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CHARACTERIZATION OF NEURAL STEM CELLS IN THE DORSAL VAGAL COMPLEX OF ADULT RAT BY *IN VIVO* PROLIFERATION LABELING AND *IN VITRO* NEUROSPHERE ASSAY

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Abstract—The dorsal vagal complex, located in the brainstem, is the major integrative center of the autonomic nervous system. By combining *in vivo* bromodeoxyuridine incorporation and phenotypic immunolabeling, we have previously reported that neurogenesis occurs in the adult rat dorsal vagal complex [Bauer S, Hay M, Amilhon B, Jean A, Moyse E (2005) In vivo neurogenesis in the dorsal vagal complex of the adult rat brainstem. Neuroscience 130:75–90.]. In the present study we asked whether adult dorsal vagal complex contains proliferative and/or neural stem cells. Using Ki-67 immunolabeling and cyclin D1 Western blot, we showed intrinsic cell proliferation in the dorsal vagal complex and its stimulation by vagotomy. Detailed time-course analysis revealed that vagotomy-induced proliferation in the dorsal vagal complex peaked three days after lesion. In order to directly assess the presence of intrinsic stem cells, primary cell cultures from adult rat dorsal vagal complex were performed in the presence of epidermal growth factor and basic fibroblast growth factor (neurosphere assay). A discrete subpopulation of dorsal vagal complex cells proliferated as neurospheres, self-renewed when passaged, and differentiated into neurons, astrocytes and oligodendrocytes. Proliferation and neuron-differentiating potentials of dorsal vagal complex neurospheres were both lower than those of subventricular zone neurospheres from the same rats. The relationship between *in vitro* neurosphere-forming cells of dorsal vagal complex and *in vivo* dorsal vagal complex neurogenesis is discussed and remains to be directly addressed. The present data demonstrate the occurrence of neural stem cells in the dorsal vagal complex of adult rat brain. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: aCSF, artificial cerebrospinal fluid; AP, area postrema; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; DMNV, dorsal motor nucleus of the vagus nerve; DVC, dorsal vagal complex; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; NSCs, neural stem cells; NST, nucleus of the solitary tract; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; PSA-NCAM, polysialylated form of the neural cell adhesion molecule; SVZ, subventricular zone; VGX, vagotomy.

Key words: autonomic nervous system, brainstem, vagotomy, proliferation, neurosphere, adult neurogenesis.

Continuous generation of new neurons in the CNS of adult mammals has been thoroughly characterized in two areas: the hippocampus, where new neurons are added to the granule cell layer of the dentate gyrus (Altman and Das, 1965; Cameron et al., 1993; Kuhn et al., 1996) and the olfactory bulb, where new neurons integrate among granular and periglomerular interneurons (Altman, 1969; Kaplan et al., 1985; Carleton et al., 2003). In the hippocampus, neurogenesis proceeds from local neural stem cells (NSCs) or progenitors (Palmer et al., 1997; Gage et al., 1998; Seaberg and van der Kooy, 2002; Aberg et al., 2003). In the olfactory bulb, neurogenesis proceeds from migrating neuroblasts originating from NSCs located in the subventricular zone (SVZ) (Luskin, 1993; Lois and Alvarez-Buylla, 1994). NSCs are characterized by their continuous proliferation, self-renewal and propensity to generate both neurons and neuroglia (Reynolds and Weiss, 1992). These characteristics can be tested *in vitro* on primary cultures from adult nervous tissue, through the so-called neurosphere assay (Campos, 2004).

Additional populations of NSCs have been revealed with the neurosphere assay in several non-neurogenic areas of the adult neuraxis (Weiss et al., 1996). Neurogenesis has also been detected *in situ* in the rat substantia nigra (Lie et al., 2002; Zhao et al., 2003) and in the primate amygdala (Bernier et al., 2002) and neocortex (Gould et al., 1999), although these findings remain a matter of controversy (Frielingsdorf et al., 2004; Kornack and Rakic, 2001; Rakic, 2002).

By combining bromodeoxyuridine (BrdU) labeling and a variety of phenotypic markers, neurogenesis was recently identified in the adult rat brainstem within the dorsal vagal complex (DVC) (Bauer et al., 2005). The DVC comprises three anatomical nuclei. The nucleus of the solitary tract (NST) is the major recipient of afferent viscero-sensory neurons of the vagus nerve. The dorsal motor nucleus of the vagus nerve (DMNV) contains the cell bodies of efferent vagus nerve fibers. The area postrema (AP) is a circumventricular organ that sprawls on the middle third of the rostro-caudal extent of the DVC. These interrelated nuclei in the floor of the fourth ventricle make up the main integrative center of cardiovascular, respiratory and gastro-intestinal reflexes (Jean, 1991; Blessing, 1997).

In the DVC, newly formed cells include equal proportions of neurons and astrocytes, which were identified *in*

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situ one to three weeks after they had incorporated BrdU (Bauer et al., 2005). These results raised the question of whether the DVC contains intrinsic NSCs. This issue was addressed by the present study by assessing the occurrence of cell proliferation within the DVC using immunohistochemistry of the dividing cell marker Ki-67 and Western blot identification of the cell-cycle triggering molecules cyclins D. In a second step, the neurosphere assay was applied to DVC primary cultures according to well-established procedures (Weiss et al., 1996; Campos, 2004).

EXPERIMENTAL PROCEDURES

Animals

Animals were handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (N.R.C., 1996) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols were carried out in compliance with institutional Ethical Committee guidelines for animal research. All efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats (Charles River, Les Oncins, France), weighing 220–240 g at the beginning of the experiment, were used in this study.

Vagotomy (VGX)

Unilateral (right) cervical VGX was performed under ketamine (80 mg/kg i.p.; Merial, Lyon, France)-xylazine (12 mg/kg i.p.; Bayer, Puteaux, France) anesthesia and under atropine (0.5 mg/kg s.c.; Meram, Melun, France). The rats were laid on their back and a 2 cm-long incision was made in the neck skin. The exposed muscles were pushed aside to yield access to the common carotid artery and a 1 cm-long segment of the right carotid and adjacent nerves were gently separated from connective tissues with blunt forceps. The vagus nerve was then dissociated from the carotid artery and a thread tied around the nerve to enable cutting and removal of a 3–4 mm-long segment. Each rat was immediately sutured and housed individually in a warmed cage with food and water *ad libitum*. In sham-operated animals, the carotid trunk was exposed, but the vagus nerve was not touched.

Tissue processing and *in situ* histochemistry

The animals were killed under deep anesthesia (ketamine–xylazine), by intra-aortic perfusion of 100 mL ice-cold phosphate-buffered saline (PBS) 0.1 M, pH 7.4, followed by 400 mL ice-cold fixative solution (3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were immediately removed, post-fixed for 1 h at 4 °C in the same fixative, rinsed for 24 h at 4 °C in PBS and cryoprotected 24–48 h at 4 °C in 25% sucrose-containing PBS. Brains were then snap-frozen for 30 s in liquid isopentane at −40 °C and stored at −80 °C. Coronal cryostat sections, 20 μm thickness, were sampled serially on quadruplets of slides, using each slide alternatively so that each slide contained sections spaced at 80 μm. Sections were air-dried, then stored at −20 °C until use.

For immunohistochemistry, in an initial step, the sections were incubated in 10 mM sodium citrate buffer pH 5.5 for 10 min at 95 °C to unmask antigens. The remaining endogenous peroxidase activity was then quenched with a 10 min incubation in 3% H_2O_2-containing PBS. The sections were incubated in blocking buffer (0.1 M PBS, 0.1% Triton X-100 and 5% normal goat serum) for 1 h at room temperature and then with the primary antibody (either mouse anti-Ki-67 [BD Biosciences, Heidelberg, Germany, 550609] diluted 1/600 or mouse anti-nestin [BD Biosciences 556309] diluted 1/100) in the same buffer overnight at 4 °C. Secondary biotin-conjugated antibody (goat anti-mouse, Vector, Burlingame, CA, USA) was diluted 1/200 in the same buffer and applied for 2 h at room temperature. Each step was followed by three rinses in PBS. Nonspecific labeling was assessed on alternate slides processed without the primary antibody.

For *in situ* labeling of apoptotic nuclear DNA fragmentation (TUNEL), frozen cryostat sections were brought to room temperature, rinsed 30 min in PBS containing 0.2% BSA and 0.1% Triton-X-100, incubated 15 min at room temperature with proteinase K at 20 μg/mL PBS, rinsed 3×5 min in PBS, treated with 2% H_2O_2 in PBS for 10 min in order to block endogenous peroxidases and rinsed 2×5 min in PBS containing 0.2% BSA and 0.1% Triton-X-100. After 5 min preincubation in 300 mM Tris–HCl, pH 7.5, containing 140 mM sodium cacodylate and 1 mM cobalt chloride, slides were incubated in the same buffer with terminal-transferase (TdT, Roche-Diagnostics, Meylan, France) at 150 U/mL and biotinylated d-UTP (Roche) at 6 nM, for 2 h at 37 °C. Slides were then rinsed 15 min at room temperature in 300 mM sodium chloride–30 mM sodium citrate solution, further rinsed in PBS–0.2% BSA–0.1% Triton-X-100, and incubated 10 min with 2% BSA in PBS. Additional sections of olfactory bulbs from the same animals were included as positive controls (Bauer et al., 2003).

Immunolabelings and TUNEL staining were revealed by incubating sections for 30–45 min with the avidin–peroxidase complex (Elite-ABC kit, Vector), and then with the diaminobenzidine reagent (DAB kit, Vector). After 5–7 min reaction at room temperature, slides were rinsed in cold buffer, dehydrated-defatted through graded ethanols and xylene and coverslipped with Depex.

Ki-67 labeling quantification

Photographs of the Ki-67 and nestin labelings and of the Nissl stainings were taken on a Nikon Eclipse E600 light microscope (Nikon-France, Champigny-s/Marne, France) using ACT-1 software. The Ki-67+ nuclei were counted and the areas of the DVC subnuclei were measured on these pictures by computer-assisted morphometry using the NIH Scion software (Bethesda, MD, USA). Labeling and countings were performed on every fourth coronal section throughout the rostro-caudal extent of the DVC. The results were reported in three ways: with the raw counts of Ki-67+ cells, with the density of Ki-67+ cells per volume of tissue (considering the volume of tissue equals the measured area multiplied by the thickness of the sections), and with the proportion of Ki-67+ cells versus the total number of neurons (estimated counting Nissl-stained cells).

Protein extraction and Western blots

For Western blots, animals were deeply anesthetized with halothane and decapitated. The brain was extracted from the skull, the meninges were removed under a dissecting microscope and the brainstem was cut into 500 μm-thick slices with a tissue-chopper. Slices were kept in ice-cold PBS while DVC was microdissected. The tissue samples were immediately placed in sterile Eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C until protein extraction. The tissue samples were homogenized with a piston pellet in RIPA lysis buffer (1% NP-40, 0.1% SDS, 0.5% desoxycholate sodium in PBS) supplemented with protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), orthovanadate (1 mM), leupeptin (10 μg/μl) and aprotinin (10 μg/μl). The mixture was incubated for 30 min at 4 °C and centrifuged at 13,000×*g* for 20 min to recover the supernatant. The protein concentration was measured with Bradford reagent (BioRad, Issy-les-M^x, France). For each sample, 15 μg proteins were resolved through a 10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Nonspecific sites of the membranes were saturated by 1 h incubation in PBST (phosphate-buffered saline with 0.05% Tween

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