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 allo-species of *Spalax* in Israel are adapted to different climatic regimes. The strongest differences in ecological conditions are observed between *Spalax galilii* (karyotype 2n = 52), inhabiting the northern cool-humid Upper Galilee Mountains with heavy soil, which often becomes flooded, and *Spalax judeae* (2n = 60), which reside in the warm-dry south with light-aerated soil. The most efficient hypoxic adaptation has consequently been demonstrated in *S. galilii*, with higher normoxic breathing and heart rate as well as higher hematocrit and Hb levels as compared with *S. judeae* (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 Htr, 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).


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1015379107/-/DCSupplemental.
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 O2 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. 1A). 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. 1B and C). Neuronal perikarya exhibited Ngb immunofluorescence that was often also seen in cell processes, whereas the cell nuclei remained free of signal (Fig. 1C). 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% O2) *Spalax*. Additional Ngb immunoreaction was observed in astrocytes. Fig. 1D 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 *Stallax* 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. 2A). 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. 2B), being highest 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 O2 supply, we performed immunostaining applying von Willebrand factor antibody (Fig. 2C). Results showed that *Spalax* blood vessels are present in OPL, INL, IPL, and GCS, suggesting O2 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. 2D). The Cygb signal colocalized with immunoreactivity toward neuronal NOS (nNOS) (Fig. 2E).

**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 [Vogl. 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. 3A). 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 ≤ 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. 3B).
We studied the changes of Ngb expression under short-term severe hypoxia (5 h, 6% O2) and longer-term moderate hypoxia (22 and 44 h, 10% O2). We observed an almost 2-fold decrease of Ngb mRNA in all species (most significantly at the P < 0.05 level), irrespective of the different hypoxic conditions (Fig. 3A). A notable exception was S. galili after 5 h of 6% O2, 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. 3C).

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. 4A). 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. 4B). 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% O2). 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% O2) 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 O2 (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 O2 supply or
Individuals were tested, numbered preparation 1 and Hb levels and a high O2 affinity in subterranean burrow systems by means of increased hematocrit and protein expression in the normoxic brain is substantially higher in Spalax (2n = 60; S60), and Spalax hypoxia (2.5-fold) than in rat. Only Spalax additionally increases brain Hb mRNA under hypoxia (44 h, 10% O2). In heart and liver, normoxic Cygb mRNA levels are the same as 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.

A

B

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 Hb mRNA under hypoxia (44 h, 10% O2). 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.

Increased Globin Levels in Spalax Indicate a Function in Hypoxia Tolerance. The role of Hb and Mb in O2 supply is well established. Spalax is known to cope with reduced O2 availability in its subterranean burrow systems by means of increased hematocrit and Hb levels and a high O2 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 Spalax 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? In-ferring adaptive significance by comparing traits of just two distinctly 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 O2 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 related, hypoxia-sensitive species nHbs reside in neurons. 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 hemoglobinins (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 hypoxia-tolerant goldfish 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 O2 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. 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 O2 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 \( \text{O}_2 \) and/or by scavenging ROS (20). The subretinal mene rod is blind and possesses only nonpigmented 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 function that does not require constitutively elevated expression levels of Ngb.

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 \( \text{O}_2 \) 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 \( \text{O}_2 \) 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 \( \text{O}_2 \)-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 Clil 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 S/S RACE products were cloned and sequenced (Starseq). GenBank/European Molecular Biology Laboratory accession numbers are AM419202 (S. judaei Ngb), AM419201 (S. galili Ngb), AM498450 (S. carmeli Ngb), FN821091 (S. galili Ngb), AM419204 (S. judaei Cygb), AM419203 (S. galili Cygb), AM498449 (S. carmeli Cygb), and FN821092 (S. galili Cygb).

Immunostaining and Western Blotting. For immunohistochemistry and Western blandering, 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 (\( P < 0.1 \), \( P < 0.05 \), \( P < 0.01 \)).

Acknowledgments. We thank Mrs. Ulrike Maas for technical support to F.G. and T.H. and Scott Permut for language editing. Financial support was provided by the European Union (QLG3-CT-2002-0154, to A.A., T.B., E.N., and T.H.), the Deutsche Forschungsgemeinschaft (DFG Ha2103/3, Bu956/12), the United States-Israel Binational Science Foundation (2005346, to A.A. and Dr. Mark Band). E.N. was supported by the Anchell-Teich Research Foundation for Genetics and Molecular Evolution.


Supporting Information

Avivi et al. 10.1073/pnas.1015379107

SI Materials and Methods

Cloning of *Spalax* Neuroglobin (Ngb).

- Coding sequence (CDS) Ngb
  
  MM Ngb 2F: 5′-AGC ATG GAG CGC CCG GAG-3′; MM Ngb 4R: 5′-TTA TCT SCC ATC CCA GCC TCG-3′.

- 5′ RACE reaction *Spalax* Ngb
  
  5′ RACE-GSP1-Ngb 1R (RT reaction): 5′-GCA TCA CCT TCC TTA TGT GAT C-3′; 5′ RACE-GSP2-Ngb 2R: 5′-GGA GAA CTG GCG GCC ATT GTA CTG-3′; 5′ RACE-nested GSP-Ngb 3R: 5′-GCA AAG AGT CTG GCG AAC AGG ACA-3′.

- 3′ RACE reaction *Spalax* Ngb
  
  3′ RACE-GSP-Ngb 4F: 5′-AGT CCC TGC TCT ACA TGC TGG-3′; 3′ RACE-nested GSP-Ngb 5F: 5′-GTG AGG TTC TCC TGC AGG ACC-3′; Ngb 6F (primer walking): 5′-GTC TTG ACT GCT TAG ATG CC-3′.

Cloning of *Spalax* Myoglobin (Mb).

- CDS Mb
  
  Myo deg CDS for: 5′-ATG GGG CTC AGY GAY GGG GA-3′; Myo deg CDS rev: 5′-AGC CCT GGA AGC CYA GCT CCT-3′.

- 5′ RACE reaction *Spalax* Mb
  
  Myo deg CDS rev (GSP1): 5′-AGC CCT GGA AGC CYA GCT CCT-3′; Quantitative PCR (qPCR) Myo deg rev (GSP2): 5′-CGG AAC AGC TCC AGG GCC T-3′; SS8 Myo 5′ RACE rev (nested GSP): 5′-TGC TTG CTC TGC AGG ACC TG-3′.

- 3′ RACE reaction *Spalax* Mb
  
  Myo deg CDS for (GSP): 5′-ATG GGG CTC AGY GAY GGG GA-3′; qPCR Myo deg for (nested-GSP): 5′-GCG ACC AAC CAC AAG ATC C-3′.

Cloning of *Spalax* Cytoglobin (Cygb).

- CDS Cygb
  
  HMR Cygb deg for: 5′-GCG GTT CAG GCT AGC TG-3′; HMR Cygb deg rev: 5′-CCA GCC CAC TTT CTT GTA GG-3′.

- Obtaining the 5′ end of *Spalax* Cygb
  
  Because our 5′ RACE was unsuccessful, the 5′ end of the Cygb coding sequence was obtained by genomic PCR walking.

- 3′ RACE of *Spalax* Cygb
  
  qPCR Cygb for (forward GSP): 5′-CCA ACT GCG AGC ACG TGG-3′

Cygb RACE 4F (forward GSP nested): 5′-TCA TCT ACA GCC ACC ACG TGA CCG-3′

Quantitative Real-Time RT-PCR.

- Primers (accession numbers according to the rat genes) and PCR cycling conditions
  
  Primers were synthesized by Biomers or Operon, MGB (minor groove binding) TaqMan probes at Applied Biosystems.

  Ngb (NM_033359)
  
  qPCR Ngb for: 5′-GAA GCA YCG GGC AGT G-3′ (190 nM) qPCR Ngb rev: 5′-AGR CAC YTC TCC AGT TAC AG-3′ (190 nM) qPCR Ngb MGB-probe: 6-FAM-5′-CTC AGC TCC TTC TGG ACA GT-3′-NFQ (200 nM)

  Cygb (NM_130744)
  
  qPCR Cygb for: 5′-CCA ACT GCG AGG ACG TGG-3′ (190 nM) qPCR Cygb rev: 5′-ACT GCG TGA AGT ACT GCT TGG C-3′ (190 nM) qPCR Cygb Mgb-probe: 5′-CTG GTG AGG TCC TTT GTG TGG-3′-NFQ (200 nM)

  Mb (NM_021588)
  
  qPCR Myo for: 5′-GCC ACC AAC CAC AAG ATC C-3′ (70 nM) qPCR Myo rev: 5′-CGG AAC AGC TCC AGG GCC T-3′ (70 nM) 15 min 95 °C, 40× (15 s 94 °C, 30 s 60 °C)

  Acidic ribosomal phosphoprotein P0 (ARP, syn. Rplp0, NM_022402)
  
  qPCR ARP for: 5′-AGG GCC ACC CAC GAG TGG-3′ (190 nM) qPCR ARP rev: 5′-GCA TCT GCT TGG AGC CCA-3′ (190 nM) 15 min 95 °C, 40× (15 s 94 °C, 30 s 60 °C)

  Cyclophilin A (Cyph, XM_578076)
  
  qPCR Cyph for: 5′-GGG ACC TTT GAG CGT GTC ACC ATG-3′ (150 nM) qPCR Cyph rev: 5′-GGT GTC TTC AAC TGC TCA CCA-3′ (150 nM) 15 min 95 °C, 40× (15 s 94 °C, 30 s 60 °C)

  Gapdh (XM_579386)
  
  qPCR GAPDH for: 5′-GCC ACC TCT CTA AGC TGA GCC GAC-3′ (190 nM) qPCR GAPDH rev: 5′-GCC AGC ATG TGA TGG AAG-3′ (190 nM) 15 min 95 °C, 40× (15 s 94 °C, 30 s 60 °C)

Avivi et al. www.pnas.org/cgi/content/short/1015379107
qPCR Vegf rev: 5’-CACCGCCCTGGTGTGCACAT-3’
15 min 95 °C, 40× (15 s 94 °C, 30 s 60 °C, 30 s 72 °C)

- Adrenomedullin (Adm; NM_012715)

qPCR Adm for: 5’-CAG GAC AAG CAG AGC TC-3’
qPCR Adm rev: 5’-TCT GGC GTG AGC GTT TGA C-3’
15 min 95 °C, 40× (15 s 94 °C, 30 s 60 °C, 30 s 72 °C)

To precisely adjust equal RNA amounts for reverse transcription, RNA solutions were treated with RNase-free DNase I (Fermentas), equilibrated to room temperature, and vortexed before optical density measurements (NanoDrop ND-1000, Peq-Lab). RNA integrity was checked on formaldehyde agarose gels. RNA quality had to be optimal (A260/A280 > 1.9). Total RNA was adjusted to 1 µg per 20 µL RT assay volume. RT reactions were conducted according to the SuperScript II protocol (Invitrogen) using oligo-dT primers. qRT-PCR was carried out on an ABI Prism 7000 SDS (Applied Biosystems). We used cDNA equivalent to 100 ng of total RNA in a 20-µL PCR volume. Reactions were run in duplicates or triplicates, and all experiments were repeated at least twice. We used one to three pooled RNAs, each prepared from at least three animals, for all genes and tissues tested. Ngb and Cygb expression was determined by TaqMan assays (Quantitect Probe PCR Kit; Qiagen). mRNA levels of Mb, the reference genes Rplp0, Cyph, and Gapdh and the control genes Vegf and Adm were measured using the QuantiTect SYBR Green PCR Kit (Qiagen). Amplicon specificity was checked by dissociation curves. For cross-species PCR, we usually used identical primers matching conserved regions in the Spalax and rat genes. To use a conserved, optimized TaqMan probe in the Ngb assay, we used PCR primers with one and three degenerate positions, respectively. These primers had the same optimal amplification efficiency (slope of -3.34) as perfectly matching ones.

Gene expression levels were normalized to total RNA, the most reliable method in absence of universally applicable housekeeping genes. However, we also identified reference genes displaying constant expression under specific conditions and in specific tissues of the species compared. Rplp0 (NM_022402) and Cyph (XM_351991) were unregulated in Spalax brain under normoxia and hypoxia. Gapdh (BC059110) showed constant expression in hypoxic and normoxic rat brains. Normalizing Ngb expression levels in brain by these genes yielded results equivalent to total RNA normalization.

qRT-PCR data were evaluated by the standard curve method. Standard curves of all genes analyzed had slopes of -3.4 ± 0.1. For standard curve preparation, plasmids containing the appropriate amplicon were diluted 10-fold (10² to 10⁰ copies). 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 of the experiments. Confidence intervals were calculated by Student’s t tests at different levels of significance (P < 0.1, P < 0.05, P < 0.01).

Immunohistochemistry.

- Antibodies used

Ngb: “α-Ngb TB1”: rabbits were immunized with synthetic peptide NH₂-CLSSPEFLDHKVM-COO (conserved across all mammals; Eurogentec); Cygb: “α-Cygb2-M”: rabbits were immunized with synthetic peptide NH₂-MEDPLEMERSPOLRK-COO (one change at pos. 3 in Spalax Cygb; Eurogentec).

The Ngb peptide was fully conserved in Spalax Ngb, whereas the Cygb peptide contained one amino acid exchange in Spalax. Animals were killed by ether overdose and fixed by transcardial perfusion. Tissue sections (40 µm) were used for Ngb detection with Cy3 as reporter (1). For double-immunofluorescence, a mouse monoclonal antibody to GFAP (1:100; Abcam) and a respective IgG coupled to Cy2 were used in addition to the Ngb antibody. The Ultra Vision Detection System Anti-rabbit, HRP/AEC (Dianova) was used for detection of Cygb immunoreactivity. Documentation was performed with a BX51 research microscope (Olympus) equipped with a Colorview-12 digital camera (Soft Imaging Solutions).

Western Blotting.

- Antibodies used

Ngb: “α-NgbTB4”: rabbits were immunized with recombinant mouse Ngb (own production); Cygb: “α-Cygb3-V”: rabbits were immunized with synthetic peptide NH₂-VVENLHDPDKVSSVL-COO (conserved in Spalax, human, mouse, and rat).

For Western blot analysis, half brains from three individuals of each species were homogenized in 1 volume of lysis buffer [10 mM Tris (pH 7.4), 10 mM NaCl, 5 mM MgCl₂, and 0.1% Nonidet P-40] supplemented with Complete (Roche) protease inhibitor. Protein was quantified by the bicinchoninic acid assay. Total protein extract (135 µg of for Ngb, 100 µg for Cygb) was denatured with SDS-containing sample buffer and separated on 15% polyacrylamide gels. Proteins were transferred to a Protran BA85 nitrocellulose membrane (0.2 µm pore size; Whatman) using transfer buffer containing 52 mM Tris, 6.67 mM glycine, and 20% methanol. Nonspecific binding was inhibited with 5% blocking reagent (Amersham Biosciences) for 1 h, and membranes were incubated overnight with the antisera. The ECL Plus Western Blotting Detection Reagent (Amersham Biosciences) was used for signal detection after 30 s to 10 min exposure to Kodak BioMax Light Film (Sigma-Aldrich). Equal loading of gel lanes was controlled by Ponceau S staining of the blotting membrane.

A. Amino acid alignment of Ngb

Fig. S1. (Continued)
**B. Amino acid alignment of Cygb**

![Amino acid alignment of Cygb](image_url)

Fig. S1. (Continued)
C. Amino acid alignment of Mb

![Amino acid alignment of Mb](image)

Fig. S1. Amino acid alignments of (A) Ngb, (B) Cygb, (C) and Mb proteins. (A) Amino acid alignment of Ngb. Comparison of *Spalax* Ngb with its vertebrate orthologs revealed only a single *Spalax*-specific amino acid replacement at position H2 (α-helix H, second amino acid: Leu), where most other taxa either have Pro or Ala. Another nonconservative amino acid replacement (ThrA14) was observed in *Spalax* and the amphibian *Xenopus tropicalis*. Helix position B1 showed an Asp in *Spalax* and most fishes, replacing the Ser typically present in other mammals. (B) Amino acid alignment of Cygb. Cygb proteins of *S. carmeli* and *S. galli* 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 *S. judaei* Cygb contains a unique, unprecedented amino acid substitution (GlyE13→GluE13). At position A4, *Spalax* shares an Asp with several fish Ngbs, whereas other mammals possess the isofunctional amino acid Glu. Additionally, *S. carmeli* and *S. galili* Cygb show unique substitutions Gly→Arg at B6 and His→Arg at CD7. (C) Amino acid alignment of Mb. For Mb, one amino acid position (Lys13) was found to be *S. carmeli* specific, whereas all other changes, for example at positions 20 and 49, also occur in other mammalian taxa. Sga, *S. golani*; Sju, *Spalax judaei*; Mmu, *Mus musculus*; Rno, *Rattus norvegicus*; Hsa, *Homo sapiens*; Cpo, *Cavia porcellus*; Ptr, *Pan troglodytes*; Cfa, *Canis familiaris*; Oca, *Oryctolagus cuniculus*; Ssc, *Sus scrofa*; Bta, *Bos taurus*; Mdo, *Monodelphis domestica*; Xtr, *Xenopus tropicalis*; Gga, *Gallus gallus*; Fru and Tru, *Fugu rubripes*; Tni, *Tetraodon nigroviridis*; Ola, *Oryzias latipes*; Omy, *Oncorhynchus mykiss*; Cca, *Cyprinus carpio*; Mlu, *Myotis lucifugus*; Oan, *Ornithorhynchus anatinus*; Ppr, *Pimephales promelas*; Gac, *Gasterosteus aculeatus*; Eeu, *Echinus europaeus*; Xia, *Xenopus laevis*; Sja, *Scomber japonicus*; Pca, *Physeter catodon*; Pvi, *Phoca vitulina*; Afo, *Aptenodytes forsteri*.

Avivi et al. www.pnas.org/cgi/content/short/1015379107
Fig. S2. Homology models of Spalax Ngb and Cygb proteins were built applying SwissModel (http://swissmodel.expasy.org/) using the crystal structures of human NGB and CYGB as templates and visualized by POLYVIEW-3D. (A) Animated model of Spalax Ngb. Relevant amino acid substitutions are displayed in red: ThrA14, SerB1, and ProH2. (B) Model of Spalax Cygb. Relevant amino acid substitutions are displayed in red: AspA4, ArgB6, and ArgCD7.
Fig. S3. Cygb immunostaining in brain, liver, and heart. (A and B) In brain, the hippocampus formation, including the dentate gyrus (DG) and Ammon’s horn (AH), shows Cygb staining in neuronal nuclei, cytoplasm, and cellular processes. (C) In liver, only the tiny hepatic stellate cells (HSC) are stained, whereas hepatocytes (HC) remain undecorated. (D) Corresponding negative control without primary antibody is devoid of HSC staining. (E) In heart muscle, Cygb expression is observed in fibroblasts (FB). (F) Corresponding negative controls without primary antibody are unstained.

Fig. S4. Real-time RT-PCR amplification results for genes Vegf (vascular endothelial growth factor) and Adm (adrenomedullin), serving as known positive controls for gene up-regulation under hypoxia. All normoxic values were set to 100%. (A) Adm levels increased in rat brain after 4 h of 6% oxygen by 4.1-fold. Bernaudin et al. (1) described a 9-fold (qRT-PCR) and 3.5-fold up-regulation (microarray analysis) after 3 h of 8% O2 in rat brain. (B) Vegf mRNA levels increased 2- to 3-fold in hypoxic Spalax brain and rat, as reported by Avivi et al. (2).

Fig. 55. Mb mRNA expression in neck muscle and heart, measured by qRT-PCR. (A) S. galili and S. judaei have a 27- to 42-fold higher Mb expression in normoxic neck muscle than rat. Expression level difference between the Spalax species is 1.6-fold. The expression value of rat was set to 100%. (B) Upon short-term severe hypoxia (5 h, 6% O2), rat increases its Mb mRNA expression 2.7-fold, whereas both Spalax species keep their Mb mRNA levels constant in neck muscle. Long-time moderate hypoxia (22 h, 10% O2) triggers a decreased expression of Mb mRNA in rat and S. judaei but not in the most hypoxia-resistant, S. galili. After 44 h, a strong reduction is observed in all species. (C) Normoxic Mb mRNA levels in heart are higher in Spalax than in rat by a factor of 2.7. In rat and Spalax heart, Mb mRNA expression rises slightly (1.5- to 1.7-fold) after moderate hypoxia (22 or 44 h at 10% O2).