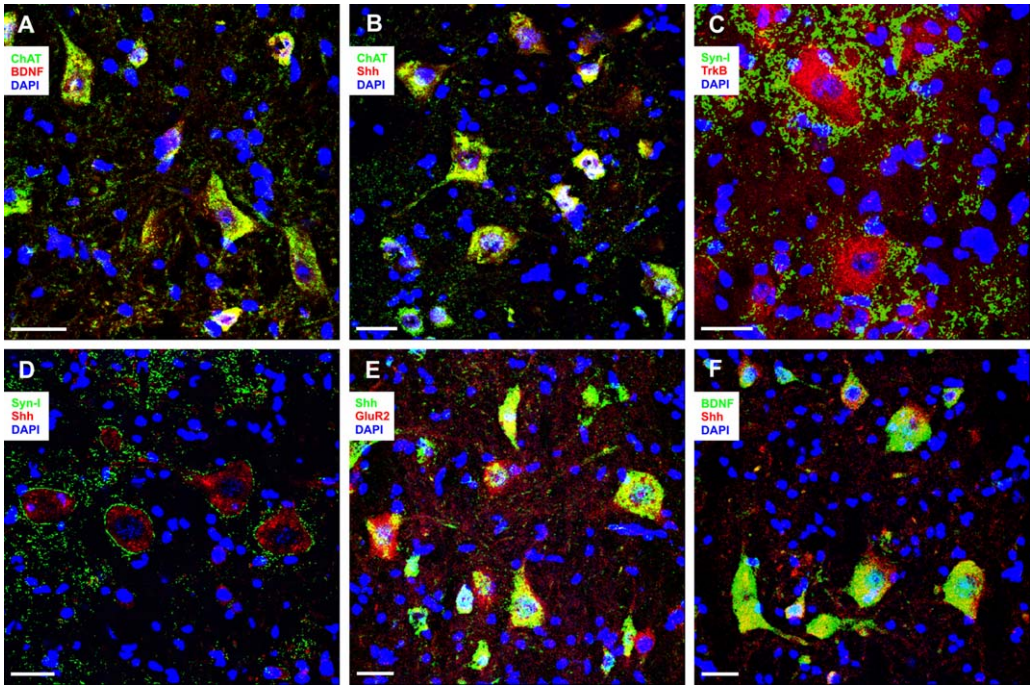

Fig. 5. Panel of confocal images showing examples of double-immunostained motoneuron-like cells, within the lesioned spinal cord. (A and B) ChAT-immunopositive motoneurons also show BDNF- (A) and sonic hedgehog-like (B) immunoreactivity. (C and D) Examples of TrkB-positive (C) and Shh-positive (D) motoneuron surrounded by synapsin-I positive puncta. (E and F) Sonic hedgehog-immunopositive motoneuron profiles are also positive for GluR2- (E) and BDNF-like (F) immunoreactivity. Nuclei have been stained with DAPI (blue). The original images have been adjusted in brightness and contrast. Scale bars: 25 μm.

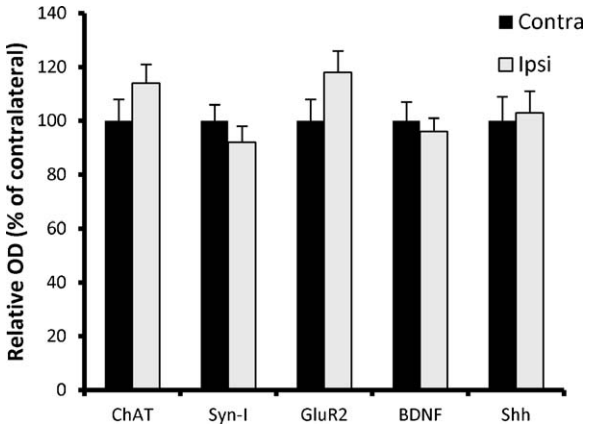

Fig. 6. Relative optical density (OD) measured in the intracellular and pericellular space of lumbar motoneurons, in the unilaterally lesioned animals ($n = 4$). Values are visualized as percent of the average values measured in the intact side and expressed as mean ± s.e.m.

Notch-1 and ChAT expression. Moreover, this analysis has demonstrated that GluR2 expression could depend from that of Shh only. Multivariate analysis is the appropriate statistical method to evaluate the strength of correlations between a single dependent with several independent variables. The method is more correct and accurate than doing separate single correlations, and it is widely used in the literature (Gulino et al., 2007b; Valle et al., 2007). The linkage between BDNF and synapsin-I is not surprising since several authors have demonstrated that synapsin-I is a mediator of activity-dependent BDNF-induced plastic changes (Jovanovic et al., 2000) in the hippocampus (Gómez-Pinilla et al., 2001a; Vaynman et al., 2003) as well as in the SC (Gómez-Pinilla et al., 2004) and, in particular, in different SC lesion models (Gómez-Pinilla et al., 2002; Ying et al., 2005; Gulino et al., 2007a,b; Ying et al., 2008). Here, we have shown that a similar mechanism could also take place in the SC, after the selective partial removal of lumbar motoneurons. Unlike BDNF, the finding of an involvement of Shh in the same mechanisms of synaptic plasticity represents a novelty. We have previously found that Shh could affect synaptic plasticity in the motoneuron-depleted SC by modulating synapsin-I expression (Gulino et al., 2010b). Here, new findings advise that Shh could also interact with the AMPA receptors and collaborate with BDNF in modulating synaptic plasticity. In particular, among AMPA receptor subunits, the relationship with Shh is probably mediated by GluR2, confirming the central role of this protein and its functional importance (Brené et al., 2000; Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007; Bassani et al., 2009). Sonic hedgehog is traditionally known as a morphogenetic factor involved in development (Traiffort et al., 1999; Fuccillo et al., 2006), but its role in adult neurogenesis, as well as in several disease conditions has been recently well documented (Ming and Song, 2005; Dellovade et al., 2006). Conversely, the involvement of Shh in synaptic plasticity by modulating synapsin-I and GluR2 expression levels is absolutely novel and interesting, and it merits further studies to elucidate the underlying mechanism, also by dissecting the role of its receptors. It is known that Shh binds to its receptor Patched, that regulates the activity of the co-receptor Smoothened (Dellovade et al., 2006). However, the expression of these proteins after SC lesion, as well as the possible effects of agonists or antagonists are completely unknown and could be matter for future studies.

In contrast to our present results (see also Gulino et al., 2010b), a transient increase in Shh and Notch-1 expression has been observed after contusion injury (Yamamoto et al., 2001; Chen et al., 2005). The reasons for this difference are elusive but it seems likely that it could depend from differences in cell and tissue response to the lesion. Our lesion model is characterized by a depletion of motoneurons and a mild glial reaction (Gulino et al., 2010b), whereas contusion injury causes the death of several cell populations, alterations of blood circulation, inflammation and a strong proliferation of ependymal and glial cells, which also express these molecules (Chen et al., 2005; Gulino et al., 2010b).

Statistical correlations are anatomically supported by co-localization studies, that have been carried out by fluorescence immunohistochemistry and laser confocal microscopy, on unilaterally lesioned SC sections. The results showed that ChAT-positive large motoneurons were also positive for both BDNF and Shh. Moreover, we observed that motoneurons expressing Shh, were also positive for GluR2 and surrounded by a large number of synapsin-I positive puncta, and that these neurons also express the BDNF receptor TrkB. Similar co-localization have been observed in the intact SC side (not shown). The observed co-localization of statistically correlated proteins suggests that these proteins could functionally interact each other in the same or adjacent cells. Interestingly, the motoneuronal expression of ChAT, synapsin-I and GluR2 was found unchanged in spite of the overall reduction observed in the whole lumbar SC. Similarly, the expression of BDNF and Shh by the spared lumbar motoneurons was also unaffected by lesion, whereas the expression levels in the whole tissue were found slightly reduced, although not significantly. Together, these results could suggest a possible compensatory increase of the activity of spared motoneurons but, as suggested above, plastic changes occurring in the entire SC circuitry could participate to this attempt to restore motor function.

The involvement of the other glutamate receptor subunits, NMDAR1, GluR1 and GluR4 in the above described plastic changes appears less clear. In fact, the expression levels of these proteins after the lesion were associated to those of ChAT only, suggesting that they could be simply affected by motoneuron depletion. However, an involvement in plasticity could not be excluded, given that ChAT is linked with motoneuron function and that, moreover, NMDAR1 and GluR1 were not reduced after the lesion. As previously stated, the modifications of glutamate receptor subunits could also reflect changes of the stoichiometry of glutamate receptors located at synapses, thus causing synaptic plasticity (Cull-Candy et al., 2006; Isaac et al., 2007; Bassani et al., 2009).

The role of Notch-1, which is fundamental in learning and memory processes (Costa et al., 2003; Presente et al., 2004; Wang et al., 2004; Costa et al., 2005), appeared also less clear in the present lesion model.

The functional linkage between BDNF and Shh is interesting but requires further investigation. It is known, for instance, that the neuroprotective effects of Shh after sciatic nerve injury could involve BDNF expression (Hashimoto et al., 2008) and that conversely, the BDNF-induced neuroprotection is mediated by Shh (Wu et al., 2009).

It is therefore likely that an experimental approach aimed at artificially increase BDNF and Shh signalling into the SC, during the first few weeks after the lesion, could stimulate synaptic plasticity, as well as provide trophic and neuroprotective effects to the damaged tissue (Akazawa et al., 2004) and then strengthen functional recovery.

In conclusion, our data suggest that BDNF and Shh are likely important regulators of synaptic plasticity within the SC, at either pre- or post-synaptic level. Given the rapidly increasing knowledge about SC plasticity, we believe that further efforts to achieve SC repair by stimulating the intrinsic potential of SC will produce interesting results. In this regard, we believe that our model of SC motoneuron degeneration represents a useful tool for future studies aiming at investigating compensatory

changes within the SC, in the presence of only neurodegenerative processes, without other micro-environmental cues such as inflammation, tissue damage, disruption of SC white matter and blood circulation.

Conflict of interest

The authors disclose no conflicts of interest.

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