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# COMPARATIVE EXPRESSION PROFILES OF ShcB AND ShcC PHOSPHOTYROSINE ADAPTER MOLECULES IN THE ADULT BRAIN

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**Abstract—Shc family of adaptor molecules has been demonstrated to play an important role during the transition from proliferating neural stem cells to postmitotic neurons. Previous studies from our group demonstrated a progressive decrease of ShcA levels occurring in coincidence with the end of embryonic neurogenesis and neuronal maturation, being ShcB and ShcC the major Shc molecules expressed in the mature brain. A growing body of evidence indicates that ShcB and ShcC are neuronal specific molecules exerting important roles in neuronal survival and phenotypic stability thus becoming potential attracting target molecules for development of drugs for interfering with brain demises.**

**Here, we examine the expression pattern of ShcB and ShcC in neuronal populations composing the adult central and peripheral nervous system, in order to better elucidate their roles *in vivo*. We found a heterogeneous and peculiar presence and subcellular localization of ShcB and ShcC in specific neuronal populations, enlightening a potential specific requirement of these two molecules in the survival/maintenance of defined neuronal subtypes. © 2005 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: Shc molecules, signal transduction, adult brain, mature neurons, neuronal survival.**

The role of several growth factors in the control of the proliferation, survival and differentiation of neural stem cells and post-mitotic neurons has been deeply investigated (Gage, 2000). Noteworthy, growth factors like epidermal growth factor or fibroblast growth factor can be mitogenic for the immature neural stem/progenitor cells (Reynolds and Weiss, 1992) but act as survival and pro-differentiative agents for postmitotic neurons (Morrison et al., 1987).

Previous studies have identified the regulated expression and activity of Shc(s) (*S*rc *h*omologue and *c*ollagene homologue) adapter molecules, which couple the signal from the activated receptor to the downstream effectors as a "simple" mechanism that the cell has developed to generate diversity in biological responses to growth factors. Shc(s) proteins, indeed appear to play a central role in the control of the proliferation and maturation of mitotically active neural stem/progenitor cells into postmitotic neurons (Cattaneo and Pelicci, 1998; Conti et al., 2001). Up to date, three Shc(s) genes have been identified, named ShcA, ShcB/Sli and ShcC/Rai/N-Shc with a consistent homology (Pelicci et al., 1992, 1996; O'Bryan et al., 1996; Luzi et al., 2000). These three Shc(s) molecules are characterized by the presence of phosphotyrosine regulatory residues and the PTB (phosphotyrosine binding domain), CH1 (collagene homology domain, a proline rich domain) and SH2 domains (Src homologue 2 domain) in the presented order. Three isoforms are known for ShcA (of 66, 52 and 46 kDa), two isoforms for ShcB (of 52 and 47 kDa) and two for ShcC (of 54 and 69 kDa). $p66^{ShcA}$ displays a further N-terminal CH domain (CH2), that contains important regulatory serine residues. They share elevated homology in both the C terminus SH2 domain and the N terminus PTB domain, the most divergent sequence being in the proline and glycine rich CH1 region. Previous results from our group demonstrated that during brain development ShcA expression is restricted to the population of dividing neural stem/progenitor cells (Conti et al., 1997) while more recently identified SHC members, ShcC and ShcB are predominantly expressed in the adult brain (O'Bryan et al., 1996; Pelicci et al., 1996; Nakamura et al., 1998; Conti et al., 2001). More detailed analyses from our group revealed that, in the brain, ShcC gradually replaces ShcA during the progression from a stem/progenitor state cell to a postmitotic/mature phenotype, and remains confined in the neuronal lineage, being excluded by the glial cells (Conti et al., 2001). ShcC is therefore expected to function as neuronal adapter instead of ShcA in many mature neurons. Recent studies (Conti et al., 2001; Pelicci et al., 2002) indicate that ShcC positively affects viability and neuronal maturation of neural cells by enhanced activation of PI3K-PKB and Erk1/2 pathways, respectively.

Similar differential expression of the three Shc has been reported during retinal development (Nakazawa et al., 2002). Indeed, early in development ShcA is highly expressed, whereas, in late development to adulthood ShcC, and to a lesser extent ShcB, are expressed in the

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*Abbreviations:* CH, collagene homology domain; DAB, 3,3′diaminobenzidine; Erk1/2, extracellular regulated kinases 1 and 2; PI3K-PKB, phosphatidylinosotol kinase–protein kinase B; PTB, phosphotyrosine binding domain; Shc, *S*rc *h*omologue and *c*ollagene homologue; SH2, Src homologue 2 domain.

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mature retinal ganglion cell layer. *In situ* analyses showed that ShcC is located in the retinal ganglion cells and amacrine cells. Since the close colocalization of ShcC with TrkB receptor, it has been speculated that ShcC could be a potential phosphotyrosine adapter among the Shc family members for brain-derived neurotrophic factor signaling and function during retinal development and regeneration *in vivo*.

Single and double ShcB/C null mice have been recently described (Sakai et al., 2000). While ShcC null mice appear not to show gross anatomical abnormalities, ShcB-deficient mice exhibit a loss of peptidergic and nonpeptidergic nociceptive sensory neurons. Noteworthy, mice lacking both ShcB and ShcC exhibit a significant additional loss of neurons within the superior cervical ganglia. This aspect may emphasize that the lack of phenotype in ShcC null mice could be due to a compensation by ShcB or others Shc members during development, thus partially masking ShcC real function in neural tissues. To this respect, a recent study reported a function of ShcC in the regulation of the neuronal adaptive response to environmental stresses (Troglio et al., 2004). In this study, it has been demonstrated that in ShcC$^{-/-}$ mice, ischemia–reperfusion injury induces severe neurological deficits, increased apoptosis and size of the infarct area, and leads to a significantly higher mortality. These data confirm the previously suggested ShcC functional neuroprotective role in brain injury, with possible implications in the treatment of stroke.

Altogether, these data indicate that ShcB and ShcC represent important molecules for the maintenance of postmitotic neurons. Nevertheless, apart from a general statement about their presence in neurons of the mature nervous system, further analysis is essential to characterize the expression of ShcB and ShcC at cellular level, in order to better elucidate their roles *in vivo*. In particular, here we show a heterogeneous distribution of ShcB and ShcC in the adult central and peripheral nervous system, enlightening their specific presence and subcellular localization in distinct neuronal subpopulations.

## EXPERIMENTAL PROCEDURES

### Tissue preparation

All experiments on animals have been carried out in strict accordance with the Italian Ministry of Health guidelines for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. Twenty adult (3 months old; Charles River, Milan, Italy) Wistar rats maintained in a controlled environment (14 h light/10 h dark) with food and water *ad libitum* were used. Animals were anesthetized (pentothal sodium; Gellini, Italy; 60 mg/100 g i.p.) and perfused intracardially with a heparinized saline solution (25 IU/ml in 0.9% NaCl, during 2–3 min) followed by a freshly prepared solution of 4% paraformaldehyde, or the same fixative with the addition of 2% picric acid, in 0.1 M sodium phosphate buffer, pH 7.4. Brains, spinal cords, eyes, olfactory mucosae, spinal and cranial ganglia were dissected and post-fixed overnight (brains) or for 2–4 h (other tissues), then cryoprotected, frozen in liquid nitrogen-cooled isopentane at −80 °C, and cryostat sectioned in series. Cryostat sections (12 μm thick) were collected onto (3-aminopropyl) triethoxysilane (Fluka, USA)-coated slides.

### Immunohistochemistry

Immunohistochemistry was carried out on cryostat sections incubated overnight at 4 °C and revealed by using single and double indirect peroxidase techniques with biotinylated secondary antibodies (Vector, UK), detected with 3,3′diaminobenzidine (DAB) and intensified with the glucose oxidase–nickel–DAB method (see Bonfanti et al., 1997; Conti et al., 2001). The following primary antisera and antibodies were used: i) anti-ShcC, diluted 1/100 (monoclonal mouse; Transduction Laboratory, USA); ii) anti-ShcB diluted 1/3000 (polyclonal rabbit; Sigma-Genosys, USA). All antibodies were diluted in 0.01 M PBS containing 0.5% Triton X-100. Controls included omission of the primary antibody, incubation with inappropriate secondary antibodies, and incubation with preimmune serum (Shc-B).

Double labeling has been performed sequentially by using indirect peroxidase methods with the elution technique (Tramu et al., 1978). The immunolabeling for the first antigen was revealed with biotinylated secondary antibodies (Vector, UK), detected with DAB. The slides were rinsed with bidistilled water, and then the antibodies were eluted by immersion for 1 min in a freshly made solution of potassium permanganate and sulfuric acid (1 ml of 2.5% $KMnO_4$ and 1 ml of 5% $H_2SO_4$ in 250 ml of distilled water). The slides were then incubated for 15 s in sodium metabisulphitum ($Na_2S_2SO_5$ 0.5%) and rinsed for 15 min in running tap water. After incubation overnight with the antibody for the second antigen as described above, the reaction was revealed with DAB and intensified with the glucose oxidase–nickel–DAB method (Shu et al., 1988). For observation, all sections were dehydrated and mounted in DPX (Raymond A. Lamb, UK).

### ShcA *in situ* analysis

For ShcA *in situ* hybridization histochemistry fixed and permeabilized sections were acetylated by 10 min incubation in a solution made of 250 ml of water with 3.5 ml of triethanolamine (Merck, Germany) and 625 μl of acetic anhydride (Merck, Germany) added dropwise. Prehybridization was performed at room temperature in the hybridization buffer (50% formamide, 5× SSC and 2% blocking reagent; Boehringer Mannheim, Mannheim, Germany) for 3 h. The hybridization mixture was prepared by adding 50 ng/ml of a full-length digoxigenated riboprobe synthesized from $p52^{ShcA}$ cDNA-containing plasmid that was heated first for 5 min at 85 °C and then rapidly chilled on ice. The hybridization was done overnight at 68 °C. Slides were washed in 5× SSC at 68 °C. Stringency washing was performed in 0.2× SSC at 68 °C for 60 min. For the immunological detection of digoxigenin-labeled hybrids the slides were incubated for 1 h at room temperature in 1% blocking reagent made in maleic acid, then incubated for 1 h in anti-digoxigenin antibody (Boehringer Mannheim, Germany) diluted 1:5000 in 1% blocking reagent and finally washed twice for 30 min in maleic acid. To perform the color reaction, we incubated the slides with 2.4 mg of levamisole, 45 μl of 4-nitroblue tetrazolium and 35 μl of 5-bromo-4-chloro-3-indolyl-phosphate (all from Sigma, USA) diluted in 10 ml of a buffer made of 0.1 M Trizma base, 0.1 M NaCl and 0.005 M $MgCl_2$, pH 9.5. The color reaction was developed in the dark for 1–24 h and stopped in a buffer made of 0.01 M Trizma base and 0.001 EDTA, pH 8, when the desired intensity was reached. The sections were mounted in phosphate-buffered saline–glycerol and coverslipped.

### Western blotting

Brain dissection, tissues processing dissected and proteic samples prepared in lysis buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% Bromophenol Blue) were performed