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The carotid body, a neurogenic niche in the adult peripheral nervous system

R. PARDAL, P. ORTEGA-SÁENZ, R. DURÁN,
A. PLATERO-LUENGO, J. LÓPEZ-BARNEO

Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/ Universidad de Sevilla, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Sevilla, Spain

ABSTRACT

We have described a new population of adult neural stem cells residing in the carotid body, a chemoreceptor organ in the peripheral nervous system. These progenitor cells support neurogenesis in vivo in response to physiological stimuli like hypoxemia, and give rise to multipotent neurospheres in culture. Studying the biology of CB stem cells helps to understand the physiological adaptations of the organ, and might shed light on the pathogenesis of CB tumors. Understanding proliferation and differentiation of these cells will enable their use for cell therapy against neurodegenerative diseases.

Key words

Carotid body • Neural crest-derived stem cells • Hypoxia • Glomus cells • Peripheral neurogenesis

Introduction

The discovery of multipotent neural stem or progenitor cells in adult neural tissues has overcome a long-standing dogma in neurobiology, which postulated the lack of neuronal regeneration in the mammalian central nervous system (CNS). The last decade has witnessed the discovery of neural progenitor cells, which are able to proliferate and differentiate into new neurons that can contribute to the structural plasticity of the CNS or even repopulate damaged areas of the brain (Altman, 1962; Eriksson et al., 1998; Doetsch et al., 1999). It is well established that adult neurogenesis takes place in specific centers of the brain, such as the subventricular zone (SVZ) and the dentate gyrus of the hippocampal formation (subgranular zone, SGZ) (Doetsch, 2003a). Neurogenesis is also known to persist throughout life in the mammalian olfactory neuroepithelium (Calof et al., 1998). However, whether neurogenesis also occurs in the neural crest-derived organs of the adult mammalian peripheral nervous system (PNS) has been studied in lesser detail. Identification of germinal centers in the adult PNS has biomedical interest and therapeutic potential, as PNS stem cells, more accessible than those in the CNS, could be used for tissue repair after injury or in the treatment of neurological diseases.

Neurons and glia of the PNS derive from the neural crest, a collection of progenitors originated in the dorsal side of the neural tube that migrate at midgestation to form, among other structures, the sympathoadrenal system. Cells of the sympathoadrenal lineage give rise to the enteric nervous system (ENS), the autonomic ganglia, and several associated paraneural organs such as the adrenal medulla or the carotid body (Le Douarin, 1986; Jiang et al., 2000). Postmigratory rat neural crest-derived stem cells (NCSCs) persist into late gestation within peripheral nerves and the ENS (Morrison et al., 1999). In culture, these NCSCs are able to self-renew and differentiate into neurons, glia, and smooth muscle

Corresponding Authors: Ricardo Pardal, José López-Barneo, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Edificio de Laboratorios, 2 planta, Avenida Manuel Siurot s/n, 41013 Sevilla, Spain - Tel.: +34-955 012648 - Email: rpardal@us.es (Ricardo Pardal); lbarneo@us.es (José López-Barneo)

cells (Bixby et al., 2002). Multipotent neural crest-derived progenitors have also been isolated from the postnatal rat gut (Kruger et al., 2002) or the heart region (Tomita et al., 2005), although neurogenesis *in vivo* has not yet been demonstrated in any structure of the neural crest-derived adult PNS.

An attractive place to look for adult neurogenesis is the carotid body (CB), a paired organ located at the carotid bifurcation (Fig. 1A), that is a principal component of the homeostatic acute oxygen (O_2) sensing system required to activate the brainstem respiratory center to produce hyperventilation during hypoxia (López-Barneo et al., 2001; Weir et al., 2005). Unlike other neural crest derived tissues, the CB grows in conditions of chronic hypoxemia (e.g. in high altitude residents or in patients with chronic obstructive pulmonary diseases). The CB is one of the most irrigated organs in the body and receives blood through a branch arising from the external carotid artery. The CB parenchyma is organized in glomeruli, clusters of cells in close contact with a profuse network of capillaries, and afferent sensory fibers joining the glossopharyngeal nerve (Fig. 1B). The most abundant cell types in the CB glomeruli are the neuron-like, glomus or type I cells, which are enveloped by processes of glia-like, sustentacular or type II cells (Fig. 1B and C). Glomus cells, the chemosensory components of the organ, are electrically excitable and function as presynaptic-like elements establishing contacts with the postsynaptic sensory nerve fibers. Upon exposure to hypoxia, CB glomus cells detect the decrease in the level of blood O_2, and release neurotransmitters to activate the adjacent nerve fibers, which in turn carry the information to the respiratory centers in the brain to trigger the appropriate counter-regulatory responses (Ureña et al., 1994; Peers and Buckler, 1995; Lopez-Barneo, 2003). Type II cells (approximately 15-20% in the CB neural parenchyma) are non-excitable and lack most of the voltage-gated channels characteristic of type I cells (Ureña et al., 1989). The molecular interactions between type I and type II cells, possibly critical for the physiology of the organ, are basically unknown. Classically, type II cells were considered to belong to the peripheral glia with a supportive role. However, our recent experimental data have shown that the adult CB is a functionally active germinal niche where type II cells might act as dormant stem cells that in response to physiological hypoxia can proliferate and differentiate into new glomus cells, thus explaining the characteristic adaptive growth of the organ (Fig. 1C and D; Pardal et al., 2007). In this review we discuss the experimental evidences that led us to the discovery of progenitor cells in the CB, the first neurogenic center identified outside the CNS. We will also summarize our current knowledge on the physiology of these neural crest-derived stem cells.

Physiological adaptation of the carotid body to sustained hypoxemia

Acclimatization to long-term hypoxia takes place at high altitude and allows gradual improvement of the ability to tolerate the hypoxic environment. This adaptive physiological response depends on the interplay between the O_2 sensors (peripheral chemoreceptors) and the effectors (brainstem centers and respiratory system). During a long-term (i.e., weeks or months) sojourn at high altitude, the global O_2 sensitivity of this pathway increases gradually, leading to hypoxic ventilatory acclimatization (HVA), defined as a gradual elevation of ventilation despite a continuously increasing arterial O_2 tension (pO_2), i.e. a decrease of the initial stimulus (Smith et al., 1986). Peripheral chemoreceptors are considered a key component of HVA. In a hypoxemic situation, the carotid bodies are able to maintain a constant neural drive for lung ventilation and O_2 delivery within vital boundaries. At the cellular level the process depends on the activity of type I cells in the carotid body, which signal through the afferent projections onto brainstem respiratory nuclei to induce hyperventilation. Previous studies have reported that chronic hypoxia increases hypoxic sensitivity of the carotid bodies through an increased excitability of type I cells (Stea et al., 1995). The CB cells contain a population of T-type Ca^{2+} channels (Ureña et al., 1989; Ortega-Saenz et al., 2010), which in other paraneural tissues are up regulated by hypoxia and increase cell excitability (Carabelli et al., 2007; Levitsky and Lopez-Barneo, 2009), thus providing a cellular explanation to HVA. Moreover, this functional modification of the excitability of type I cells in the CB is accompanied by drastic structural changes in the organ that are also necessary for the capacity to achieve HVA. Few days after expo-

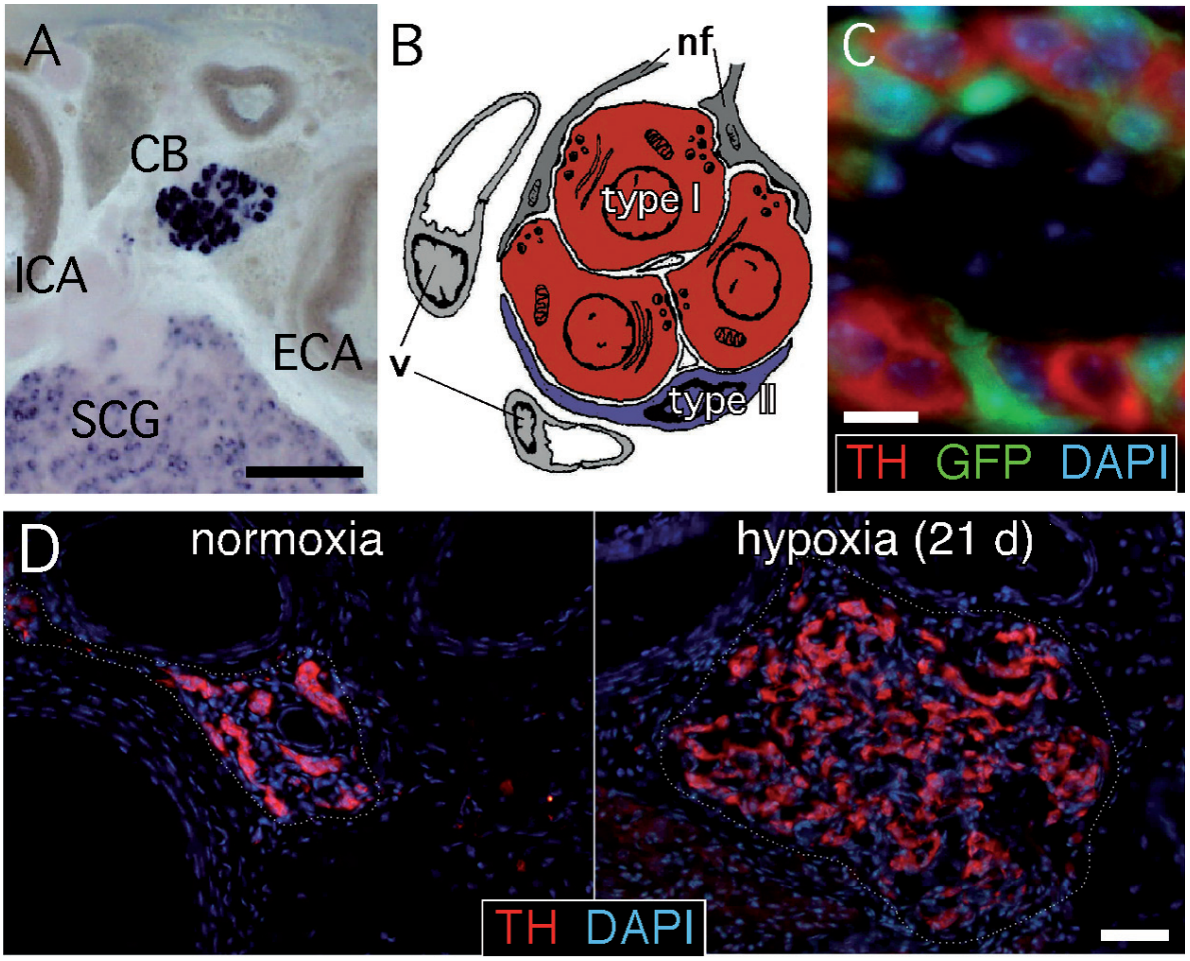

Fig. 1. - Structure of the adult carotid body and organ growth in chronic hypoxia.
A. *In situ* hybridization to detect tyrosine hydroxilase (TH) mRNA in the carotid body (CB). ICA, internal carotid artery; ECA, external carotid artery; SCG, superior cervical ganglion. B. Scheme of a CB glomerulus, with neighboring blood vessels (v) and afferent nerve fibers (nf). Neuron-like type I cells are enveloped by glia-like type II cells. C. Immunocytochemical detection of tyrosine hydroxilase and green fluorescent protein (GFP) in the CB of a GFAP-GFP transgenic mouse. D. Immunocytochemical analysis of carotid bodies removed from control mice (left) or from mice exposed to chronic hypoxia (10% O_2) (right). The typical growth of the TH+ glomus cell mass under hypoxia (21 days) is shown. Scale bars: 300 μm in panel (A), 10 μm in (C), and 50 μm in (D). Modified from Pardal et al., 2007.

sure to the hypoxic environment, the CB suffers a marked hypertrophy due to an increased number and volume of chemosensitive type I cells, and an enhanced vascularization due to both vasodilatation and growth of new blood vessels (Arias-Stella and Valcarcel, 1976; McGregor et al., 1984; Pequignot et al., 1984). The increase in number of neuronal type I cells is crucial for the physiological adaptation of the organ during hypoxemia. However, the cellular and molecular mechanisms involved in this peripheral neurogenic process have remained almost completely unknown.

We have recently studied in detail the hypoxia-induced growth of the CB at the cellular level. In rodents subjected to isobaric hypoxia we have quantified the increase in number of tyrosine hydroxylase (TH)-positive type I cells within the neural parenchyma of the organ (Pardal et al., 2007). To demonstrate the appearance of new neuronal cells in response to the hypoxic stimulus, animals were treated with the thymidine analog BrdU, which is incorporated to DNA and marks proliferative cells and their derivatives (Pardal et al., 2007). Although previous studies had suggested that neuron-like type I cells might retain some capacity for cell division (Nurse and Vollmer, 1997; Paciga et al., 1999; Paciga and Nurse, 2001; Lopez-Barneo et al., 2008), the delay in the appearance of newly generated glomus cells

suggested the existence in the CB of a collection of progenitor cells that, upon exposure to hypoxia, can proliferate and differentiate into mature cells, thus explaining the marked growth of the organ.

Identification of multipotent and self-renewing carotid body progenitor cells

To study the presence of neural progenitors within the CB parenchyma, we dissociated the tissue to single-cell level and cultured the cells in a medium designed to allow the growth of neural crest progenitors (Morrison et al., 1999). In this type of medium, neural crest-derived stem cells clonally generate floating neurospheres, spherical colonies of proliferating progenitors and their derivatives. CB cell cultures were maintained under moderate hypoxia (3% O_2) (Morrison et al., 2000), a condition that mimics the hypoxic stimulation of CB growth *in vivo*. In these experiments, about 1% of the plated CB cells gave rise to neurospheres (Fig. 2A). In contrast with the typical spherical shape of neurospheres derived from neurogenic centers in the CNS or from different neural crest progenitors in the PNS (Doetsch et al., 1999; Molofsky et al., 2003), most of the CB-derived neurospheres had large blebs budding out of the main core (arrowheads in Fig. 2A). Immunocytochemical analysis of thin sections of CB neurospheres revealed nestin+ progenitors within the central core, and TH+ neuronal cells organized in the external blebs (Fig. 2B and C). These TH+ blebs resembled in shape the glomeruli characteristic of the *in situ* CB. When the neurospheres were attached to adherent plates, progenitors migrated out of the core and differentiated into smooth muscle actin (SMA)-positive myofibroblasts, a typical derivative of neural crest stem cells (Fig. 2D and E; Kruger et al., 2002). Since neurospheres were also obtained from single-cell deposition experiments, the existence of the different cell types *in vitro* supported the multipotency of CB progenitors. It remains to be elucidated whether this multipotency also occurs *in vivo*, where CB stem cells might contribute to vasculogenesis by undergoing differentiation into smooth muscle cells, in addition to the typical neurogenesis. The self-renewal capacity of CB progenitors was assayed by dissociating primary neurospheres and replating the cells to obtain secondary neurospheres. The shape and cell content of secondary neurospheres were similar to those of primary neurospheres. Therefore, CB progenitors behave similar to other NCSCs (Bixby et al., 2002), being able to produce clonal colonies, to self-renew, and to differentiate into multiple cell types.

The glial identity of carotid body stem cells

Besides their ability to proliferate, self renew and differentiate *in vitro*, we have also studied the nature of the adult CB stem cell. The first hint on the nature of these cells came from *in vivo* experiments showing that the GFAP staining of sustentacular type II cells progressively vanished upon exposure to hypoxia, as the number of proliferating BrdU+ cells increased, and prior to the appearance of newly generated TH+ cells. GFAP+ cells reappeared once the animals returned to a normoxic atmosphere, and the CB resumed to its original size (Fig. 3A). Hence, these data suggested that GFAP-expressing type II cells might get activated under hypoxia and function as progenitors in the CB.

To further evaluate whether type II cells are indeed the CB stem cells, we used a transgenic mouse approach. We crossed two mouse lines that had on one hand the floxed ROSA26-STOP-LacZ construct, and on the other hand the cre recombinase under the control of the GFAP promoter. Animals with both constructs would excise the STOP sequence from the ROSA26-LacZ construct, allowing the expression of the reporter enzyme galactosidase, only in cells expressing the GFAP promoter (Fig. 3B). As a result, we were labeling not only GFAP+ cells but also their derivatives, since the recombination event is irreversible. After a simple X-gal reaction to highlight the blue precipitate typical of the expression of the β-galactosidase enzyme, we were able to confirm the presence of the labeling in the newly formed BrdU+ type I cells, thus suggesting that they derive from GFAP+ type II cells (Fig. 3C-E). Moreover, neurospheres generated from these transgenic mice showed labeling in every cell type, including progenitors within the core, type I cells generating the peripheral blebs, and SMA+ myofibroblasts obtained upon adherence (Fig. 4A-D).

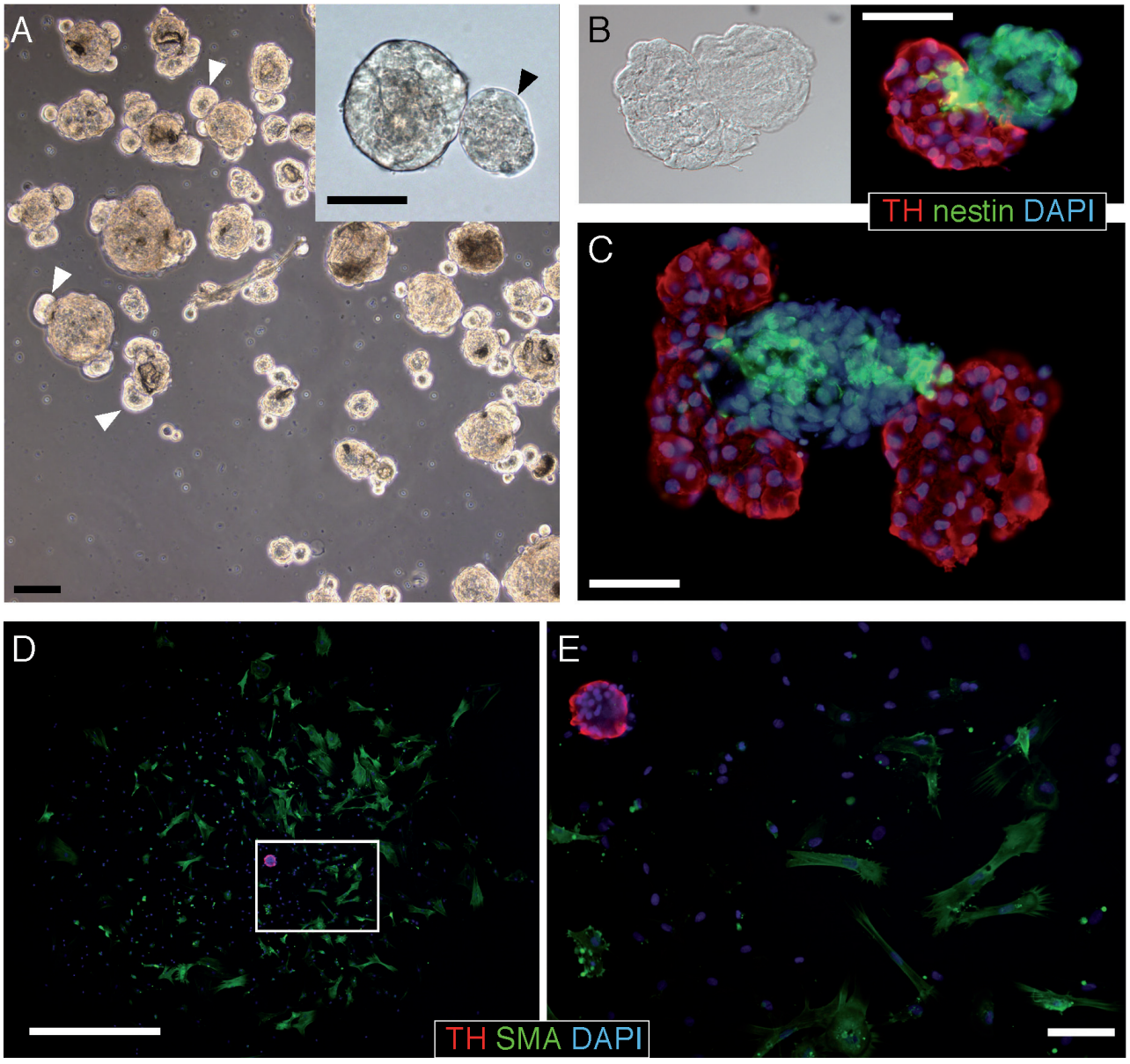

Fig. 2. - Formation of neurospheres and multipotency of carotid body stem cells *in vitro*.
A. Bright field picture of neurospheres formed by dispersed CB cells after 10 days in culture. The inset shows an example of the typical blebs (arrowheads) appearing from these neurospheres. B. Immunohistochemical analysis of a neurosphere thin section (bright field on the left), illustrating the presence of nestin+ progenitors within the neurosphere core, and TH+ glomus cells within the bleb (right). C. Grown neurosphere (20 days in culture) with two large blebs containing differentiated TH+ cells. D. Differentiation into smooth muscle cells (SMA+) of the carotid body progenitors upon replating of the neurospheres to adherent substrate. A higher magnification image of the area depicted in (D) is shown in panel (E). Note the presence of TH+ glomus cells in the center of the colony. Scale bars: 100 μm in panels (A) and (E), 50 μm in panels (B) and (C), and 1 mm in panel (D). Modified from Pardal et al., 2007.

Overall, the data suggest that type II cells in the CB are responsible for the progenitor activity both *in vivo* and *in vitro*.

As a second method to confirm the role of type II cells as adult CB progenitors, we performed the prospective isolation of GFAP+ CB cells by flow cytometry. We freshly dispersed rat CB cells and transfected them with an enhanced version of GFP under the control of the GFAP promoter (pGFAP-EGFP expression plasmid). A population of intensely fluorescent GFP+ cells, clearly observable 36-48 hr later, was separated by flow cytometry. Most

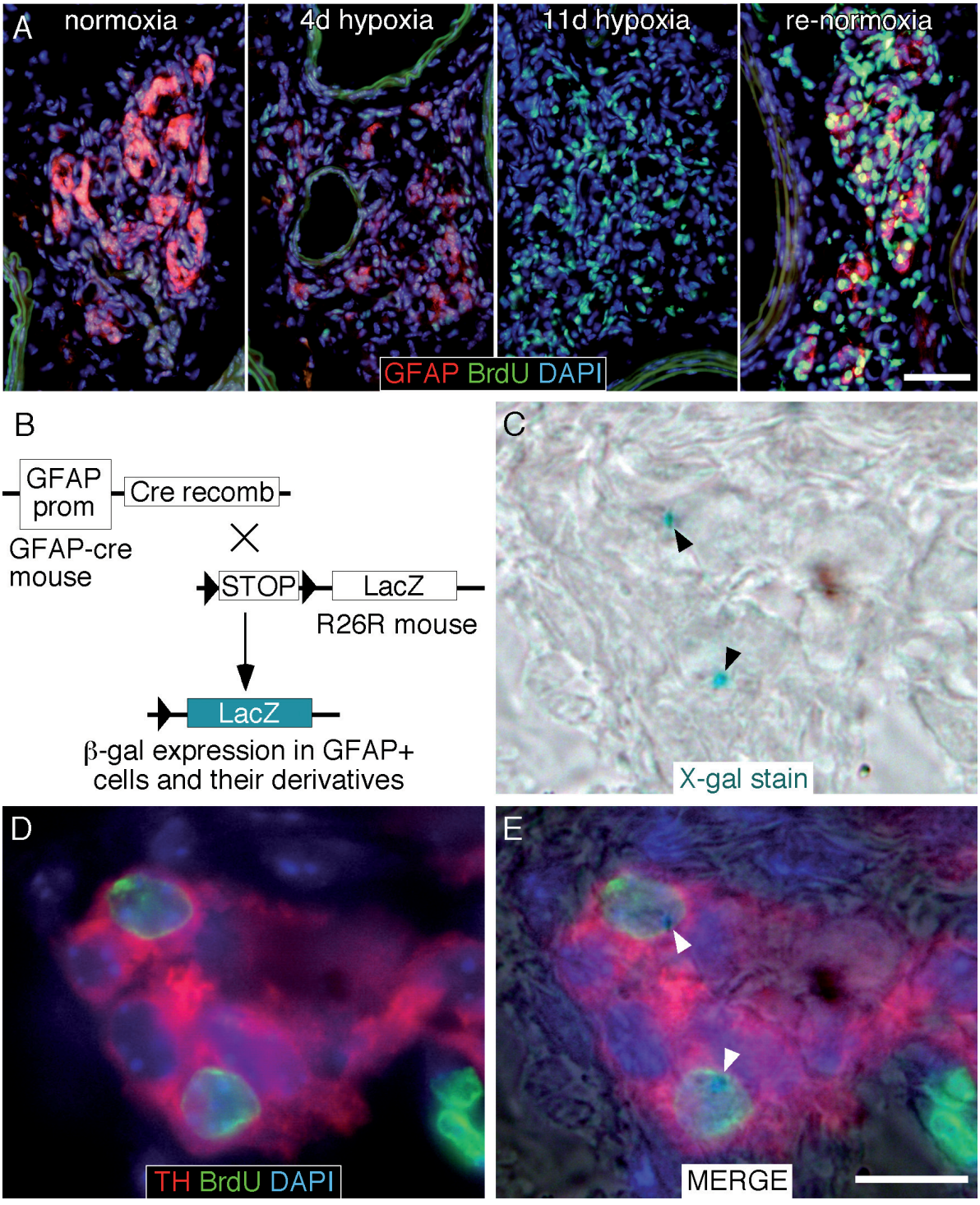

Fig. 3. - Glia-like type II cells differentiate into neuron-like type I cells under hypoxia.
A. Immunohistochemical detection of the glial marker GFAP, and of BrdU, within the carotid bodies of normoxic, hypoxic and re-normoxic mice. The disappearance and subsequent appearance of the GFAP staining suggested that glia-like type II cells, activated by hypoxia, are the carotid body progenitors. B. Investigation of the glial lineage of the carotid body progenitors giving rise to glomus cells. The carotid bodies of hypoxic GFAP-cre/floxed LacZ transgenic mice were analyzed by immunohistochemistry to look for type II cell derivatives, which appear as LacZ+. C. X-gal staining of a thin section of the hypoxic transgenic carotid body, indicating the presence of blue precipitate (arrowheads) in two different cells. D. Immunohistochemical detection of TH, BrdU and nuclei (DAPI). E. Merged image illustrating that the two newly formed BrdU+ glomus cells within this glomerulus are derived from GFAP+ type II cells. Scale bars: 50 μm in (A) and 10 μm in (C-E). Taken from Pardal et al., 2007.

of the sorted GFP+ cells were GFAP+ (75%) and highly efficient to induce the formation of multipotent neurospheres in clonal cultures (20% of plated cells formed neurospheres). In contrast, the ability to generate neurospheres of the GFP negative cell population (with only 7% of GFAP+ cells) was drastically reduced (0.7% of plated cells formed neurospheres). Therefore, enrichment of the GFAP+ CB cell population increases 30-fold the efficiency of neurosphere formation. Taken together, these data strongly suggest that GFAP+ type II cells are the stem/progenitor cells in the adult CB. Hence, GFAP+ type II cells are viewed as quiescent (or slowly dividing) CB stem cells that can be reversibly converted to nestin+ intermediate progenitors. Upon exposure to hypoxia, the equilibrium is displaced toward the nestin+ population, giving rise to TH+ glomus cells (Fig. 4E). It remains to be elucidated whether these same nestin+ progenitors are able to give rise *in vivo* to smooth muscle cells (Fig. 4E).

Conclusions and perspectives

The adult mammalian nervous system harbors multipotent stem or progenitor cells, which reside and participate in specialized niches that support self-renewal and differentiation. Neural progenitors exhibit features of differentiated glial cells at the ultrastructural and molecular levels including expression of glial intermediate filaments, such as glial fibrillary acidic protein (GFAP) (Doetsch, 2003a). During development of the CNS, the last waves of neurogenesis depend on radial glial cells, a population of progenitors derived from neuroepithelial cells in the neural tube, that present features characteristic of mature glia, such as the expression of GFAP or the presence of glycogen granules (Choi and Lapham, 1978). During perinatal life, radial glial cells adopt the final astrocytic phenotype that will be maintained along adult life. These specialized astrocytes are responsible for adult neurogenesis and reside within CNS neurogenic niches, the SVZ and SGZ areas (Doetsch, 2003a). We have now demonstrated that neurogenic centers in the PNS also respond to this glial phenotype of adult neural progenitors. CB stem cells or type II cells express GFAP and have cellular projections surrounding neuronal type I cells, which originally induced researchers to think about these cells as supportive glial cells. Moreover, the glial phenotype of adult neural progenitors seems to reflect quiescence in both CNS and PNS neurogenic niches, since once proliferation is activated, these cells convert into intermediate progenitors expressing nestin but not GFAP (Doetsch, 2003a; Pardal et al., 2007). We have further shown in the CB that nestin+ intermediate progenitors are able to revert to the GFAP+ quiescent type II cell phenotype upon re-exposure to normoxia (Pardal et al., 2007). Whether intermediate progenitors in the SVZ and SGZ areas are also capable of resuming back to the quiescent astrocytic phenotype upon stimulus ending is an important aspect that remains to be elucidated. The described behavior of adult neural stem cells seems to be tightly controlled by niche cells and factors in both CNS and PNS (Doetsch, 2003b). Understanding the *in vivo* adult neural stem cell niche is crucial to elucidating the function of neural stem cells and their progeny and ultimately defining their therapeutic potential.

The presence of adult neural progenitors with *in vivo* neurogenic capacity in neural crest derived structures might not be exclusive of the CB. In fact, various PNS organs contain cell types that resemble CB type II cells on their glia-like appearance and sustentacular shape. GFAP and vimentin expressing satellite cells have been described in other sympathoadrenal structures like the adrenal medulla or the sympathetic ganglia (Kameda, 1996; Shi et al., 2008). Furthermore, some of these populations of satellite glial cells, like those in the dorsal root ganglia (DRG), have been shown to behave as progenitors *in vitro*, being activated *in situ* in response to injury (Li et al., 2007). Hence, it remains to be clarified whether satellite glial cells are indeed adult PNS stem cells with the capacity to undergo neurogenesis, at least in pathological conditions.

CB stem cells have a clear physiological role sustaining the growth of the organ in chronic hypoxia. However, their discovery raises the question of their possible role in the pathophysiology of CB chemodectomas, a tumor subtype belonging to the relatively frequent paragangliomas affecting the autonomous nervous system. The incidence of CB paragangliomas increases in high-altitude residents (Astrom et al., 2003), and the tumors have histological features characteristic of the CB in individuals

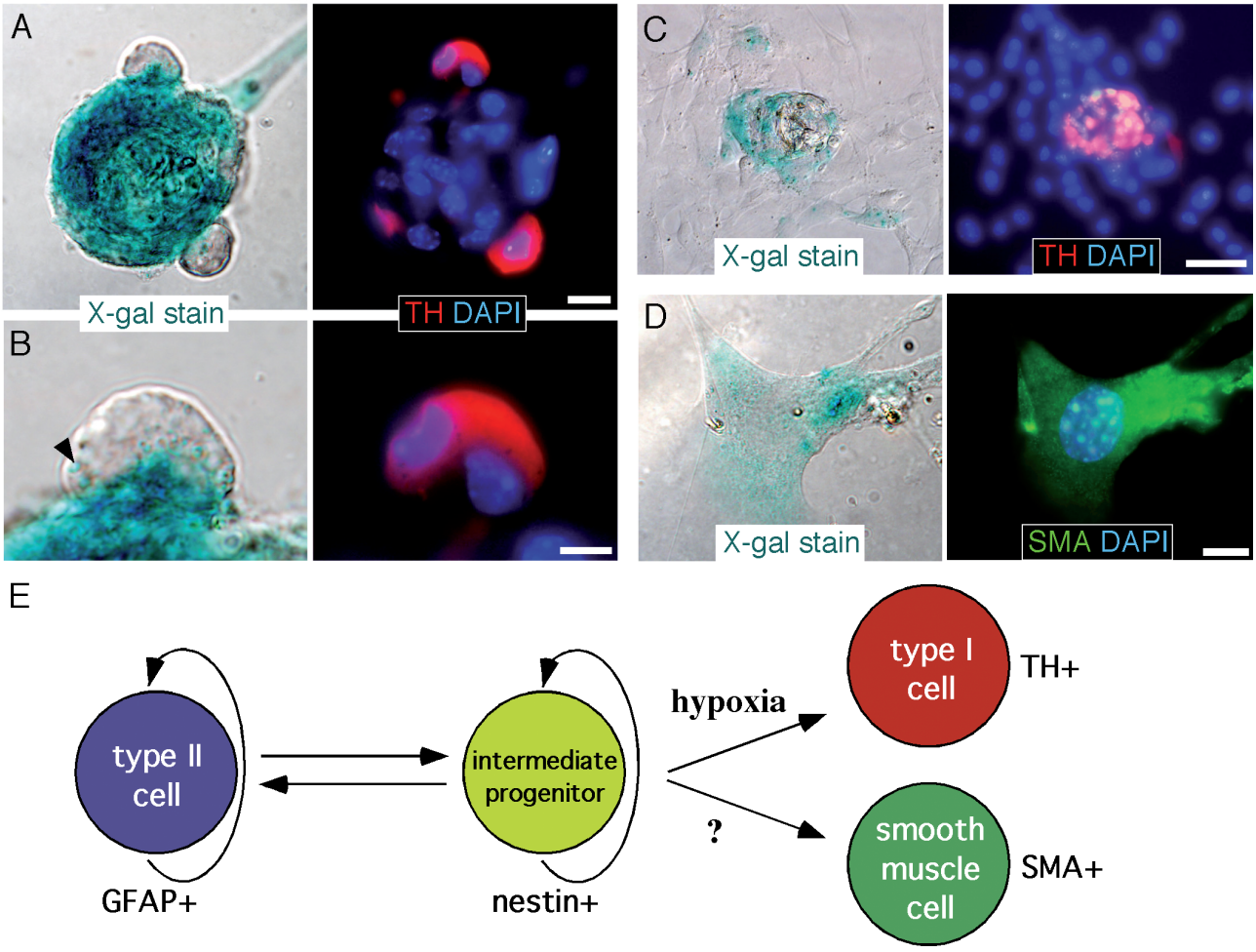

Fig. 4. - Formation of multipotent neurospheres from isolated GFAP+ carotid body stem cells *in vitro*.
A. Left, Bright field picture of a neurosphere derived from a GFAP+, X-gal+ cell dispersed from CBs of GFAP-cre/floxed LacZ mice. Right, Immunocytochemical identification of TH+ cells. B. Magnified images illustrating the co-localization of X-gal deposits on TH+ cells (arrowhead). C. Left, Bright field picture of a neurosphere derived from a GFAP+, X-gal+ cell, cultured on adherent substrate. Right, Immunocytochemical identification of TH+ cells. D. Magnified images illustrating the co-localization of X-gal deposits on SMA+ smooth muscle cells. E. Schematic drawing of cellular events taking place in the carotid body stem cell niche. Scale bars: 10 μm in panel (A), 5 μm in panels (B) and (D), and 50 μm in panel (C). Modified from Pardal et al., 2007.

with chronic hypoxemia (Arias-Stella and Valcarcel, 1976; Heath et al., 1982). Thus, it is possible that disruption of CB stem cell homeostasis leads to tumor transformation. Chemodectomas are most frequently benign and have low incidence of mitosis, with an immunocytochemical profile characterized by numerous neuron-like (TH+ and enolase+) glomus type I cells and GFAP+ sustentacular type II cells. Abundance of type II cells could offer good prognosis of paragangliomas, since GFAP immunoreactivity is inversely correlated with the grade of malignancy (Kliewer et al., 1989; Achilles et al., 1991). In addition, GFAP+ cells are absent in the rare cases of CB tumors with metastasis to cervical lymph nodes (Robertson and Cooney, 1980). Hence, a plausible hypothesis is that conversion of GFAP+ latent stem cells to aberrant GFAP negative proliferating progenitors could give rise to cancer stem cells leading to CB tumorigenesis.

Because of their dopaminergic nature, CB cells have been used for transplantation studies in Parkinson's disease (PD). Intrastriatal transplantation of CB cells yields good histological and functional recovery in rodent (Espejo et al., 1998; Toledo-Aral et al., 2003) and monkey (Luquin et al., 1999) models of PD. On the other hand, glomus cells contain unusually large

amounts of GDNF (Villadiego et al., 2005), and intrastriatal delivery of this trophic factor is believed to be effective for antiparkinsonian therapy because it promotes dopaminergic neuron survival and sprouting of the nigrostriatal pathway (for references, see Kirik et al., 2004; Pascual et al., 2008). Autotransplantation of CB cell aggregates ameliorates the symptoms in PD patients (Arjona et al., 2003), but a major limiting factor of this therapy is the histological integrity of the CB at advanced age and the scarce amount of tissue available for transplantation. The identification of CB stem cells offers a new tool with potential clinical applicability. We have shown that in long-term (> 3 weeks) cultures glomus cell clusters in a single neurosphere can reach the size of a whole CB. The newly produced glomus cells are highly catecholaminergic, synthesize GDNF, and have normal chemoreceptive and electrophysiological properties (Pardal et al., 2007). Therefore, to determine the efficacy and safety of intrastriatally transplanted CB neurospheres in parkinsonian animal models, and to investigate the existence of adult human CB progenitors and their ability to proliferate and differentiate *in vitro* are promising scientific objectives that should be addressed in future experimental work.

Acknowledgments

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