Neuroglobin, cytoglobin, and myoglobin contribute to hypoxia adaptation of the subterranean mole rat *Spalax*

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The subterranean mole rat *Spalax* is an excellent model for studying adaptation of a mammal toward chronic environmental hypoxia. Neuroglobin (Ngb) and cytoglobin (Cygb) are O_2-binding respiratory proteins and thus candidates for being involved in molecular hypoxia adaptations of *Spalax*. Ngb is expressed primarily in vertebrate nerves, whereas Cygb is found in extracellular matrix-producing cells and in some neurons. The physiological functions of both proteins are not fully understood but discussed with regard to O_2 supply, the detoxification of reactive oxygen or nitrogen species, and apoptosis protection. *Spalax Ngb* and *Cygb* coding sequences are strongly conserved. However, mRNA and protein levels of *Ngb* in *Spalax* brain are 3-fold higher than in *Rattus norvegicus* under normoxia. Importantly, *Spalax* expresses Ngb in neurons and additionally in glia, whereas in hypoxia-sensitive rodents Ngb expression is limited to neurons. Hypoxia causes an approximately 2-fold down-regulation of Ngb mRNA in brain of rat and mole rat. A parallel regulatory response was found for myoglobin (Mb) in *Spalax* and rat muscle, suggesting similar functions of Mb and Ngb. *Cygb* also revealed an augmented normoxic expression in *Spalax* vs. rat brain, but not in heart or liver, indicating distinct tissue-specific functions. Hypoxia induced *Cygb* transcription in heart and liver of both mammals, with the most prominent mRNA up-regulation (12-fold) in *Spalax* heart. Our data suggest that tissue globins contribute to the remarkable tolerance of *Spalax* toward environmental hypoxia. This is consistent with the proposed cytoprotective effect of Ngb and Cygb under pathological hypoxic/ischemic conditions in mammals.

Oxygen levels that are inadequate to sustain cellular energy production constitute a life-threatening condition for mammals. Metabolically most active tissues (e.g., nerve cells) are exquisitely sensitive to a reduction of O_2 (hypoxia), and humans are severely affected by hypoxic disease conditions like stroke or myocardial ischemia. It is therefore mandatory to investigate the specific adaptations evolved by mammals that live in naturally hypoxic environments where low ambient O_2 tensions limit the availability of O_2 to the organism (1).

The blind mole rat *Spalax* spends its entire life in underground burrows that can be extremely hypoxic/hypercapnic (2, 3). The Spalacidae, originating 25–40 million years ago, have evolved physiological strategies enabling their respiratory and cardiovascular systems to cope with hypoxia more efficiently than other mammalian species (2, 4). The four karyotypically distinct allospecies of *Spalax* in Israel are adapted to different climatic regimes. The strongest differences in ecological conditions are observed between *Spalax galili* (karyotype 2n = 52), inhabiting the northern cool-humid Upper Galilee Mountains with heavy soil, which often becomes flooded, and *Spalax judaei* (2n = 60), which reside in the warm-dry south with light-aerated soil. The most efficient hypoxic adaptation has consequently been demonstrated in *S. galili*, with higher normoxic breathing and heart rate as well as higher hematocrit and Hb levels as compared with *S. judaei* (2, 5). Another two *Spalax* species, *Spalax golani* (2n = 54) and *Spalax carmeli* (2n = 58), are intermediate in their hypoxia adaptation. Compared with the hypoxia-sensitive rodent *Rattus norvegicus*, *Spalax* survives substantially longer at low ambient O_2 levels and high CO_2 without serious deleterious effects or behavioral changes (6).

Hypoxia tolerance mechanisms identified in *Spalax* as compared with *R. norvegicus* include blood properties, anatomical and biochemical changes in respiratory organs (2, 4), and differences in the structure and function of a growing list of gene products (7–10). Transcription patterns of genes related to hypoxic stress differ interspecifically in *Spalax* (5, 11) and between *Spalax* and rat, involving key players such as erythropoietin (*Epo*) and its receptors, and hypoxia-inducible factor-1α (*Hif-1α*) (12, 13). An important adaptation of *Spalax* to hypoxic habitats is mediated by an increased blood vessel density, which is triggered by a constitutively higher expression (compared with rat) of vascular endothelial growth factor (*Vegf*), *Hif-1α*, and *HuR*, a post-transcriptional stabilizer of *Vegf* mRNA (6, 14).

The aerobic metabolism of mammals relies on respiratory proteins that function in the delivery and storage of O_2. Hb in erythrocytes transports O_2 from the lungs to inner organs (15). Myoglobin (Mb) in cardiac and striated muscles acts as a local O_2 store and facilitates intracellular diffusion of O_2 (16). Ten years ago, neuroglobin (Ngb) and cytoglobin (Cygb) were discovered as unique members of the mammalian globin family (17). The physiological functions of Ngb and Cygb are still uncertain. In most mammals, Ngb resides in neurons of the central and peripheral nervous systems, as well as in endocrine organs (18, 19). Ngb may have an Mb-like role in supplying O_2 to the mitochondrial respiratory chain (18, 20, 21). Alternatively, it may function as a scavenger of reactive oxygen or nitrogen species (ROS/RNS) (22, 23) or protect cells from cytochrome *c*-induced apoptosis (24, 25). Regardless of its ultimate role, there is conclusive evidence that Ngb localization is tightly linked to active oxidative metabolism and mitochondria (19, 20). The highest Ngb level was found in the neuronal retina, which also has the highest O_2-consuming rate in the body (20, 26). Several studies have shown that Ngb is cyto- and neuroprotective (27–29). Recently it was demonstrated that in the hooded seal, a diving mammal that tolerates prolonged hypoxia of the brain (30), Ngb is expressed in astrocytes (31). This may indicate an unusual shift of oxidative metabolism from neurons to glial cells.

Cygb occurs predominantly in the fibroblast cell lineage, as well as in some neurons (32–34). The function of Cygb is even less clear than that of Ngb but has been interpreted in terms of ROS defense or O_2 supply to certain enzymes (17, 33, 35).

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Globins are candidates that may enable *Spalax* to better survive low ambient oxygen conditions. To understand the particular role of Ngb, Cygb, and Mb in hypoxia tolerance, we have studied their sequences, expression patterns, and gene regulation in different *Spalax* species in comparison with rat. The data provide indirect evidence to the physiological function(s) of Ngb and Cygb in mammals and point to the biomedical significance of these proteins.

Results

Sequence Analysis of *Spalax Ngb*, *Cygb*, and *Mb*. *Ngb* and *Cygb* gene sequences from *S. carmeli* were reported previously (36). Here we have cloned, sequenced, and compared the coding regions of *Ngb* and *Cygb* from all four *Spalax* species (Fig. S1). As in other mammals (18, 37), the *Spalax Ngb* cDNA encodes a strongly conserved protein of 151 amino acids. The four *Spalax* species maximally differ by two amino acids, and the best match to other genera was found with mouse Ngb (94% identity, 96% similarity). *Spalax Cygb* cDNAs encode a protein of 190 amino acids, as typical for most other mammals. Cygb proteins of *S. carmeli* and *S. galili* are identical and show two amino acid differences to *S. golani* and *S. judaei*, which has one additional substitution. *S. golani* Cygb is 95% identical/99% similar to mouse Cygb. Only very few *Spalax*-specific amino acid replacements were observed for Ngb and Cygb (Fig. S1). Homology modeling showed that these substitutions are on the surface of the Ngb and Cygb proteins (Fig. S2). Except for the minor differences, *Spalax* Ngb and Cygb include the conserved sequence hallmarks of functional O_2 carriers [e.g., the proximal and distal His (F8 and E7) and PheCD1, which are involved in heme and ligand binding].

The coding region of *Spalax Mb* (462 bp) translates into a protein of 154 amino acids. Its sequence—here obtained from *S. carmeli* (2n = 58)—matched the published *S. judaei* Mb protein sequence (38) with 98.1% identity. Again, all functional positions are conserved, and the observed replacements are found in other mammals as well.

Expression Patterns of Ngb and Cygb in *Spalax* Tissue. We analyzed the localization of Ngb and Cygb proteins by immunostaining in the brain and other organs of *Spalax.* We observed a ubiquitous and homogeneous Ngb expression in *Spalax* brain (Fig. 1*A*). Stronger signals were obtained from regions with higher neuronal density (such as piriform cortex or hippocampal formation), but fluorescence intensity of single neurons was similar throughout brain sections (Fig. 1 *B* and *C*). Neuronal perikarya exhibited Ngb immunofluorescence that was often also seen in cell processes, whereas the cell nuclei remained free of signal (Fig. 1*C*). The Ngb signals in *Spalax* neurons were basically identical in regional distribution and intensity to those obtained from mice (19, 39). Immunofluorescence detection of Ngb did not differ between brains prepared from normoxic and hypoxic (5 h, 6% O_2) *Spalax*. Additional Ngb immunoreaction was observed in astrocytes. Fig. 1 *D* and *E*, taken from the corpus callosum, show cells that exhibit immunostaining of both Ngb and GFAP.

Cygb immunofluorescence was observed in fibroblasts and related, extracellular matrix-producing cell types in various *Spalax* organs, including heart muscle and liver (Fig. S3). As in mouse (33), Cygb was localized in the cytoplasm of these fibroblast-type cells. In both *Spalax* and mouse (33), we observed Cygb staining in the nucleus and cytoplasm of distinct neuronal cell populations.

We also investigated the expression patterns of Ngb and Cygb in the *Spalax* retina. The atrophied eye of the blind *Spalax* still comprises similar retinal layers as in other rodents. Ngb staining was mainly detected in the ganglion cell layer (GCL) of the *Spalax* retina (Fig. 2*A*). Additional weak signals were observed in the inner and outer nuclear layers (INL, ONL). Photoreceptors (PL; inner and outer segments) and plexiform layers (IPL, OPL) were devoid of the intense Ngb staining observed in other rodents (20, 26). To investigate the possible involvement of Ngb in retinal oxidative metabolism, we analyzed the distribution of cytochrome *c* as a marker for mitochondria. Cytochrome *c* protein was observed in most layers of the *Spalax* retina (Fig. 2*B*), being highest

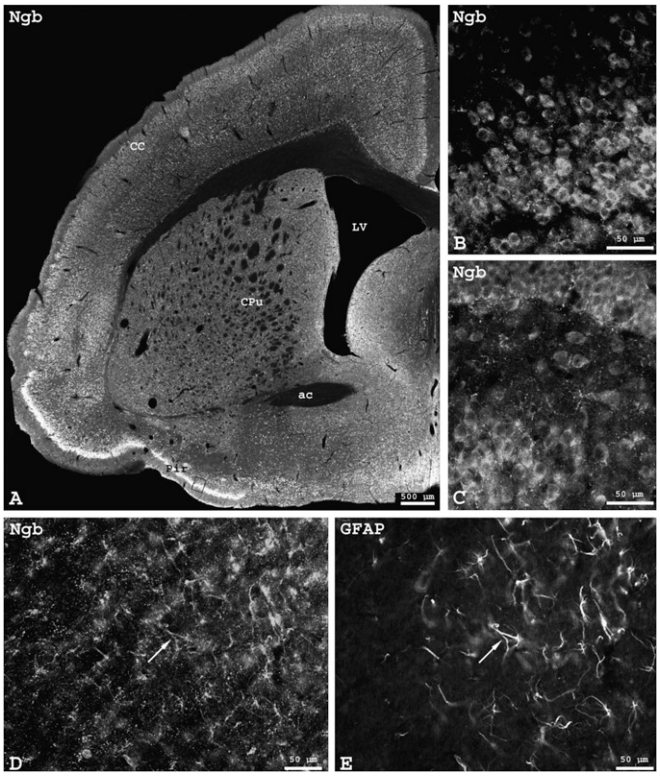

Fig. 1. Ngb immunofluorescence in *Spalax* brain. (*A*) Frontal section of *Spalax* brain at the level of the lateral ventricle (LV) and anterior commissure (ac). Ngb staining of neurons was found in the piriform (Pir), insular, sensory, and motor regions of the cerebral cortex (CC) and in the striatum (caudate nucleus-putamen, CPu), as well as medial and basal forebrain regions. Higher magnifications show Ngb-positive neurons from the piriform cortex (*B*) and dentate gyrus (*C*). Ngb signal was also seen in astrocytes identified by GFAP staining in the minor forceps of the corpus callosum (*D* and *E*; arrows: double-labeled astrocytes). Note cross-sectioned axons positive for Ngb (*D*) and negative for GFAP (*E*).

in PL and GCL and lowest in ONL and INL. Ngb and cytochrome *c* patterns thus do not strictly overlap in *Spalax* retinal layers. To investigate whether the pattern of Ngb staining in the *Spalax* retina is determined by a distinct mode of capillary O_2 supply, we performed immunostaining applying von Willebrand factor antibodies (Fig. 2*C*). Results showed that *Spalax* blood vessels are present in OPL, INL, IPL, and GCs, suggesting O_2 supply by deep retinal and superficial capillaries as in the vascularized retina of mouse and rat (20).

In contrast to the presence of Ngb in various retinal layers, positive Cygb staining was exclusively found in the GCL, and with weaker intensity in IPL (Fig. 2*D*). The Cygb signal colocalized with immunoreactivity toward neuronal NOS (nNOS) (Fig. 2*E*).

Quantitative Analyses of Gene Expression. Globin mRNA levels were compared by quantitative RT-PCR (qRT-PCR) and protein levels by Western blotting, between *Spalax* and rat at normoxia and at different hypoxic conditions. Two positive control genes [*Vegf, adrenomedullin* (40)] were used to confirm the effects of hypoxia treatment at the mRNA level (Fig. S4).

We first compared Ngb mRNA levels in *R. norvegicus*, *S. judaei*, and *S. galili* brain (Fig. 3*A*). At normoxia, Ngb expression in *S. judaei* accounted for 180% of the mRNA level in rat. The most hypoxia-resistant species, *S. galili*, exhibited a relative Ngb level of 280% ($P \leq 0.05$). This increased expression of Ngb mRNA under normoxic conditions was confirmed by Western blotting, which revealed up to 3.5-fold more Ngb protein in *Spalax* than in rat (Fig. 3*B*).

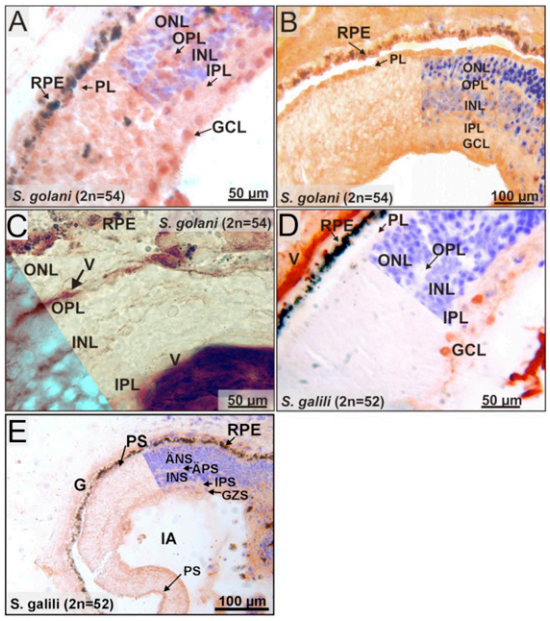

Fig. 2. Immunostaining of *Spalax* retina cryosections. (*A*) Ngb expression (red-brown signals) is mainly found in the GCL. The PL, ONL, and INL show weak additional signals (RPE, retinal pigment epithelium). (*B*) Cytochrome *c* is found in each layer of the retina with the most prominent expression in PL and GCL. (*C*) Endothelial cells of the blood vessels (V) were stained with a von Willebrand factor antibody. Staining was found in OPL, INL, IPL, and GCs, indicating the presence of deep retinal and superficial capillaries. (*D*) Cygb protein is present in ganglion cells and their processes and shows weak expression in the IPL. All other retinal layers are unstained. (*E*) nNOS signal is also detected in GCL and IPL.

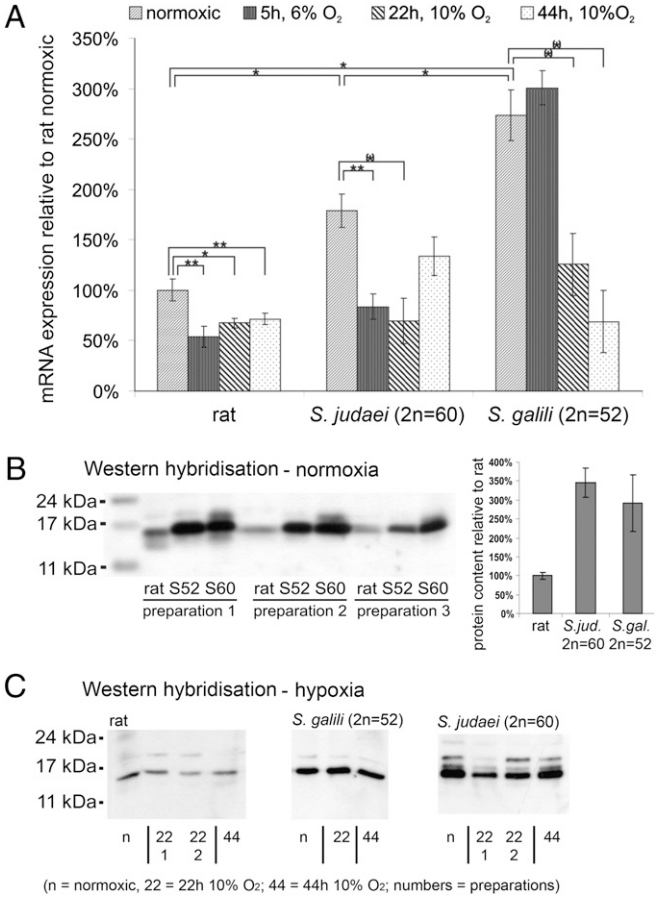

Fig. 3. *Ngb* expression quantification. (*A*) Ngb mRNA expression in total brain, quantified by qRT-PCR. Under normoxia, Ngb expression is 1.8- and 2.8-fold higher, respectively, in *S. judaei* and *S. galili* than in rat. In *S. judaei* and rat, severe short-time hypoxia (5 h, 6% O_2) decreases Ngb mRNA to half of its normoxic value, whereas the amount in *S. galili* is unchanged. Longer-term moderate hypoxia (22 and 44 h, 10% O_2) decreases Ngb expression to 40–75% of the normoxic condition in all three species. Significance levels, indicated by asterisks and horizontal brackets, were obtained by the Student's *t* test: $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $^{(*)}P \leq 0.1$. (*B*) Western blot analysis of Ngb protein expression in rat, *S. judaei* (2n = 60; S60), and *S. galili* (2n = 52; S52) normoxic total brain. Three individuals of each species were tested (preparations 1–3). The blot, containing equal amounts of protein per lane, indicates an up to 3.5-fold higher Ngb protein level in the *Spalax* species as compared with rat. (*C*) Western blot analysis of Ngb in hypoxic vs. normoxic (n) animals. In rat we observe a slight down-regulation after 22 or 44 h of moderate hypoxic stress (10% O_2). In *S. galili* (S52) and *S. judaei* (S60), protein levels do not proportionately reflect the decreasing mRNA but show that there is no hypoxic up-regulation of Ngb.

We studied the changes of Ngb expression under short-term severe hypoxia (5 h, 6% O_2) and longer-term moderate hypoxia (22 and 44 h, 10% O_2). We observed an almost 2-fold decrease of Ngb mRNA in all species (mostly significant at the $P < 0.05$ level), irrespective of the different hypoxic conditions (Fig. 3*A*). A notable exception was *S. galili* after 5 h of 6% O_2, which still showed unchanged Ngb mRNA levels. On the protein level, a parallel slight decrease in Ngb was observed after moderate hypoxia in rat, but not so clearly in *Spalax* (Fig. 3*C*).

Cygb mRNA levels of rat and *Spalax* were compared in brain, heart, and liver. The results revealed differences between species, as well as between organs (Fig. 4*A*). At normoxia, a higher Cygb mRNA level was found in *Spalax* vs. rat only in the brain (up to 2.5-fold) but not in heart and liver. This result in normoxic brain was confirmed on the protein level (Fig. 4*B*). Whereas Cygb mRNA expression under hypoxia remained essentially unchanged in rat brain, it increased almost 2-fold in *Spalax* brain after 44 h of moderate hypoxia (10% O_2). In hypoxic heart muscle tissue, both rodents up-regulated Cygb mRNA, although to markedly different extents (rat: 2.5-fold after 44 h; mole rat: up to 12-fold after 44 h). A moderate, approximately 2-fold increase of Cygb mRNA was noted in hypoxic liver in both species.

We additionally analyzed Mb mRNA expression in neck muscle, used by *Spalax* for digging, and in heart tissue (Fig. S5). In normoxic neck muscle, *S. galili* and *S. judaei* contained 42- and 27-fold more Mb mRNA than rat, respectively. In normoxic heart, both *Spalax* species had 2.7-fold more Mb mRNA than rat. Short-term severe hypoxia (5 h, 6% O_2) did not alter *Mb* transcription in neck muscle of *Spalax*, whereas rat Mb mRNA increased 2.7-fold. After longer-term moderate hypoxia, Mb mRNA was found strongly down-regulated at 22 h in rat and *S. judaei*. The most hypoxia-tolerant species, *S. galili*, showed the same tendency only after 44 h of hypoxia. Interestingly, Mb mRNA expression in hypoxic heart gave a different picture, increasing slightly, 1.5- to 1.7-fold, in *Spalax* and rat.

Discussion

In humans a lack of oxygen leads to loss of consciousness within minutes. Acute insults such as cerebral ischemia have a devastating impact on the brain, which is essentially impossible to repair (41). Some mammals, however, can tolerate even prolonged periods of ambient hypoxia; for example, diving mammals show morphological and physiological adaptations that allow them to tolerate periodic hypoxia better than their terrestrial relatives (1, 42). *Spalax* survives severe chronic hypoxia in its underground burrows and thus is an excellent model system for studying the adaptation of a mammal toward the lack of O_2 (2). In fact, genes such as *Epo*, *Hif-1α*, or *Vegf* are instrumental in alleviating hypoxia in *Spalax* (6, 12–14). Likewise, globin proteins, which enhance O_2 supply or

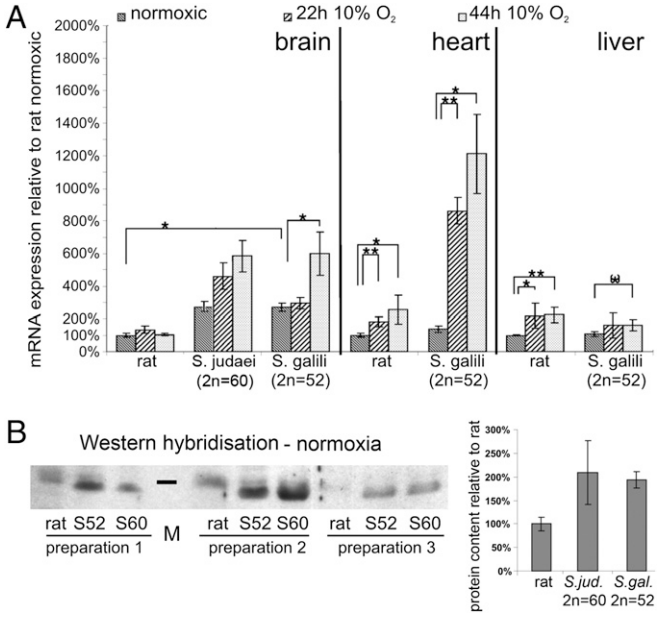

Fig. 4. Comparative analyses of *Cygb* expression. (*A*) Cygb mRNA expression levels in brain, heart, and liver, determined by qRT-PCR. In brain, constitutive normoxic levels of Cygb mRNA are higher in *Spalax* (2.5-fold) than in rat. Only *Spalax* additionally increases brain Cygb mRNA under hypoxia (44 h, 10% O_2). In heart and liver, normoxic Cygb mRNA levels are the same in rat and *Spalax*. In hypoxic heart, Cygb mRNA increases only 2.5-fold in rat but 12-fold in *Spalax*. In hypoxic liver, both species reveal a moderate 1.5- to 2-fold increase in Cygb mRNA. mRNA levels cannot be compared directly between tissues in the figure, because they are substantially different: normoxic brain and heart express ≈10-fold more Cygb than liver in both species. (*B*) Western blot analysis of Cygb protein expression in rat, *S. judaei* (2n = 60; S60), and *S. galili* (2n = 52; S52). In normoxic brain, Cygb protein expression is approximately 2-fold higher in *Spalax* than in rat. Three individuals were tested, numbered preparation 1–3. Marker bar (M) indicates a molecular mass of 21 kDa.

decrease hypoxia-caused injuries by other means, may promote viability of *Spalax* under hypoxic stress. By comparing mRNA and protein expression of *Ngb*, *Cygb*, and *Mb* in two *Spalax* species with different degrees of hypoxia tolerance and the hypoxia-sensitive rat, we made the following major observations: (*i*) Ngb mRNA and protein expression in the normoxic brain is substantially higher in *Spalax* than in rat. (*ii*) In *Spalax*, but not in rat, Ngb is expressed in astrocytes in addition to its usual presence in neurons. (*iii*) Ngb mRNA (and to a lesser extent also the protein) is decreased upon hypoxia in *Spalax* and rat. A parallel down-regulation after hypoxia is observed for Mb mRNA in *Spalax* and rat muscle. (*iv*) Cygb mRNA and protein are also constitutively increased in normoxic brain of *Spalax* compared with rat and can be further augmented by hypoxia in *Spalax*. In other tissues, Cygb mRNA is present at comparable levels in normoxic *Spalax* and rat, and it is up-regulated after hypoxic stress in both taxa.

Increased Globin Levels in *Spalax* Indicate a Function in Hypoxia Tolerance. The role of Hb and Mb in O_2 supply is well established. *Spalax* is known to cope with reduced O_2 availability in its subterranean burrow systems by means of increased hematocrit and Hb levels and a high O_2 affinity of the Hb (43). Likewise, Mb protein levels in *Spalax* skeletal muscle were reported to be 3-fold higher than in rat (4). This ratio may even be higher in the mole rat neck muscle used for underground digging, as suggested by the 11-fold enhanced Mb mRNA level. The amino acid replacements observed in mole rat Hb and Mb sequences do not seem to be prime mediators of hypoxia adaptation (38, 43). The same is probably true for Ngb and Cygb, which display high sequence conservation in the mole rat. In parallel to Hb and Mb, however, we detected significantly higher Ngb and Cygb mRNA and protein levels in *Spalax* tissues as compared with rat (Figs. 3, 4), which points to an important role of both globins in hypoxia adaptation. Our findings thus corroborate in vitro and in vivo studies using ectopic globin overexpression, which at least for Ngb conclusively report a survival-enhancing effect in neuronal cells after hypoxic and ischemic insult (27–29). The data also emphasize the importance of gene regulatory changes (vs. sequence changes) as a major adaptive mechanism in *Spalax*.

Glial Expression of Ngb: A Key Feature of Hypoxia Adaptation? Inferring adaptive significance by comparing traits of just two distantly related taxa such as *Spalax* and rat can be dangerous. Therefore it is most important that we observe intriguing parallels between the *Spalax*–rat data and other animal models, which significantly strengthens our interpretations. Interspecific differences in Ngb expression levels have been reported before in fish: the hypoxia-tolerant goldfish (*Carassius auratus*) has approximately 5-fold more Ngb protein in the brain than the more hypoxia-sensitive zebrafish (*Danio rerio*) (44). This quantitative difference can be interpreted in terms of an O_2 supply function and/or an ROS detoxification role of Ngb as an adaptive strategy to alleviate hypoxic stress. In *Spalax*, the interpretation is more complicated: we show that Ngb in the mole rat is localized in neurons and astrocytes, whereas in the brain of hypoxia-sensitive rodents like mouse or rat, Ngb is localized primarily in neurons (19, 39, 45). Thus, both cell types contribute to the elevated Ngb expression level in *Spalax* brain, and we are currently unable to separately quantify neuronal and glial expression. Interestingly, however, in the brain of the deep-diving hooded seal (*Cystophora cristata*) Ngb is predominantly present in astrocytes (31). Glial expression of nerve hemoglobins (nHbs) has also been observed in hypoxia-tolerant invertebrates, whereas in related, hypoxia-sensitive species nHbs reside in neurons (46, 47). It is therefore tempting to assume that the glial expression of Ngb in the brains of the hooded seal and *Spalax* is a common feature of hypoxia tolerance. In the seal, the glial expression of Ngb has been interpreted in terms of a shift of oxidative metabolism from neurons to astrocytes, whereas neurons essentially rely on anaerobic fermentation (31). In fact, seal neurons are more hypoxia tolerant than those of rat (30). It remains to be shown whether this also applies to *Spalax* neurons.

Adaptation to Chronic Hypoxia: Alleviating the Need for an Acute Hypoxic Up-Regulation of Ngb and Mb in *Spalax*? The possible involvement of Ngb and Mb in *Spalax* hypoxia tolerance led us to study their gene regulatory response after experimental O_2 deprivation. In fact, hypoxia causes an increase of Ngb gene expression in zebrafish (mRNA and protein level) (48) and turtle (mRNA) (49). By contrast, no significant changes of Ngb mRNA were found in the brains of mice after prolonged hypoxia (50) or in brains of rats after global ischemia (51), suggesting that Ngb fulfills a constitutive function rather than being an acute stress-response protein. Even more surprising is our observation that experimental hypoxia triggers a significant decrease of Ngb mRNA in rat and *Spalax* brains, even though protein levels are less affected. At first glance, this result is difficult to reconcile with previously proposed functions of Ngb (17, 21). However, *Spalax* must adapt to chronic hypoxia in its underground burrows, and thus there may be no evolutionary pressure to evolve pathways, which enable an acute up-regulation of Ngb. Rather, the higher Ngb mRNA/protein content of *Spalax* brain (and the glial plus neuronal Ngb localization) reflect a constitutive, intrinsic hypoxia tolerance of *Spalax* tissues.

Interestingly, Mb mRNA is also down-regulated after prolonged hypoxia in *Spalax* neck muscle, but indicates adaptation to chronic hypoxic conditions by the constitutive normoxic higher Mb mRNA [and protein (4)] expression. This parallel mode of regulation of Ngb and Mb might imply similar functions of the two respiratory proteins (i.e., in O_2 supply and ROS/RNS detoxification) (52). The 2.5-fold up-regulation of Mb mRNA under acute strong hypoxia in *Rattus* neck muscle (and to a lesser extent in heart) in turn indicates that the hypoxia-sensitive spe-

cies is adapted to deliver an acute stress response, in agreement with a recent study on the molecular pathways of *Mb* hypoxia regulation in mouse (53).

Evidence for Distinct Tissue-Specific Functions of Ngb and Cygb. The eye is central to the discussion of Ngb function because in the neuronal retina of sighted rodents, Ngb protein is expressed in substantial amounts in the plexiform layers, ganglion cells, and inner segments of photoreceptors (26). The subcellular colocalization of Ngb with mitochondria and the spatial correlation with retinal vasculature strongly suggest an involvement of Ngb in the intense oxidative metabolism of the retina by supplying O_2 and/or by scavenging ROS (20). The subterranean mole rat is blind and possesses only minute regressed eyes covered by skin (54). The *Spalax* retina, however, still reveals all typical cell layers, although less organized. The outer segments of the photoreceptors are rudimentary and it is considered that the *Spalax* retina has been restructured to evolve a function in photoperiodic sensing (55). Although we show here that the distribution of blood vessels in *Spalax* fits a typical "vascular-type" retina, Ngb expression in the *Spalax* retina is extremely reduced and almost limited to the GCL. An intense Ngb expression in several retinal layers is therefore positively selected to sustain visual processes in sighted mammals. The retained Ngb expression in *Spalax* ganglion cells may indicate an additional, residual role of this globin in nerve cells, perhaps operating at lower expression levels.

The physiological function of Cygb is currently even less well understood than the role of Ngb (17). The *Spalax* data, revealing distinct modes of *Cygb* gene regulation in brain vs. heart and liver, confirm the notion from other rodents that Cygb may have different roles in neurons and in fibroblast-related cell types of diverse organs (33, 34). In brain, the elevated normoxic level of Cygb in *Spalax* vs. rat suggests involvement in the chronic hypoxia tolerance, as seen for Ngb and Mb. The colocalization of Cygb with nNOS in the GCL of the *Spalax* retina has been observed before in specific neuronal populations of the mouse brain (47, 56) and may indicate that Cygb and nNOS interact functionally, for example by a delivery of O_2 from Cygb to nNOS during the production of NO or by scavenging excess NO.

In heart and liver, interspecific Cygb mRNA levels are similar at normoxia. In these organs, however, *Cygb* responds to O_2 deprivation by stress-induced mRNA up-regulation. This points to a Cygb function that does not require constitutively elevated expression levels but operates in an O_2-dependent regulated mode in conserved cellular processes (e.g., collagen maturation) (32, 33).

Conclusions. Quantitative changes in gene regulation seem to be a major adaptive mechanism in the chronic hypoxia tolerance of the mole rat. *Spalax* can thus be regarded as a "natural" alternative to transgenic animal models. In globin research, the suggestions of very diverse molecular functions of Ngb and Cygb, mostly obtained in vitro, have to take into consideration observations from natural animal models. For example, hypotheses claiming an involvement of Ngb in complex neuronal signal transduction processes should explain the shift during mammalian evolution into another cell type (glia) in hypoxia model organisms like *Spalax* and seal. Together, the *Spalax* data strengthen the argument that Ngb functions in oxidative cellular metabolism, whereas Cygb may have distinct tissue-specific functions.

Materials and Methods

Animals. *Spalax* was captured in the field and housed in the Institute of Evolution, Haifa. Sprague-Dawley rats were used. After hypoxic treatment, animals were killed by Ketaset CIII injection (Fort Dodge Animal Health) at 5 mg per kg of body weight. The Ethics Committee of the University of Haifa approved all experiments.

Cloning and Sequencing of *Spalax* Ngb, Cygb, and Mb cDNAs. *Spalax* cDNAs for Ngb, Cygb, and Mb were isolated by RT-PCR from total RNA of brain, liver, and muscle tissues, respectively. PCR primers (*SI Materials and Methods*) were derived from published globin sequence alignments and the *Spalax* Mb protein sequence (38). RT-PCR and 5′/3′ RACE products were cloned and sequenced (Starseq). GenBank/European Molecular Biology Laboratory accession numbers are AM419202 (*S. judaei* Ngb), AM419201 (*S. galili* Ngb), AM489450 (*S. carmeli* Ngb), FN821091 (*S. golani* Ngb), AM419204 (*S. judaei* Cygb), AM419203 (*S. galili* Cygb), AM489449 (*S. carmeli* Cygb), and FN821092 (*S. golani* Cygb).

Immunostaining and Western Blotting. For immunohistochemistry and Western hybridization, we used established polyclonal rabbit antisera raised against synthetic peptides of Ngb and Cygb (26, 33; see *SI Materials and Methods* for experimental details).

Quantitative Real-Time RT-PCR. mRNA quantities were determined by standard real-time RT-PCR using either Taqman chemistry (Quantitect Probe PCR Kit) or QuantiTect SYBR Green detection (Qiagen). Experimental details (primers, probes, reagent concentrations, PCR conditions, normalization, and reference genes) are given in *SI Materials and Methods*. We used one to three pooled RNAs, each prepared from at least three animals, for all genes and tissues tested. Data were evaluated by the standard curve method. Graphs show fold changes of expression levels relative to normoxic conditions in the hypoxia/normoxia comparisons, and relative to rat in the interspecies comparisons. Error bars indicate SEM, calculated for biological replicates. Confidence intervals were calculated by Student's *t* tests at different levels of significance ($P < 0.1$, $P < 0.05$, $P < 0.01$).

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