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Carotid body oxygen sensing

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ABSTRACT: The carotid body (CB) is a neural crest-derived organ whose major function is to sense changes in arterial oxygen tension to elicit hyperventilation in hypoxia. The CB is composed of clusters of neuron-like glomus, or type-I, cells enveloped by glia-like sustentacular, or type-II, cells. Responsiveness of CB to acute hypoxia relies on the inhibition of O_2-sensitive K^+ channels in glomus cells, which leads to cell depolarisation, Ca^{2+} entry and release of transmitters that activate afferent nerve fibres. Although this model of O_2 sensing is generally accepted, the molecular mechanisms underlying K^+ channel modulation by O_2 tension are unknown. Among the putative hypoxia-sensing mechanisms there are: the production of oxygen radicals, either in mitochondria or reduced nicotinamide adenine dinucleotide phosphate oxidases; metabolic mitochondrial inhibition and decrease of intracellular ATP; disruption of the prolylhydroxylase/hypoxia inducible factor pathway; or decrease of carbon monoxide production by haemoxygenase-2. In chronic hypoxia, the CB grows with increasing glomus cell number. The current authors have identified, in the CB, neural stem cells, which can differentiate into glomus cells. Cell fate experiments suggest that the CB progenitors are the glia-like sustentacular cells. The CB appears to be involved in the pathophysiology of several prevalent human diseases.

KEYWORDS: Acute hypoxia, carotid body, chronic hypoxia, ion channels, oxygen sensing, stem cells

The carotid body (CB), a small neural crest-derived paired organ located at the carotid bifurcation (fig. 1a), is a principal component of the homeostatic acute oxygen-sensing system required to activate the brainstem respiratory centre to produce hyperventilation during hypoxaemia (*e.g.* in high-altitude residents or in patients with chronic obstructive pulmonary diseases) [2–4]. 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 organised into glomeruli: clusters of cells, in close contact with a profuse network of capillaries, and afferent sensory fibres joining the glossopharyngeal nerve (fig. 1a and b). 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 type-II cells (fig. 1c). The CB also contains some autonomic neurons and fibres, which seem to have an efferent regulatory action on glomus cells [5].

Glomus cells are physiologically complex, as they express a broad variety of voltage- and ligand-gated ion channels, as well as transient receptor potential and background K^+ channels. They contain secretory vesicles packed with neurotransmitters, notably ATP, dopamine and acetylcholine, among others [6]. Voltage-gated ion channels have been studied in detail in patch-clamped glomus cells from several species [2]. Macroscopic ionic currents recorded from these cells are composed of outward (mediated by several classes of K^+ channels) and inward (mediated by Na^+ and/or Ca^{2+} channels) components (as discussed hereunder). Quantitatively, the proportion of the different subtypes of K^+, Na^+ and Ca^{2+} channels expressed in glomus cells greatly varies among the mammalian species studied. Owing to the presence of voltage-gated membrane channels, glomus cells are electrically excitable and can repetitively generate action potentials. This property is particularly evident in rabbit glomus cells, with relatively large

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voltage-dependent Na^+ currents [7]. Glomus cell membrane depolarisation induces a reversible neurosecretory response, dependent on extracellular Ca^{2+} influx, which can be easily monitored by amperometry [8, 9]. Thus, glomus cells behave as presynaptic-like elements that establish contact with the postsynaptic sensory nerve fibres.

The precise functional significance of the numerous neurotransmitters that exist in the CB is still under debate. The CB is among the most dopaminergic structures in the body and, as extracellular dopamine inhibits the Ca^{2+} channels in glomus cells, it has been suggested that this transmitter has an autocrine role [10]. In contrast, ATP and, possibly, acetylcholine appear to be the major active neurotransmitters at the glomus cell-afferent fibre synapse [6, 11]. There are other amines and several neuropeptides in the CB whose functional significance is, as yet, not well known. Glomus cells also have high levels of neurotrophic factors, which seem to exert a local autocrine and paracrine action [12]. Among these factors, the glia cell line-derived neurotrophic factor (GDNF) has attracted particular attention because it is highly expressed in adult glomus cells [13–15]. As GDNF can promote the survival of dopaminergic neurons, CB transplants have been used for intrastriatal delivery of dopamine and GDNF in parkinsonian animal models and in some pilot clinical studies on Parkinson's disease patients (see section Carotid body function and mechanisms of disease).

Of the cells in the CB parenchyma, ~15–20% are type-II cells, which *in vivo* exhibit long processes surrounding type-I cells (fig. 1b and d). Type-II cells are nonexcitable and lack most of the voltage-gated channels characteristic of type-I cells [7, 16]. 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, recent experimental data have shown that the adult CB is a functionally active germinal niche. In this regard, it has been strongly suggested that type-II cells are indeed dormant stem cells that in response to physiological hypoxia can proliferate and differentiate into new glomus cells (see section Carotid body plasticity in chronic hypoxia: adult carotid body stem cells) [1].

RESPONSES OF GLOMUS CELLS TO ACUTE HYPOXIA: MODEL OF CAROTID BODY O_2 SENSING

Glomus cells are polymodal arterial chemoreceptors, activated not only by hypoxia but also by other stimuli, most notably hypercapnia, extracellular acidosis and hypoglycaemia [2, 17]. It is, however, the sensitivity to acute changes of O_2 tension what makes the CB essential for the classical adaptive hyperventilatory reflex in response to hypoxaemia. Although the function of the CB as an acute oxygen sensor has been known since the first half of the 20th century, it was during the past 15–20 yrs that the basic cellular events underlying this physiological process were unveiled. It is now broadly accepted that glomus cells are the chemoreceptive elements in the CB and that they contain several classes of O_2-sensitive K^+ channels whose open probability decrease during hypoxia [2–4]. Voltage-dependent K^+ channels were initially reported to be O_2 sensitive in rabbit CB cells [18], but other K^+ channel types (particularly Ca^{2+}-dependent maxi-K^+ and twin pore acid-stimulated K^+ channel-like background channels) have been also found to be modulated by O_2 in several CB preparations [19, 20]. Inhibition of the K^+ channels leads to glomus cell membrane depolarisation and increase in the firing frequency of the cells, thus resulting in Ca^{2+} channel opening, transmembrane Ca^{2+} influx and transmitter release. The major steps in the chemotransduction process are: hypoxic inhibition of the K^+ currents (fig. 2a) and inhibition of single K^+ channel activity (fig. 2b) [18, 21], external Ca^{2+}-dependent increase of cytosolic Ca^{2+} in hypoxia (fig. 2c) [8, 23], and catecholamine release from hypoxic glomus cells (fig. 2d) [8, 9]. The dose-dependent cellular responses to hypoxia (increase of cytosolic Ca^{2+} concentration and catecholamine release) almost perfectly match the characteristic hyperbolic correlation between arterial O_2 tension and the afferent discharges of the CB sinus nerve or the increase in ventilation seen *in vivo*. Within the context of this broadly accepted ''membrane model'' of CB O_2 sensing, schematically summarised in figure 3, it is worth remarking

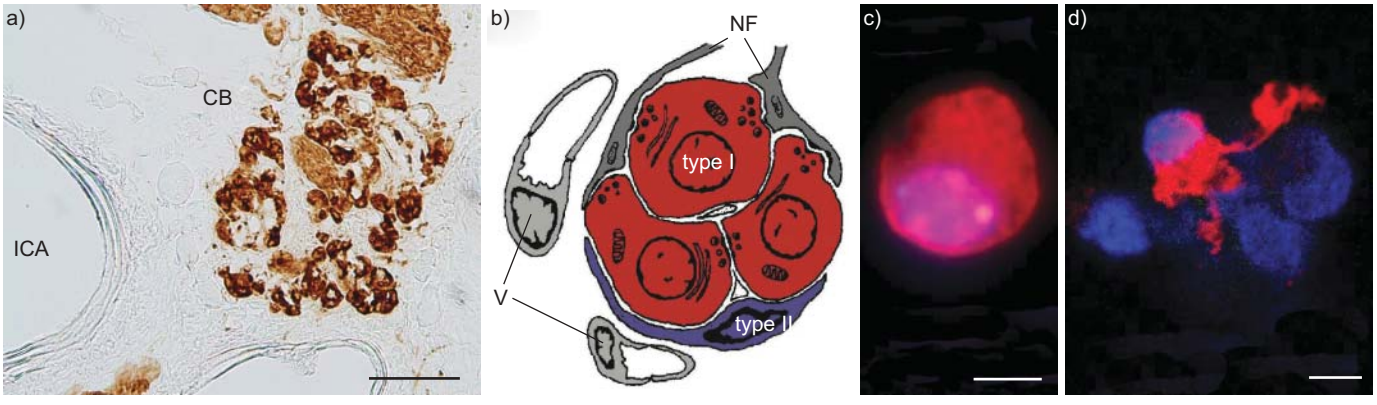

FIGURE 1. Anatomical organisation of the carotid body (CB). a) Histological section of the mouse carotid bifurcation immunostained with an antibody against tyrosine hydroxylase (TH; brown colour). The clusters (glomeruli) of TH+ cells forming the CB can be easily identified. b) Schematic representation of a CB glomerulus with indication of type-I and type-II cells, blood vessels (V) and afferent sensory nerve fibres (NF). c) Appearance of a rat TH-positive type-I glomus cell. d) Appearance of a rat glial fibrillary acidic protein (GFAP)-positive type-II cell with processes surrounding GFAP-negative cells. Cell nuclei are stained with 4′,6-diamidino-2-phenylindole. ICA: internal carotid artery. Scale bars=100 μm (a) and 5 μm (c and d). Modified from [1].

that, although the participation of mitochondria in CB O_2 sensing is under debate (see section Mechanisms of carotid body acute O_2 sensing), the experimental data available unequivocally indicate that they do not contribute to the hypoxia-induced rise of cytosolic Ca^{2+} concentration necessary to trigger glomus cell secretion.

The membrane model of CB oxygen sensing discussed in the preceding paragraph has been generalised to other neurosecretory or contractile cells acutely responding to hypoxia. Among those are neonatal adrenomedullary chromaffin cells [25–27], cells in the neuroepithelial bodies of the lung [28] or PC12 cells [29], as well as pulmonary arterial myocytes [30, 31]. These cells belong to the homeostatic acute O_2-sensing system that allows mammalian fast adaptation to hypoxic environments [4].

MECHANISMS OF CAROTID BODY ACUTE O_2 SENSING

Despite progress in CB cellular physiology, the molecular mechanisms underlying glomus cell O_2 sensing, *i.e.* how the change in O_2 tension is translated into decrease of K^+ conductance, remain essentially unknown. Several possible O_2-sensing mechanisms, including the direct interaction of O_2 with the ion channels or their indirect modulation through O_2-sensing molecules, have been postulated [2, 24]. Since numerous K^+ channel types have been reported to be O_2 sensitive, it is assumed that there could be several O_2 sensors coexisting in the same cell or distributed among the different O_2-sensitive cell types, even in closely related animal species. Understanding CB O_2 sensing at the molecular level is, however, challenged by the small size of the organ, which precludes elaborated biochemical and molecular biology experiments, and the gaseous nature of the detected molecule, which is easily diffusible across cell membranes and difficult to keep under strict control in the open chambers normally used for *in vitro* studies. In addition, O_2 responsiveness of isolated CB glomus cells is often lost, because of damage during the vigorous enzymatic and mechanical treatment needed for their dispersion. Finally, some possible O_2-sensing mechanisms have been inferred from pharmacological experiments using compounds (as, for example, mitochondrial inhibitors) that might have nonspecific effects [32, 33], hence providing misleading conclusions. The mechanisms of CB O_2 sensing are summarised in the following sections, emphasising the proposals that are currently under debate and the knowledge generated by the current authors' experimental work.

Redox-based O_2 sensor: reduced nicotinamide adenine dinucleotide phosphate oxidase

A plausible form of CB O_2 sensing is the conversion of O_2 into reactive oxygen species (ROS), which would in turn alter the redox status of signalling molecules and the function of membrane ion channels. The two ROS-producing sites postulated as O_2 sensors are the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria systems.

NADPH oxidase is found in neutrophils and histochemically localised in the CB, although its presence in the chemosensitive glomus cells is not well documented. This enzyme has been proposed to transduce O_2 levels by changing the rate of superoxide anion production, which, after conversion to hydrogen peroxide, oxidises ion channels and other molecules. The neutrophil oxidase is an oligomer composed of the membrane-bound catalytic complex (formed by gp91phox and p22phox), a cytochrome, and several cytosolic regulatory subunits (p47phox and others). Although impaired O_2 sensitivity of airway chemoreceptor cells has been reported in

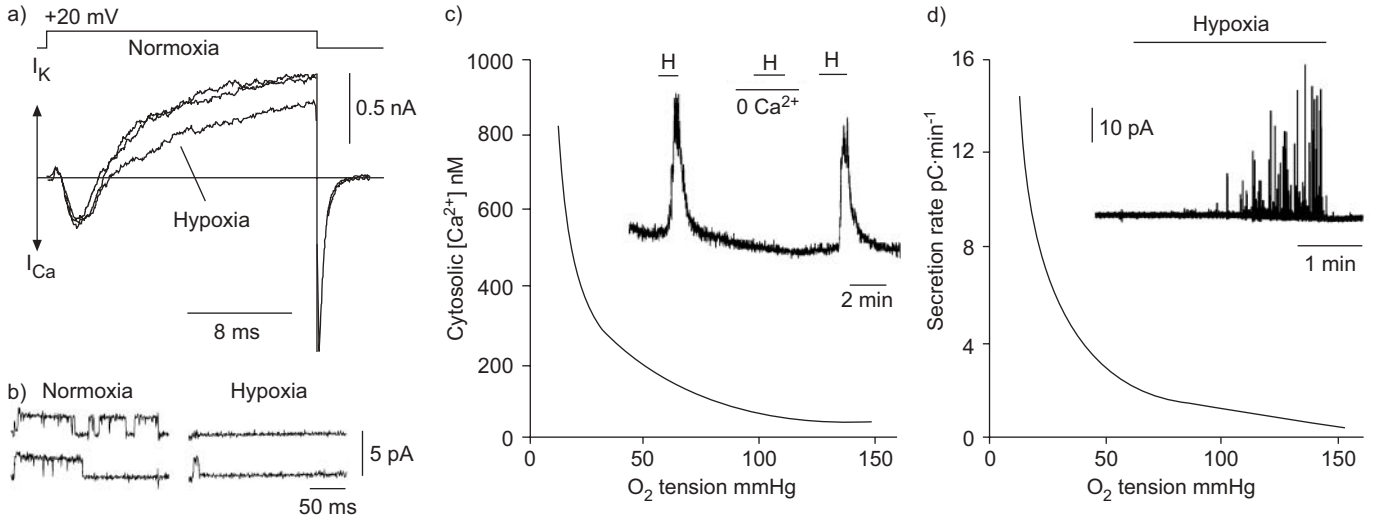

FIGURE 2. Responses of glomus cells to hypoxia. a) Ionic currents recorded from a patch-clamped rabbit glomus cell during a depolarising pulse from -80 mV to +20 mV. Inward calcium (I_{Ca}) and outward potassium (I_K) currents are indicated. Voltage-gated sodium channels were blocked with tetrodotoxin. Note the selective reversible inhibition of the K^+ current by exposure to hypoxia (switching from an O_2 tension 150 to ~15 mmHg). Modified from [9]. b) Single K^+ channel activity recorded in an excised membrane patch exposed to normoxic and hypoxic solutions. Note that lowering O_2 tension reduces the single channel open probability without affecting the single channel current amplitude. Modified from [21]. c) Changes in cytosolic calcium concentration, $[Ca^{2+}]$, in rabbit glomus cells as a function of oxygen tension. The inset shows that hypoxia (H) induces a rise of cytosolic Ca^{2+} concentration, which is strictly dependent on extracellular Ca^{2+} influx. Modified from [8, 9]. d) Catecholamine secretion from rabbit glomus cells as a function of oxygen tension. The inset illustrates the secretory response of a single cell to hypoxia as monitored by amperometry. Modified from [22].

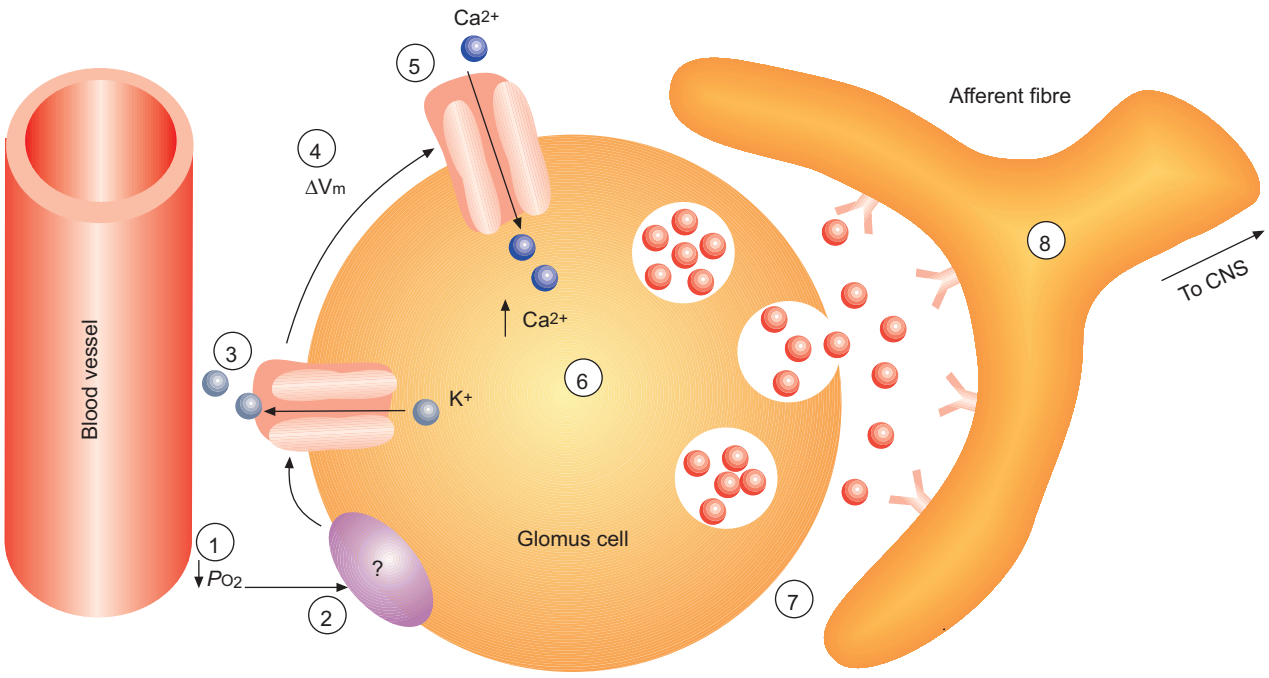

FIGURE 3. Membrane model of glomus cell oxygen sensing. The steps in chemosensory transduction are as follows. 1) Decrease of O_2 tension (P_{O_2}), 2) O_2 sensing, 3) closure of potassium channels, 4) cell depolarisation, 5) opening of calcium channels, 6) increase of cytosolic calcium concentration, [Ca^{2+}], 7) transmitter release and 8) activation of afferent fibres, which send the information to the central nervous system (CNS). Although these steps in chemosensory transduction have broad experimental support, the nature of the O_2 sensor and the mechanisms by which changes in O_2 tension regulate K^+ channel activity are still unknown (question mark). ΔV_m: change in membrane voltage. Modified from [2, 24].

gp91phox-null mutant mice [34], hypoxia responsiveness of CB and other cells appears to be unaltered [35, 36]. Moreover, in patch-clamped glomus cells from these animals the modulation of the O_2-sensitive K^+ current by O_2 tension is unchanged [37]. Surprisingly, genetic suppression of another component of the neutrophil's oxidase (p47phox) results in mice with increased basal activity in the carotid sinus nerve and exacerbated ventilatory response to hypoxia [38]. This phenotype suggests nonspecific modifications in the p47phox knockout mouse rather than the selective alteration of the O_2-sensing machinery in the CB cells. Altogether, these studies indicate that the phagocytic NADPH oxidase is not directly involved in CB O_2 sensing, although it is conceivable that other isoforms, existing in numerous tissues [39], could contribute to the hypoxia responsiveness of CB cells. The entire concept of redox-based O_2 sensing in glomus cells is, however, challenged by the finding that the reduced/oxidised glutathione ratio in CBs remains unchanged during exposure to hypoxia, despite the fact that this quotient increases after incubation of CBs with *N*-acetylcysteine, a precursor to reduced glutathione and an ROS scavenger [40].

Mitochondrial dysfunction

Several investigators have traditionally considered mitochondria to be the site of O_2 sensing in glomus cells because, similar to hypoxia, inhibitors of the electron transport chain (ETC) or mitochondrial uncouplers increase the afferent activity of the CB sinus nerve [41]. This proposal was complemented by reports that hypoxia and cyanide (an inhibitor of mitochondrial complex IV) lead to Ca^{2+} release from mitochondria in dispersed glomus cells [42]. As indicated in the previous section, the mitochondrial hypothesis of CB O_2 sensing has lost much support after the discovery of O_2-regulated K^+ channels and experimental demonstration that the Ca^{2+} ions needed for glomus cell secretion in hypoxia enter the cell *via* plasmalemmal voltage-gated Ca^{2+} channels [8, 9, 23]. The interest in mitochondria has, however, resurged more recently because mitochondria uncouplers raise cytosolic Ca^{2+} and reduce background K^+ permeability in glomus cells [43, 44]. So, it could be that in hypoxia, mitochondria generate signals that alter membrane ionic conductances (*e.g.* through modification of the cell redox status or *via* reduction of cytosolic ATP). In fact, it has been proposed that the redox modulation of membrane K^+ channels is the reason for the O_2 sensitivity of other acutely responding cells [45]. Conversely, decrease of intracellular ATP in hypoxia could result in either the direct closure of ATP-regulated background K^+ channels [46] or the increase in AMP/ATP ratio leading to AMP kinase activation. AMP kinase could, in turn, modulate membrane ion channels thus eliciting cell depolarisation [47]. In favour of this hypothesis is the existence of O_2-sensitive background K^+ channels, which appear to be modulated by mitochondrial uncouplers and ATP. In addition, mRNA of AMP kinase is detected in glomus cells (unpublished data of the current authors). In contrast with these observations, the current authors have shown that in the presence of saturating concentrations of mitochondria ETC inhibitors acting at different complexes (I, II, III and IV), hypoxia can still activate transmitter release from glomus cells, thus suggesting that mitochondrial inhibition and hypoxia might activate glomus cells through separate pathways [32]. Moreover, patch-clamped glomus cells loaded with a high concentration of

Mg-ATP (3–5 mM) still respond to hypoxia [8, 18]. Interestingly, the current authors have also observed that rotenone, but no other agents inhibiting complex I at different sites, can block hypoxia responsiveness of glomus cells, thus suggesting that a rotenone-binding site participates in O_2 sensing [32]. This effect of rotenone seems to be quite specific, as glomus cell responsiveness to hypoglucaemia is unaffected by rotenone [48]. However, the pharmacological data must be interpreted with caution, as it is highly likely that at the concentrations used, ETC inhibitors have nonspecific effects on the voltage-gated ion channels [33]. It is known, for example, that rotenone can reversibly inhibit K^+ currents in cells devoid of mitochondria [49].

Mitochondria have also been associated with CB O_2 sensing because mutations in the mitochondrial complex II (particularly in the small membrane-anchoring subunit of succinate dehydrogenase (SDHD)) are the main cause of familiar hereditary paraganglioma (PGL), a highly vascularised and often catecholamine-secreting CB tumour [50]. As PGLs display cellular hyperplasia/anaplasia similar to the CB of individuals exposed to chronic hypoxaemia [51, 52], it has been proposed that the ultimate cause of tumorigenesis is a defect in sensing environmental O_2 levels [50, 53–55]. The current authors have tested this hypothesis by generating a knockout mouse model lacking *SDHD*. Whereas null animals die early during embryonic life, heterozygous *SDHD*+/- mice develop normally without apparent signs of respiratory distress. *SDHD*+/- animals show, however, a 40–50% decrease of mitochondrial complex II activity in all the tissues tested (brain, liver, heart and kidney) and a small (~15%) increase in the number and size of glomus cells [56]. Despite these structural changes, the response to hypoxia of glomus cells in *SDHD*+/- mice was unaltered, or even augmented, in comparison with *SDHD*+/+ litter mates, indicating that partial deficiency of complex II activity does not seem to alter glomus cell responsiveness to hypoxia.

In summary, although the exact role of mitochondria in CB function is not fully clarified, the data available thus far suggest that these organelles do not directly contribute to the primary steps in CB O_2 sensing. However, mitochondrial dysfunction (*e.g.* in extreme hypoxia or after addition of ETC inhibitors) might result in metabolic alterations leading to changes in membrane ion channels that could modulate glomus cell activity. In accord with this idea, glomus cells in partially deficient SDHD mice (*SDHD*+/-), although with normal O_2 sensing, exhibit an abnormally high resting secretory activity and a constitutive ~50% reduction in total K^+ current density [56].

Prolyl/asparagyl hydroxylases and hypoxia-inducible factor pathway

The best-studied O_2 sensors are probably the prolyl/asparagyl hydroxylases, enzymes that utilise molecular O_2 (together with Fe^{2+} and α-ketoglutarate as cosubstrate) to hydroxylate specific proline/asparagine residues, respectively, of hypoxia-inducible transcription factors (HIF)-1α, and its isoforms, as well as other molecules which, in turn, regulate the expression of numerous hypoxia-sensitive genes. In the absence of O_2, the lack of hydroxyl groups in specific proline and asparagine residues of the HIF molecule prevents its degradation by the proteasome and facilitates its stabilisation, dimerisation with HIF-1β, translocation to the nucleus, and transcriptional activity [57, 58]. Hydroxylation of HIF in the presence of O_2 occurs in a few minutes, hence it is conceivable that O_2-dependent hydroxylases could also modulate ion channels and thus participate in the acute responses to hypoxia. The current authors have tested this plausible hypothesis using CB slices incubated with saturating concentrations of dimethyloxalylglycine (DMOG), a membrane-permeant competitive inhibitor of α-ketoglutarate that completely and nonselectively inhibits hydroxylases [59]. It is well known that DMOG mimics hypoxia and induces the expression of HIF-dependent genes [59, 60]. However, after incubation of CB slices with DMOG, glomus cells retain their normal responsiveness to acute hypoxia. Preliminary experiments on prolyl hydroxylase 3-null mice performed in the current authors' laboratory have also shown that their CB sensitivity to acute hypoxia is unaltered.

It has also been suggested that HIF-1α could directly participate in the acute responsiveness to hypoxia, since the plastic changes in the chemosensory activity (augmented ventilatory response and long-term facilitation) induced by sustained and intermittent chronic hypoxia are altered in HIF-1α+/- mice [61, 62]. CB cells in slices from HIF-1α+/- mice show, however, a marked secretory response to hypoxia indistinguishable from that measured in homozygous HIF-1α+/+ wild-type mice [63]. These data suggest that, although HIF-1α may contribute to CB functional plasticity, partial deficiency of the transcription factor does not significantly alter the intrinsic acute O_2 sensitivity of CB glomus cells.

Haemoxygenase-2

Haemoxygenase (HO)-2 is an antioxidant enzyme constitutively expressed in most cells, including CB cells [64–66]. This enzyme uses O_2 to convert haem into biliverdin, iron and carbon monoxide [67]. The possible involvement of HO-2 in CB acute O_2 sensing has been suggested because it co-immunoprecipitates with heterologously expressed maxi-K^+ channels and its inhibition with small interfering RNA abolishes the O_2 modulation of recombinant channels [65]. HO-2 is expressed in rat CB glomus cells and, in addition, native maxi-K^+ channels recorded in patches excised from these cells are activated by HO-2 substrates (haem and NADPH). Based on these data it has been proposed that HO-2 could act as an O_2 sensor through the production of CO, which is by itself a maxi-K^+ channel activator [67, 68].

Although the proposal that HO-2 participates in O_2 sensing is quite attractive [69] it has been challenged by experiments performed on the HO-2 knockout mouse, which develop normally, without alteration in haematocrit or signs of respiratory distress during the first postnatal 2–3 months, although they manifest cardiorespiratory alterations at advanced age [67, 70]. The current authors have studied in detail the secretory responses to acute hypoxia of glomus cells from HO-2+/+, HO-2-/- and HO-2+/- animals using CB slices [63, 22]. In all cases, secretion rate increased drastically upon exposure to low O_2 tension. The dose–response curves obtained from glomus cells exposed to different O_2 tensions were indistinguishable in HO-2-deficient and wild-type mice, suggesting that partial or complete HO-2 deficiency do not alter glomus cell O_2 sensitivity. It can be also disregarded that

the embryonic absence of HO-2 is compensated by upregulation of HO-1, an inducible HO, since the mRNA expression of this enzyme in CB tissue from HO-2-null animals is not significantly increased. Moreover, HO-1 does not seem to compensate for HO-2 deficiency, since within the CB it is expressed predominantly in blood vessels and, even in HO-2-/- animals, it is absent from the clusters (glomeruli) of tyrosine hydroxylase (TH)+ glomus cells [22].

Although glomus cell responsiveness to hypoxia is normal in HO-2-null animals, it seems that HO-2 deficiency causes CB phenotypic alterations secondary to redox dysregulation [65]. HO-2-null young adults (<3 months) showed a marked upregulation of cyclophilin and TH, the rate-limiting enzyme for catecholamine synthesis highly expressed in CB glomus cells. In contrast, CB Slo1 mRNA (the maxi-K^+ channel α-subunit gene) was significantly downregulated in HO-2-null mice in comparison with controls. These alterations in the CB gene expression profile, although unrelated to the mechanisms of CB O_2 sensing, are compatible with a subclinical cellular oxidative stress, which could also be responsible for a small, but significant, CB growth observed in HO-2-null animals [22].

In summary, no definitive conclusion can be drawn to date regarding the molecular mechanisms of CB O_2 sensing. There are numerous hypotheses and interesting proposals under debate but clarification of this important physiological process must await future experimental work.

CAROTID BODY PLASTICITY IN CHRONIC HYPOXIA: ADULT CAROTID BODY STEM CELLS

In addition to its role as an acutely responding arterial chemoreceptor, the CB is special among the adult neural and paraneural organs because it grows several-fold upon exposure to chronic hypoxia. In humans, this adaptive response occurs during acclimation to high altitude [52, 71, 72] or in patients suffering cardiopulmonary diseases presenting hypoxaemia [51, 73] (see section Carotid body function and mechanisms of disease). The current authors have recently studied in detail the morphological changes induced by chronic hypoxia in mouse and rat CB, with the aim of identifying the progenitors that could be used for *in vitro* expansion of CB dopaminergic glomus cells [1]. Mouse CBs from animals kept in normoxia (21% O_2 atmosphere) show the typical histological organisation of the organ with clusters of TH+ glomus cells (fig. 4a). Exposure of the animals to hypoxia (10% O_2 atmosphere) induces a marked CB enlargement caused by dilation and multiplication of blood vessels, as well as expansion of the parenchyma, with increased number of TH+ glomus cell clusters (fig. 4b). To analyse the origin and formation of new glomus cells, mice were treated with BrdU (a marker selectively incorporated in replicating DNA) and, subsequently, maintained several days in a hypoxic environment. After a few days in hypoxia, even before the CB growth became macroscopically obvious, numerous BrdU+ TH+ cells were observed, indicating the appearance of new glomus cells (fig. 4c). Brief (2-h) BrdU pulses were also applied to animals that had been kept in hypoxia for several days, in order to test whether some TH+ cells could be captured in the process of division. In some of these experiments, TH+ BrdU+ cells (fig. 4d) were observed, suggesting that, as reported previously [12], glomus cells might undergo mitosis upon activation by hypoxia. TH+ glomus cells cannot produce clonal neurospheres *in vitro* (as discussed hereunder), so it is likely that their mitogenic potential is limited and possibly depends on the level of hypoxia and animal age [1]. The time course of CB structural changes induced by hypoxia is shown in figure 4e. Although BrdU incorporation into the CB tissue is observed immediately after exposure to hypoxia, the newly formed glomus cells (BrdU+ TH+) were predominantly seen after few days. This time course also suggested the existence in the CB of precursors, whose proliferation in hypoxia precedes their differentiation into glomus cells.

The precursors giving rise to glomus cells have been identified using enzymatically dispersed CB cells plated in floating conditions. To stimulate clonogenic proliferation, cells are cultured under moderate hypoxia (3% O_2), a condition that mimics the hypoxic stimulation of CB growth *in vivo*. Under these conditions, ~1% of the plated CB cells give rise to neurospheres, typical colony-like structures formed by growing neural stem cells (fig. 5a and b). In contrast to the typical spherical shape of neurospheres formed by stem cells isolated from other neural (central or peripheral) areas [74, 75], most of the CB-derived neurospheres have characteristically one or two large blebs budding out of the main core (fig. 5a and b). Immunocytochemical analysis of thin-section neurospheres have revealed the presence of nestin (a typical neural stem cell marker)-positive cells within the main core, and clusters of differentiated TH+ and nestin- cells within the blebs attached through a hilus (fig. 5c and d). The TH+ blebs resembled in shape the glomeruli characteristic of the *in situ* CB and grew to a large size after several weeks in culture (fig. 5e). This morphological and immunological pattern (core of nestin+ cells preceding the blebs with TH+ cells) is consistently observed in most of the CB neurospheres studied (fig. 5f–h). The clonal origin of CB neurospheres has been confirmed by single cell deposition experiments (fig. 5i–k).

The data described in the previous section indicate that the CB contains stem cells from which TH+ cells (resembling glomus cells) can be differentiated *in vitro*. The current authors have studied the physiology of stem cell-derived TH+ cells in order to test whether they behave as true matured glomus cells. TH+ cells within the neurosphere buds generated *in vitro* were subjected to voltage clamp using the whole-cell configuration of the patch-clamp technique. The recording in figure 6a illustrates that the newly formed cells have small inward Ca^{2+} currents (I_{Ca}) followed by larger outward K^+ currents (I_K). The amplitude, time course and voltage dependence of the outward current were similar to those recorded from cells in rat CB slices or after enzymatic dispersion [17, 76]. As in normal CB glomus cells, blockade of the K^+ outward current with internal Cs^+ revealed the presence of typical inward, non- (or slowly) inactivating Ca^{2+} currents (fig. 6a). The newly formed glomus cells responded to hypoxia with an acute surge of catecholamine secretion indistinguishable from that evoked in the CB *in vivo* (fig. 6b) and they also expressed GDNF mRNA, a trophic factor characteristic of adult glomus cells (fig. 6c) [15, 76]. These data indicate that TH+ cells derived *in vitro* from CB progenitors exhibit the characteristic complex functional properties of mature glomus cells [1].

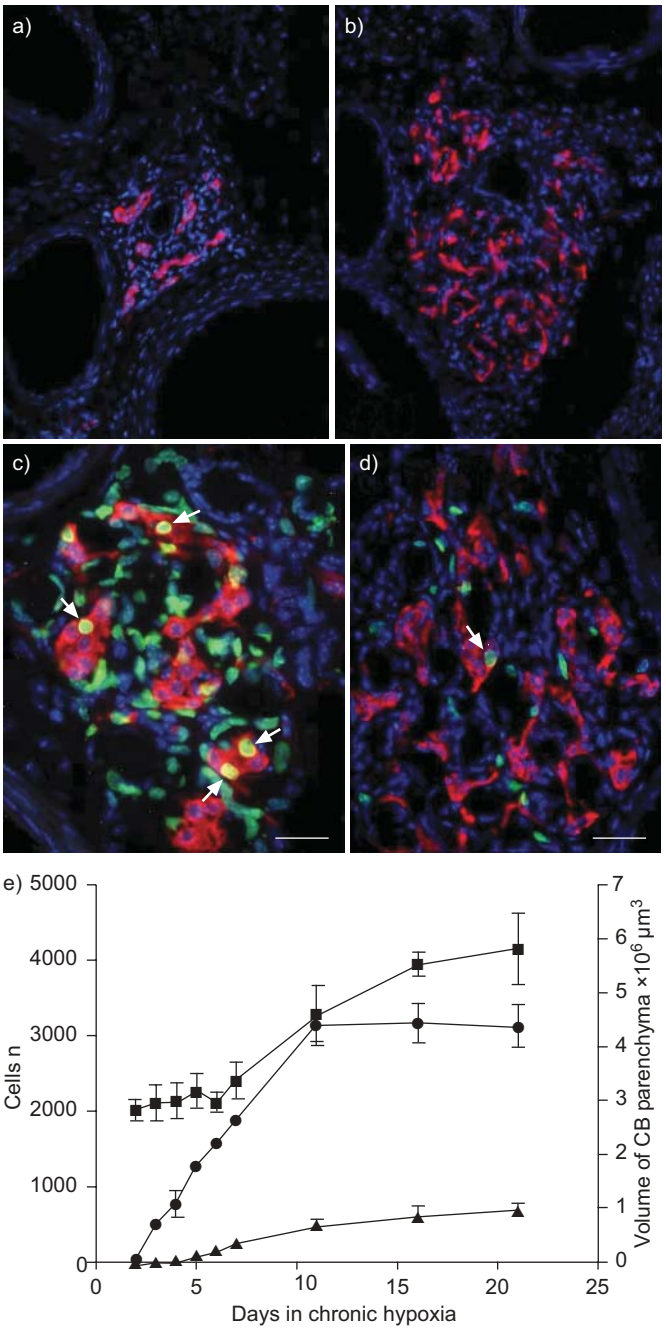

FIGURE 4. Carotid body (CB) growth in chronic hypoxia. Increase of CB size in a mouse exposed to hypoxia (10% O_2) for 21 days (b), compared with normoxia (a). c) Tyrosine hydroxylase (TH)+ and BrdU+ cells (arrowheads) in a mouse CB exposed to hypoxia for 7 days. BrdU was administered each day from the beginning of exposure to hypoxia. d) TH+ and BrdU+ cells (arrows) in a mouse CB exposed to hypoxia for 7 days. A pulse of BrdU (in d) was administered 2 h before animal sacrifice. Cell nuclei are stained with 4′,6-diamidino-2-phenylindole. TH+ cells are shown as red, and BrdU+ as green. Scale bars=50 µm. e) Progressive changes of mouse CB cell number and volume during exposure to hypoxia. ■: volume; ●: BrdU+ cells; ▲: TH+ BrdU+ cells. Reproduced and modified from [1] with permission from the publisher.

Altogether, the data summarised in the present section indicate that the adult CB is a neurogenic niche where new neuron-like glomus cells can derive from progenitors. In fact, this is the first example of neural crest-derived stem cells with a recognisable function identified in the adult peripheral nervous system. Based on numerous cell fate experiments both *in vivo* and *in vitro* [1], the current authors have proposed the model for neurogenesis depicted in figure 6d. Rat glial fibrillary acidic protein (GFAP)-positive 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 towards the nestin+ population, giving rise to TH+ glomus cells. Therefore, the adult CB is a well-identified neurogenic centre that can be used for research on the molecular mechanisms of neurogenesis. Knowledge on CB stem cell physiology could also facilitate the expansion of human CBs for use in cell therapy (see section Carotid body function and mechanisms of disease).

CAROTID BODY FUNCTION AND MECHANISMS OF DISEASE

The CB is mainly known for its role in the control of respiration; nevertheless, it also has increasing clinical significance, as there is mounting evidence that CB dysfunction is involved in the pathophysiology of several human diseases, some of them of high prevalence.

Pathologies associated with primary alterations of carotid body O_2 sensing

CB sensitivity to hypoxia develops during the early postnatal period and this correlates with an enhanced Ca^{2+} rise in response to hypoxia and increase in K^+ current amplitude. Maturation of CB chemosensitivity is particularly important in the newborn since, in addition to increasing ventilation and sympathetic tone, activation of the CBs facilitates arousal from sleep and switch from nasal to oral breathing. Loss of chemosensitivity due to CB denervation around the time of birth produces severe respiratory disturbances in rats, piglets and lambs, exposing the newborn to respiratory instability and unexpected death [77]. In several animal species, however, the hyperventilatory response to hypoxia, abolished by CB denervation, is re-established totally or partially several months after the surgery (possibly due to activation of other chemoreceptors). In contrast, glomectomy due to tumour surgery in humans results in complete and sustained lack of hypoxia responsiveness [78]. Asthmatic humans treated by bilateral CB ablation have blunted responses to hypoxia, mainly during sleep, and some have died suddenly and unexpectedly [79].

It is believed that some respiratory disorders of the newborn, such as the sudden infant death syndrome (SIDS) could be due to primary alterations of the CB chemoreceptors [80, 81]. Abnormalities in CB size or transmitter content have been reported in victims of SIDS. A common histological finding in CB from SIDS patients is the overgrowth of sustentacular/progenitor cells with decrease of glomus cell number [82, 83]. Glomus cells from patients affected by SIDS contain a lesser number of dense core vesicles and appear to have higher CB dopamine content (released to the extracellular medium) than in normal children [84]. This could be a cause of CB hypochemosensitivity, as dopamine is known to inhibit Ca^{2+} currents in glomus cells [10]. Nicotine acting on peripheral

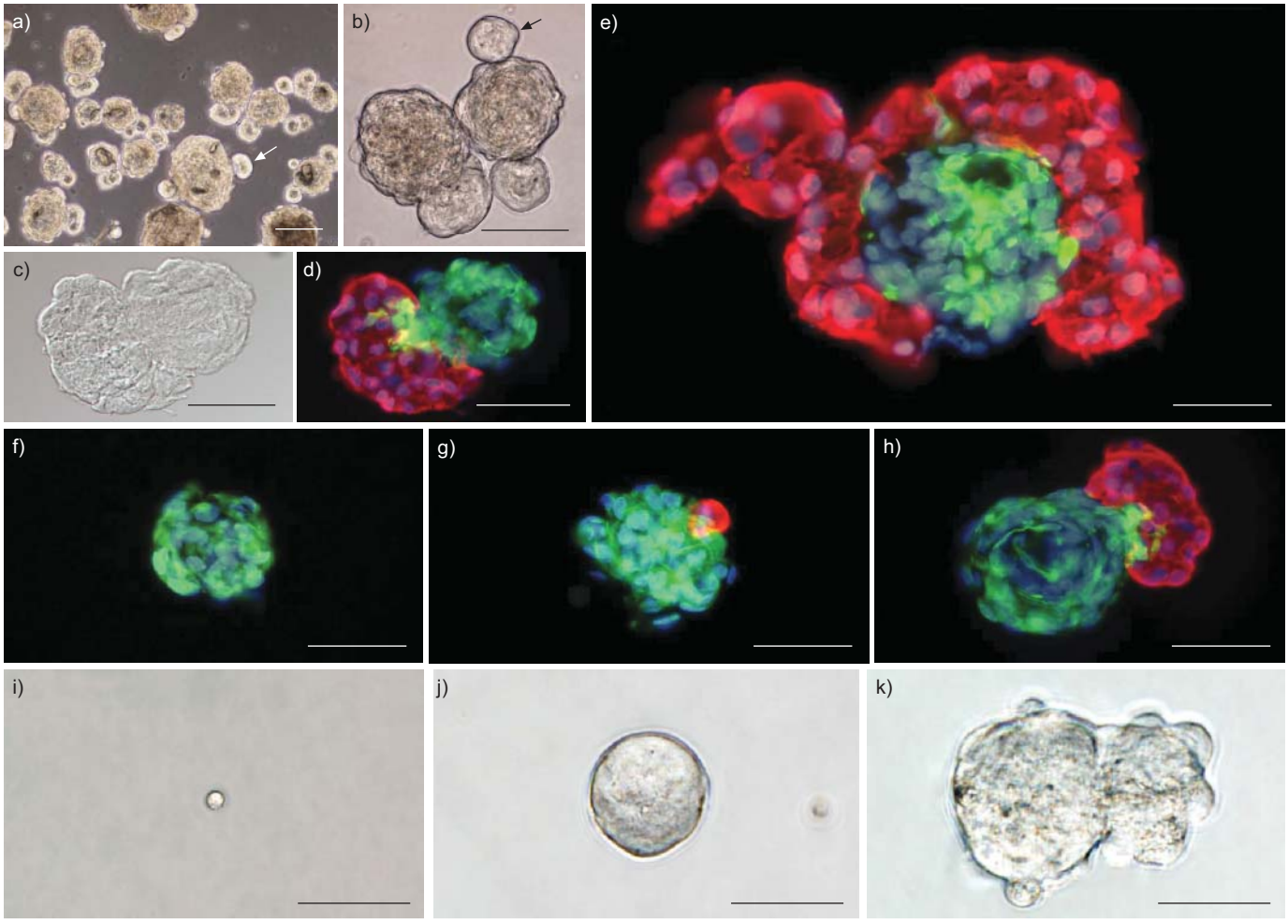

FIGURE 5. Carotid body (CB) stem cells. a) Neurospheres formed by dispersed CB cells after 10 days in culture; b) examples of the typical blebs (arrows) emerging from the neurospheres. Immunohistochemical analysis of a neurosphere thin section with bright field representation (c), and illustrating the presence of nestin+ progenitors (green) within the neurosphere core, and tyrosine hydroxylase (TH)+ glomus cells (red) within the bleb (d). e) Grown neurosphere (20 days in culture) with large blebs containing differentiated TH+ cells. Time course of rat CB neurosphere formation: f) 5 days; g) 7 days; h) 10 days. Organisation of the neurosphere core containing nestin+ progenitors precedes the appearance of TH+ glomus cells. Sequential photographs of a clonal colony illustrating the formation of a typical CB neurosphere from a single CB stem cell: i) 0 days; j) 5 days; k) 10 days. Scale bars=100 μm (a and b) and 50 μm (c–k). Modified and reproduced from [1] with permission from the publisher.

chemoreceptors may delay CB resetting after birth and attenuate the protective chemoreflex response, thus increasing vulnerability to hypoxic episodes in the newborn. This could explain the association between maternal smoking and SIDS syndrome [85]. It has been suggested that SIDS is probably not a sudden event but may be preceded by a relatively long period of hypoxia due to failure of reflex mechanisms [86]. More recently, vascular endothelial growth factor, a gene induced by chronic hypoxia, was found increased in cerebrospinal fluid of infants who died of SIDS as compared with controls [87], thus further supporting the view that SIDS is caused by a decreased sensitivity of chemoreceptors.

The congenital central hypoventilation syndrome (CCHS) is a life-threatening disorder with impaired ventilatory response to hypoxia and hypercapnia that as SIDS appears to be also related to CB dysfunction. In some CCHS patients, necropsy showed >50% decrease in the number of TH+ CB glomus cells, increase in sustentacular cells and decrease in the number of dopaminergic vesicles [88]. Numerous cases of CCHS are associated with genetic mutations. Mutations inherited from one of the parents have been found in the coding regions of endothelin-1, brain-derived neurotrophic factor and receptor tyrosine kinase (RET). All of these genes participate in development of neural crest-derived tissues [89]. Mutations in the tyrosine kinase domain of RET are particularly interesting, since they also appear in Hirschsprung's disease, which is associated with ~20% of the cases of CCHS. RET is part of the multicomponent receptor complex of GDNF, and both RET and GDNF are highly expressed in CB cells [14, 15]. A recent study has shown heterozygous *de novo* mutations in PHOX2B, another gene necessary for the early development of neural crest-derived cells and for the formation of reflex circuits in the autonomic nervous system, in 18 out of 29 individuals with CCHS [90, 91]. These data suggest that alteration of CB development and function is also associated

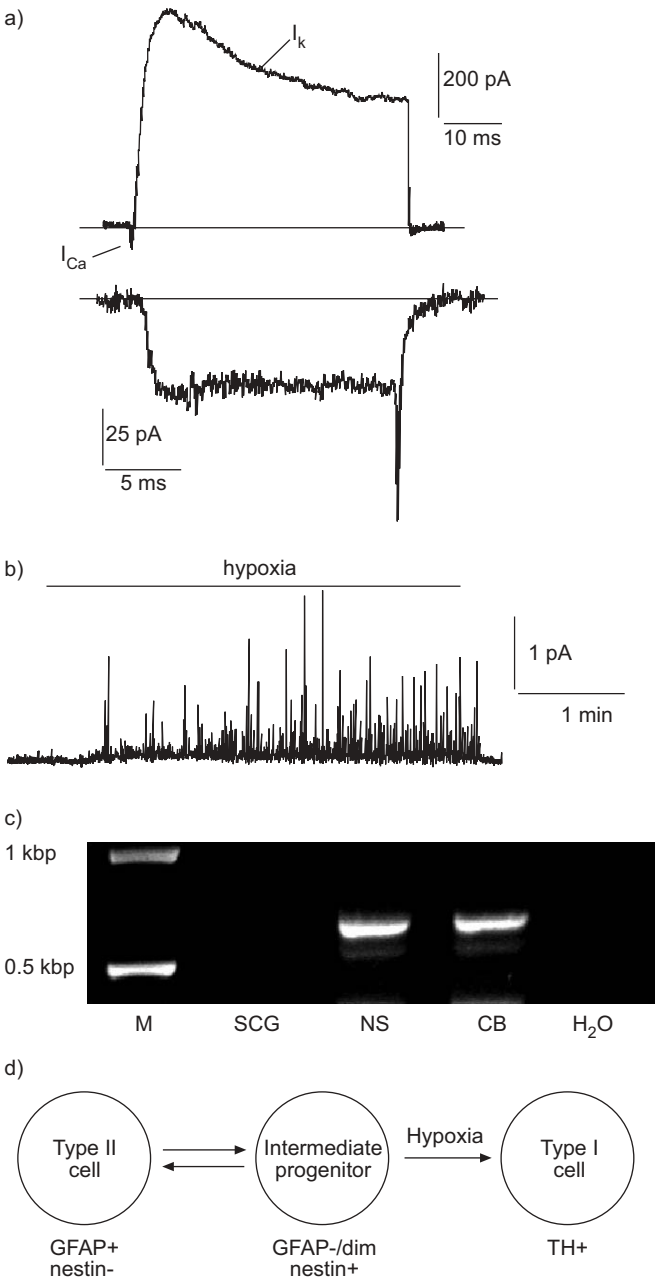

FIGURE 6. Physiological properties of *in vitro* differentiated glomus cells. a) Recording of calcium (I_{Ca}) and potassium (I_K) voltage-dependent currents obtained from a patch-clamped glomus cell in a neurosphere bleb: depolarisation from -80 mV to +20 mV (top). Patch-clamp recording of voltage-gated calcium currents after blockade of potassium channels with intracellular caesium ions (bottom). b) Glomus cell catecholamine secretory response to hypoxia (switching from a solution with O_2 tension of 145 mmHg (19.3 kPa) to another with ~15 mmHg (2.0 kPa)). Each spike represents a single exocytotic event. c) RT-PCR analysis of rat carotid body (CB), superior cervical ganglion (SCG) and CB-derived neurospheres (NS) to show the selective expression of glia cell line-derived neurotrophic factor mRNA. M: marker. d) Hypothetical sequence of cellular events occurring within the carotid body during exposure to hypoxia. Glial fibrillary acidic protein (GFAP)+ type-II cells are considered to be the progenitors activated by hypoxia to produce nestin+ cells, which give rise to tyrosine hydroxylase (TH)+ glomus cells. Modified and reproduced from [1] with permission from the publisher.

with genetic CCHS. Hence, it could be that primary alterations of the CB germinal niche are a major cause of respiratory reflex dysfunction seen in CCHS and SIDS patients.

Hypoventilation in adults with chronic obstructive pulmonary disease results in ''blue bloaters'', while those with normal ventilation are termed ''pink puffers''. Offspring of the blue bloaters have a poorer ventilatory response to hypoxia than offspring of pink puffers, suggesting a familial component [4]. The genetic influence on CB function is clear in two strains of rats, which had different CB calcium responses to acute hypoxia and different carotid sinus nerve traffic [92]. The respiratory stimulant, doxapram, mimics the effect of hypoxia by inhibiting both voltage- and Ca^{2+}-dependent K^+ currents in glomus cells [93], providing further evidence of the importance of K^+ channels in O_2 sensing.

Carotid body and pathophysiology of chronic hypoxia

Exposure to chronic hypoxia, *e.g.* living at high altitude, produces a compensatory CB hypertrophy and cellular hyperplasia (see section Carotid body plasticity in chronic hypoxia: adult carotid body stem cells). The same occurs in situations in which alveolar gas exchange is compromised as, for example, in cystic fibrosis or cyanotic heart disease [74, 75]. In these patients, stimulation of the respiratory centre by CB fibres is necessary to maintain the respiratory drive; thus, special precaution must be taken in the management of the patients to avoid excessive oxygenation and inhibition of CB activity. CB hypertrophia and cellular hyperplasia/anaplasia is also observed in CB tumours (chemodectomas or paragangliomas). These are relatively rare, mostly benign tumours in the neck that, besides the symptoms due to local compression, can also produce systemic hypertension [55]. The most frequent cause of chemodectoma is the hereditary CB paraganglioma due to mutations in *SDHD*, a gene that encodes the small membrane anchoring subunit of SDHD in the mitochondrial complex II [50]. The histological similarity between the CB growth in chronic hypoxia and paraganglioma has led to the suggestion that mitochondrial complex II participates in O_2 sensing. As discussed previously (in the Mechanisms of carotid body acute oxygen sensing section), heterozygous SDHD knockout mice show a mild CB hypertrophy without alteration in acute responsiveness to lowering O_2 tension [56]. The grade of malignancy of CB paraganglioma is inversely associated with the number of GFAP+ type-II cells, a fact that could indicate that deregulation of CB progenitors (with type-II cell phenotype) participates in tumorigenesis [1].

An important health problem related to CB function is obstructive sleep apnoea syndrome (OSAS) [94]. OSAS is a highly prevalent problem occurring at rates of 2–3% in children, 3–7% in middle-aged adults and 10–15% in healthy elderly subjects [95]. It also has 30% prevalence among patients with so-called essential hypertension. CB seems to play a critical role in the development of hypertension associated with sleep apnoea. In rats exposed to 30 days of intermittent hypoxia (7 h per day), hypertension was observed but surgical denervation of peripheral chemoreceptors prevented the increase in arterial blood pressure. Adrenal demedullation and chemical destruction of the peripheral sympathetic nervous system by 6-OH dopamine also prevented hypertension [96, 97]. In patients suffering from OSAS there is an increase in sympathetic activity, probably due

to the recurrent arousal following the periods of apnoeas. However, it is believed that hypoxia *per se* also increases the sympathetic tone. Intermittent hypoxia in rats induces plastic changes in the CB, thus increasing its sensitivity and tonic sympathetic activation without obvious morphological alterations [62, 98]. Similarly, OSAS patients have enhanced peripheral chemoreflex sensitivity and in those who experience repetitive hypoxaemia this increase might contribute to high levels of sympathetic activity even during normoxic daytime wakefulness [99, 100].

Carotid body and cell therapy

As the carotid body is a highly dopaminergic organ, it has been used in dopaminergic cell replacement for Parkinson's disease. Additional advantages of the carotid body for cell therapy rely on its survival in hypoxic environments, similar to that existing in the brain parenchyma after a tissue graft, and because it offers the possibility of autotransplantation in humans. Carotid body cell aggregates have been transplanted with excellent functional recovery in parkinsonian rats [101, 102] and monkeys [103]. In a safety pilot study performed on PD patients, carotid body autotransplantation produced a clear amelioration in some cases [104]. The beneficial effects of carotid body transplants are not only due to the local release of dopamine but also to a trophic action exerted on nigrostriatal dopaminergic neurons [14]. The carotid body contains more glia cell line-derived neurotrophic factor than any other structure in adult mice [15]. Therefore, glomus cells are ideal candidates to be used as biological pumps for the controlled endogenous release of glia cell line-derived neurotrophic factor and possibly other trophic factors with unique synergistic actions. In fact, carotid body grafting has also been shown to reduce neuronal death in an acute rat stroke model [105]. The systematic clinical applicability of carotid body dopamine- and glia cell line-derived neurotrophic factor-producing cells is under investigation [1, 106].

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