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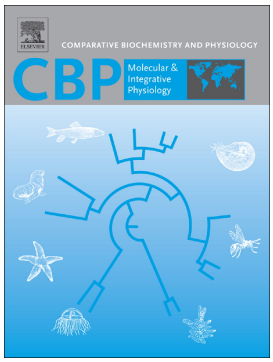

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Transcriptome analysis reveals a high aerobic capacity in the whale brain

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Abstract

The brain of diving mammals is repeatedly exposed to low oxygen conditions (hypoxia) that would have caused severe damage to most terrestrial mammals. Some whales may dive for up to two hours with their brain remaining active. Many of the physiological adaptations of whales to diving have been investigated, but little is known about the molecular mechanisms that enable their brain to survive sometimes prolonged periods of hypoxia. Here, we have used an RNA-Seq approach to compare the mRNA levels in the brains of whales with those of cattle, which serves as a terrestrial relative. We sequenced the transcriptomes of the brains from cattle (*Bos taurus*), killer whale (*Orcinus orca*), and long-finned pilot whale (*Globicephala melas*). Further, the brain transcriptomes of cattle, minke whale (*Balaenoptera acutorostrata*) and bowhead whale (*Balaena mysticetus*), which were available in the databases, were included. We found a high expression of genes related to oxidative phosphorylation and the respiratory electron chain in the whale brains. In the visual cortex of whales, transcripts related to the detoxification of reactive oxygen species were more highly expressed than in the visual cortex of cattle. These findings indicate a high oxidative capacity in the whale brain that might help to maintain aerobic metabolism in periods of reduced oxygen availability during dives.

Keywords

Cetacean, hypoxia tolerance, aerobic metabolism, marine mammals, diving

List of abbreviations

RNA-Seq, RNA Sequencing; mRNA, messenger RNA; RPKM, Reads Per Kilobase per Million mapped reads; ROS; reactive oxygen species: VC; visual cortex; CE, cerebellum; FL, frontal lobe; GO, gene ontology; PCA, principal component analysis

Introduction

In most mammals, including humans, a shortage in O_2 supply (hypoxia) leads to irreversible damage to the brain and death of neurons within a few minutes (Choi and Rothman 1990, Dirnagl et al. 1999, Peers et al. 2007, Walton et al. 1999). Without O_2, the oxidative phosphorylation stops and alternative pathways cannot produce sufficient amounts of ATP, which leads to an impairment of highly energy-consuming processes such as ion transport or neurotransmitter uptake. Depolarization and an excessive extracellular release of neurotransmitters results in overstimulation of receptors and uncontrolled Ca^{2+} influx, which triggers cell damage and, eventually, cell death (Bano and Nicotera 2007, Lipton 1999). For a limited period, ATP production can be sustained by anaerobic glycolysis, which, however, leads to the production of lactate. An excess of lactate decreases the pH, resulting in additional energy consumption and cell damage. When the O_2 supply is re-established, the excess of O_2 causes the generation of reactive oxygen species (ROS), which leads to additional damage (Cherubini et al. 2005, Murphy 2009, Therade-Matharan et al. 2005). In humans, an impaired supply of O_2 to the brain is involved in various diseases that cause millions of casualties. For example, cerebral ischemia (stroke) has a devastating impact on the nerve cells, which is largely impossible to repair (Ding et al. 2014, Horner and Gage 2000).

Diving mammals can cope with extended periods of quite severe hypoxia, apparently without damage to their brain (Butler 2004, Butler and Jones 1997, Kerem and Elsner 1973, Meir et al. 2009, Ramirez et al. 2007). Expert divers may perform dives exceeding two hours (Schorr et al. 2014), which require multiple behavioral, morphological, physiological and molecular adaptations. For example, since marine mammals cannot breathe during the dives, O_2 must be stored bound to hemoglobin, which is present in very high levels in their blood (Burns et al.

2007, Scholander 1940, Snyder 1983). The high myoglobin content of their muscles provides additional local O_2 stores (Lenfant et al. 1970, Polasek and Davis 2001, Scholander 1940, Snyder 1983). During long-duration diving, circulatory mechanisms such as peripheral arterial constriction ensure blood supply to the most O_2-sensitive organs (*e.g.*, the brain) while reducing the O_2-delivery to other organs (*e.g.*, kidney and gut) to a minimum (Blix et al. 1983, Scholander 1940, Zapol et al. 1979). Under such conditions, these tissues subsist based on a combination of hypometabolic and anaerobic processes (Butler 2004, Butler and Jones 1997, Ramirez et al. 2007, Scholander 1940).

In recent years, the first data on the molecular adaptations of marine mammals have emerged. For example, the genome of the minke whale (*Balaenoptera acutorostrata*) showed amplification of genes involved in stress-response and anaerobic metabolism, and a loss of genes involved in hair formation and sensing (Yim et al. 2014). The return into the water was accompanied by a shift in the molecular evolutionary rate (Chikina et al. 2016), including positive selection in genes involved in hypoxia adaptation (Tian et al. 2016) and energy metabolism (McGowen et al. 2012). The comparative analysis of the genomes of unrelated marine mammals, such as killer whale, walrus (*Odobenus rosmarus*) and the manatee (*Trichechus manatus latirostris*) identified multiple convergent substitutions in distinct genes, suggesting that similar molecular mechanisms and metabolic pathways are involved (Foote et al. 2015). In addition, Mirceta et al. (2013) revealed that net surface charge characteristics of myoglobin allow elevated myoglobin levels in several species of diving mammals, thus indicating a convergent molecular adaptation. Brain-specific adaptations have been investigated in the hooded seal (*Cystophora cristata*) (Fabrizius et al. 2016, Hoff et al. 2017). Comparative transcriptomics showed, for example, lower levels of enzymes involved in aerobic metabolism in the seal brain compared to the closely related ferret (*Mustela putorius*), which may reflect an energy saving strategy. The mRNA levels of certain

stress-related proteins, *i.e.*, clusterin and S100B, were found enhanced in the hooded seal brain, and it may be that these genes contribute to convey hypoxia tolerance to the seal brain (Fabrizius et al. 2016). However, it is still largely unknown how the brain of whales copes with the hypoxic stress during the dive and reperfusion after surfacing. We found that the brains of the harbor porpoise (*Phocoena phocoena*) and the minke whale have higher neuroglobin levels than the brain of cattle (*Bos taurus*), which may contribute to enhanced O_2 supply (Schneuer et al. 2012).

To better understand the molecular adaptations of the whale brain, we have sequenced the brain transcriptomes of the killer whale (*Orcinus orca*), the long-finned pilot whale (*Globicephala melas*), and cattle (*B. taurus*). Although not generally deep divers, foraging killer whales can dive to at least 700 m (Reisinger et al. 2015). The pilot whale reaches > 800 m with high speed ("cheetahs of the sea" (de Soto et al. 2008)), suggesting high energy and O_2 consumption. The available brain transcriptomes of the minke whale (*B. acutorostrata*), the bowhead whale (*Balaena mysticetus*) and additional cattle transcriptomes were integrated into the analysis of the gene expression levels by RNA-Seq.

Materials and methods

Animals

The brain samples of all animals used in this study were obtained opportunistically. The brain of a cattle (*B. taurus*) (male, 22 months) was acquired from a German butchery under the exceptional permission of the German law (*Tierische Nebenprodukte-Beseitigungsgesetz*). Dissection of the skull and sampling of the visual cortex was performed by the University of

Hamburg taxidermist. The brain of a juvenile killer whale (*O. orca*), which died after stranding alive, was transported to the Institute of Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation, in Büsum for necropsy. A brain sample of the visual cortex was preserved in RNAlater at -80 °C (Qiagen, Hilden, Germany). Samples of the visual cortex and the cerebellum from a long-finned pilot whale (*G. melas*) (male, body length 569 cm) were obtained from an animal that was harvested during traditional whaling on the Faroe Islands. The visual cortex was chosen because this tissue was used in the first electrophysiological recordings from seal brain slices, demonstrating its remarkable hypoxia tolerance (Folkow et al. 2008), later followed by further studies on potential mechanisms underlying hypoxia tolerance in the seal brain, e.g., by Fabrizius et al. (2016) and Hoff et al. (2017). The cerebellum was used as a reference tissue to address regional differences in gene expression and to detect potential global adaptations in the brain. This particular region was also largely chosen because of previous studies (e.g., Mitz et al. 2009; Schneuer et al., 2012). Regulations of the *Convention on Biological Diversity* (CBD) and *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES) were followed, and the appropriate permits were obtained (Permit number: E-01456/17).

RNA preparation and sequencing

Total RNA was extracted using peqGOLD Trifast (PEQLAB, Erlangen, Germany) or Crystal RNA Mini Kit (Biolab Products, Bebensee, Germany). The quality and quantity of the RNA were analyzed by spectrophotometry and gel electrophoresis. Total RNA from the visual cortices of the cattle (2 µg), the killer whale (9 µg), and from the visual cortex and cerebellum of the long-finned pilot whale (2.5 µg each) were used for the generation of a cDNA library. Paired-end

sequencing (PE 150) was performed on a NextSeq 500 platform (StarSEQ, Mainz, Germany) with an estimated output of 50 million reads per sample. Analysis of the data was performed with the aid of CLC Genomics Workbench, Version 10.0.1 (Qiagen, Venlo, Netherlands). For quality trimming the first 15 nucleotides from the 5′ end were removed. Additionally, all reads with more than two ambiguous nucleotides and reads with a mean Phred quality lower than 15 were discarded. The raw Illumina files of the transcriptomes are available from the NCBI Sequence Read Archive (SRA) from the visual cortex of the cattle (**SRR8305676**), the visual cortex of the killer whale (**SRR8305677**), and from the visual cortex (**SRR8305674**) and cerebellum (**SRR8305675**) of the long-finned pilot whale and under the Bioproject **PRJNA506903**.

Differential Expression analysis using RNA-Seq

To identify genes differentially expressed between the brains of cattle and whale, the transcriptome datasets of long-finned pilot whale (visual cortex and cerebellum), killer whale (visual cortex), bowhead whale (cerebellum) (*B. mysticetus*) (**SRX790347**) and minke whale (brain region not specified) (*B. acutorostrata*) (**SRX313597)** were compared with five transcriptomes of the cattle (3 × visual cortex, 2 × frontal lobe) (**SRX211675**, **SRX211674**, **SRX196362**, **SRX196353**, **SRX196362**). Mapping of the quality-trimmed reads was performed using the RNA-Seq algorithm of the CLC Genomics Workbench. Due to a high percentage of mitochondrial reads in some datasets, the mitochondrial transcripts were excluded. Whale reads matching 75 % of the read length and 75 % of the nucleotides of the cattle mitochondrial genome were excluded from further analysis. For the filtering of the cattle mitochondrial reads parameters were adjusted to 85 % read length and 85 % identity. The NCBI cattle genome (assembly ARS-UCD1.2) was used as reference genome. Only reads that matched 85 % of the read length and

85 % of nucleotides of the reference were included in the mapping of the whale reads. For the mapping of the cattle reads, parameters were adjusted to 90 % length match and 90 % similarity. Reads with non-specific matches were ignored in the mapping. The paired read distance was calculated automatically. Differential gene expression analysis was carried out using the CLC Genomics Workbench tool for statistical analysis. The unique gene read number normalized to total reads of each sample was used as count values for the "Exact Test" (Robinson and Smyth 2008). A cut-off of 5 reads and a Bonferroni-correction of the *p*-values were applied. Only genes with a fold change ≥ 2, a range (difference between the highest and the lowest expression value) ≥ 5 and a Bonferroni-corrected *p*-value ≤ 0.05 were considered significant. Gene expression was calculated as RPKM (Reads Per Kilobase per Million mapped reads) (see Additional File 1). Differential expression analysis was also conducted with two brain transcriptomes of the beluga whale (*Delphinapterus leucas*) that were available at the Short Read Archive (**SRX2585929**, **SRX2585928**) (see Additional File 2), but these data were not included in the analyses due to large differences in overall gene expression compared to other species of whales.

Gene ontology analysis

Gene Ontology (GO) analyses were performed using the PANTHER Overrepresentation Test (Protein Analysis Through Evolutionary Relationships, http://go.pantherdb.org/) version 13.1 released 2018-02-03 (Mi et al. 2017). Contigs with high expression in whales transcriptomes but annotated as pseudogenes in the cattle genome were considered functional in whales and renamed to their parental genes (Additional File 3). The annotated cattle genes in the PANTHER DB were used as a reference list, and overrepresentation was tested in the PANTHER GO-Slim terms and

Reactome pathways with Fisher's Exact Test with FDR multiple test correction. Only categories with corrected p-values < 0.05 were considered significant.

Principal component analysis

Principal component analysis (PCA) was performed by plotting the two principal components of the log10 transformed expression values of all 34,436 transcripts using the CLC Genomics Workbench Principal Component Analysis tool with default settings.

Results

Generation of transcriptomes of the whale brain

We generated ~40 million Illumina reads (150 nt, paired-end) of the transcriptomes from the brains of each, the killer whale (visual cortex), the long-finned pilot whale (visual cortex and cerebellum), and – to allow a comparative approach – of cattle (visual cortex). We included the transcriptomes of four additional cattle brains in the analyses, which were available from the SRA database at NCBI (Table 1). To trace possible adaptations of the whale brain to diving on the molecular level, we estimated the mRNA levels of the brain transcriptomes by RNA-Seq. Due to the lack of an annotated whale genome, the cattle genome was employed as a reference.

Table 1

Summary of Illumina Sequencing

Sample	SRA accession	Raw reads #	Trimmed	Trimmed without mitochondrial reads	Mapped reads %
Oor1	SRR8305677	29,964,586	29,861,576	26,448,179	39.76
Gme VC	SRR8305674	49,465,538	49,462,293	47,712,366	52.95
Gme CE	SRR8305675	41,444,974	41,442,287	42,197,185	50.84
Bac	SRR918699	51,470,260	51,341,266	50,624,402	63.7
Bmy	SRR1685414	21,182,210	17,750,937	20,935,295	49.39
Bta1	SRR8305676	32,089,626	32,086,347	24,615,661	80.37
Bta cowb	SRR594482	56,890,388	56,292,520	51,624,386	91.41
Bta cowc	SRR594491	67,256,226	64,242,921	59,311,464	89.93
Bta br1	SRR636934	53,310,044	53,119,486	48,272,031	92.16
Bta br2	SRR636935	29,597,408	29,429,907	26,871,240	90.85
Dle A**	SRX2585928	246,941,740	246,909,289	238,525,305	53.82
Dle Q**	SRX2585929	270,336,352	270,299,555	258,319,503	47.46

The number of reads per sample before and after quality trimming is given. The reads used for RNA-Seq after elimination of reads originating from mitochondrial genes and the percentage of reads mapped to the cattle genome is denoted.

*Oor = *Orcinus orca*; Gme VC = *Globicephala melas* (visual cortex); Gme CE = *Globicephala melas* (cerebellum); Bac = *Balaenoptera acutorostrata*; Bmy = *Balaena mysticetus*; Bta = *Bos Taurus*; cowb = *Bos taurus* sample b; cowc = *Bos taurus* sample c; br1 = *Bos taurus* sample br1, br2 = *Bos taurus* sample br2; Dle = *Delphinapterus leucas*

** only used for PCA

Regional differences in gene expression within the brain regions of the long-finned pilot whale

We first examined the regional differences of mRNA levels within the whale brain by comparing the transcriptomes of the visual cortex and the cerebellum from the long-finned pilot whale. In the visual cortex, the most highly expressed gene was synaptosomal nerve-associated protein 25 (SNAP25; 2,151 RPKM), followed by the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1,871 RPKM), and the proteolipid-protein 1 (PLP1; 1,431 RPKM). In the cerebellum, the highest expression also was found for SNAP25 (1,966 RPKM), followed by the glycolytic enzyme aldolase C (ALDOC; 1,327 RPKM) and S100 calcium-binding protein B (S100B; 1,277 RPKM) (see Additional File 4 Table S1).

The most highly overrepresented (highest RPKM-fold difference) transcript in the visual cortex relative to the cerebellum was the guanine deaminase (GDA; 8,594-fold difference), which plays a role in the guanine degradation (Yuan et al. 1999). The second highest difference was found for neurogranin (NRGN; 4,002-fold), a gene that interacts with the calcium regulator calmodulin (Prichard et al. 1999), followed by the mechanosensory transduction mediator homolog STUM (738-fold difference) that is essential for mechanical sensing in proprioceptive neurons in the fruit fly (Desai et al. 2014), and the G protein-coupled receptor 26 (GPR26; 721-fold difference), which is involved in responses to environmental stimuli (Jones et al. 2007) and, at last, the gamma-aminobutyric acid type A receptor alpha 5 subunit (GABRA5; 665-fold difference) that acts as receptor to GABA which is the major inhibitory neurotransmitter in the mammalian brain (Wingrove et al. 1992) (Table 2A).

The transcripts that were most highly overrepresented in the cerebellum compared to the visual cortex were: fat atypical cadherin 2 (FAT2; 10,143-fold difference), a cerebellar development protein (Nakayama et al. 2002), another GABA receptor subunit (GABRA6; 2,190-fold difference), and a glutamate ionotropic receptor NMDA type subunit 2C (GRIN2C; 1,648-fold difference) that is involved in learning, memory, and synaptic development (Ogden et al. 2017). The next two were the rho guanine nucleotide exchange factor 33 (ARHGEF33; 1,494-fold difference) and the zic family member 4 transcript (ZIC4; 1,195-fold difference), which plays a role in the cerebellar development (Blank et al. 2011) (Table 2 B).

Table 2: Transcripts overrepresented in different brain regions of the long-finned pilot whale

Gene	Gene symbol	Function	Visual cortex (RPKM)	Cerebellum (RPKM)	Fold difference
A. Visual cortex					
Guanine Deaminase	GDA	Microtubule assembly	69.46	0.01	8,594
Neurogranin	NRGN	protein kinase substrate that binds calmodulin	740.41	0.19	4,002
Stum, Mechanosensory Transduction Mediator Homolog	STUM	Essential for Mechanical Sensing in Proprioceptive Neurons	21.03	0.03	738
G Protein-Coupled Receptor 26	GPR26	Responses to environmental stimuli	8.40	0.01	721

Gamma-Aminobutyric Acid Type A Receptor Alpha5 Subunit	GABRA5	Ligand-gated chloride channel	91.53	0.14	665
B. Cerebellum					
FAT Atypical Cadherin 2	FAT2	Cerebellar development	0.01	109.15	10,143
Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit	GABRA6	Ligand-gated chloride channel	0.18	385.03	2,190
Glutamate Ionotropic Receptor NMDA Type Subunit 2C	GRIN2C	Learning, memory, and synaptic development	0.03	49.48	1,648
Rho Guanine Nucleotide Exchange Factor 33	ARHGEF33	Rho guanyl-nucleotide exchange	0.01	16.89	1,494
Zic Family Member 4	ZIC4	Transcriptional regulation	0.01	13.14	1,195

Transcripts most highly overrepresented in the visual cortex (A) vs. the cerebellum (B) of the long-finned pilot whale.

We conducted a gene ontology (GO) analysis with the PANTHER GO-Slim tool of transcripts overrepresented in either the visual cortex or the cerebellum. Only transcripts with an RPKM-fold difference > 2 and RPKM > 5 in one sample were considered. We found 1,184 transcripts higher expressed in the visual cortex and 1,020 transcripts higher expressed in the cerebellum (see Additional File 5). The 1,184 transcripts that were higher expressed in the visual cortex

showed enrichment in several GO terms in the category Biological Process (Figure 1). The highest enrichment was found for the terms: "skeletal system development (GO:0001501)" (7.1-fold, corrected p-value: 0.0103), "cell growth (GO:0016049)" (4.1-fold, p-value: 0.00001) and "JNK cascade (GO:0007254)" (3.9-fold, p-value: 0.0407). In total, 303 transcripts were found to be statistically enriched in ten GO terms in the category Biological Process (Figure 1).

In the 1,020 transcripts that were higher expressed in the cerebellum only the terms "ectoderm development (GO:0007398)" (2.2-fold, p-value: 0.0324) and "regulation of transcription from RNA polymerase II promoter (GO:0006357)" (2-fold, p-value: 0.00014) were found to be enriched in the GO-category Biological Process, totalling 75 transcripts.

Analysis of gene set enrichment in the visual cortex of whales and cattle

As we found significant regional differences in the gene expression profiles in the brain of the long-finned pilot whale, we used for the following comparative analyses only the visual cortex of pilot whale, killer whale, and cattle. Only transcripts that had an RPKM > 2 in all samples were included. The transcript most highly overrepresented in the visual cortex of the killer whale and the long-finned pilot whale was tubulin alpha-1C chain-like (LOC100141266; 44.6-fold), which may be important for microtubule cytoskeleton organization, followed by complement C1q C chain (C1QC; 20-fold), which may be involved in immune regulation (Petry 1998), and dehydrogenase/reductase 7 (DHRS7; 17.3-fold), which encodes a member of the short-chain dehydrogenases/reductases family that metabolize many compounds such as steroid hormones, prostaglandins and retinoids (Matsunaga et al. 2008). The ribosomal protein S4 y-linked 1 (RPS4Y1) showed a 16-fold difference and was followed by the solute carrier family 38 member

5 (SLC38A5; 11.2-fold), which functions as a sodium-dependent amino acid transporter (Nakanishi et al. 2001) (Table 3).

Table 3

Transcripts most highly overrepresented in the visual cortex of the long-finned pilot whale and the killer whale

Gene	Gene symbol	Function	Whale mean (RPKM)	Cattle mean (RPKM)	Fold difference	*p*-value (Bonferroni)
tubulin alpha-1C chain-like	LOC100141266	microtubule cytoskeleton organization	162	3.6	44.6	4×10^{-16}
Complement C1q C Chain	C1QC	immune response	59.6	3	20	7.5×10^{-5}
Dehydrogenase/Reductase 7	DHRS7	oxidoreductase activity	75	4.3	17.3	2.7×10^{-8}
Ribosomal Protein S4 Y-Linked 1	RPS4Y1	translation	364.9	22.8	16	1.6×10^{-5}
Solute Carrier Family 38 Member 5	SLC38A5	amino acid transport	47.6	4.3	11.2	0.517

Transcripts most highly overrepresented in the visual cortex of the killer whale and the long-finned pilot whale compared to the visual cortex of cattle are shown

For differential expression analyses, only transcripts with at least a two-fold difference of normalized unique gene reads, a range (difference between the highest and the lowest normalized unique gene reads) > 5, and a Bonferroni corrected *p*-value < 0.05, were considered. We found 560 genes that were significantly higher expressed in the visual cortex of cattle compared to the mean value for killer whale and long-finned pilot whale visual cortices. In total 613 genes were higher expressed in the mean expression values of visual cortices of the pilot whale and the killer whale (Additional File 6), compared to the visual cortex of cattle. No enrichment was found in the PANTHER Overrepresentation Test for the genes that were more highly expressed in the cattle brain. Annotating the transcripts more highly expressed in the visual cortex of whale, the PANTHER GO-Slim category Biological Process showed several enriched terms (Figure 2). The terms "oxidative phosphorylation (GO:0006119)" (7-fold, corrected p-value: 0.00158) with eight genes and "respiratory electron transport chain (GO:0022904)" (4.1-fold, p-value: 0.00406) with ten genes, showed the highest fold enrichment. These oxygen-related GO-Terms were followed by the terms "protein folding (GO:0006457)" (4.1-fold, p-value: 0.006) and "rRNA metabolic process (GO:0016072)" (3.9-fold, p-value: 0.00352), both being related to protein translation. Other significantly enriched terms were "mitochondrion organization (GO:0007005)" (3.7-fold, p-value: 0.0192), "cellular component biogenesis (GO:0044085)" (1.7-fold, p-value: 0.0304) and "biosynthetic process (GO:0009058)" (1.5-fold, p-value: 0.0215), which are related to macromolecular modifications and cell metabolism.

In the transcripts that are more highly expressed in the whale cortex, reactome pathway enrichment analyses revealed the term "detoxification of reactive oxygen species" (11.1-fold, p-value: 0.00471), followed by "L13a-mediated translational silencing of ceruloplasmin expression" (8.6-fold, p-value: 0.00437), "HSP90 chaperone cycle for steroid hormone receptors (SHR)" (6.8-fold, p-value: 0.0266), and "respiratory electron transport" (5.1-fold, p-value: 0.0472) (Figure 2). A total of 17 transcripts were found annotated in the pathway "detoxification of reactive oxygen species", including known antioxidant enzymes like peroxiredoxin 6, superoxide dismutase 1 and glutathione S-transferase P, which may be candidate genes involved in the adaptation of the whale brain (Kinnula et al. 2004, Mates et al. 1999) (Table 4).

Table 4: Candidate genes for functional analysis

Original gene symbol	Annotated gene symbol	Gene name	Whale mean (RPKM)	Cattle mean (RPKM)	Fold difference
A. Oxidative phosphorylation					
LOC101902002	COX6A1	Cytochrome c oxidase subunit 6A1	123.4	0.2	573.4
LOC112443463	NDUFB4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	28.0	0.2	170.3
LOC100847304	CYCS	Cytochrome c	6.8	0.1	59.9
LOC783502	UQCRC2	Cytochrome b-c1 complex subunit 2	53.8	0.3	176.2
COXP1	COX4	Cytochrome c oxidase subunit 4 isoform 1	40.9	0.2	199.0
COX7ALP1	COX7A1	Cytochrome c oxidase subunit 7A1	39.8	0.2	181.1
LOC101907120	UQCRB	Cytochrome b-c1	35.5	0.0	/

		complex subunit 7			
LOC783686	NDUFV2	NADH dehydrogenase [ubiquinone] flavoprotein 2	19.2	0.2	113.4
B. Respiratory electron chain					
LOC101902002	COX6A1	Cytochrome c oxidase subunit 6A1	123.4	0.2	573.4
LOC781255	GLRX3	Glutaredoxin-3	6.7	0.0	202.4
LOC112443463	NDUFB4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	28.0	0.2	170.3
LOC100847304	CYCS	Cytochrome c	6.8	0.1	59.9
LOC783502	UQCRC2	Cytochrome b-c1 complex subunit 2	53.8	0.3	176.2
COXP1	COX4	Cytochrome c oxidase subunit 4 isoform 1	40.9	0.2	199.0
COX7ALP1	COX7A1	Cytochrome c oxidase subunit 7A1	39.8	0.2	181.1
LOC101907120	UQCRB	Cytochrome b-c1 complex subunit 7	35.5	0.0	/
LOC783686	NDUFV2	NADH dehydrogenase [ubiquinone] flavoprotein 2	19.2	0.2	113.4
LOC100847677	CYP4X1	Cytochrome P450 4X1	0.2	0.0	/
C. Detoxification of reactive oxygen species					
LOC786533	PRDX6	Peroxiredoxin-6	30.5	0.0	716.3
LOC507743	SOD1	Superoxide dismutase	121.9	0.3	429.4
NCF2	NCF2	Neutrophil cytosol factor 2	13.7	1.5	9.1
GPX2	GPX2	Glutathione peroxidase	2.6	0.1	44.2
LOC100847304	CYCS	Cytochrome c	6.8	0.1	59.9

GSTP1	GSTP1	Glutathione S-transferase P	10.6	0.1	182.8
LOC107132346	TXN2	Thioredoxin	1.2	0.0	619.0

Genes of the enriched PANTHER GO-Slim category Biological Process "oxidative phosphorylation", "respiratory electron chain" and "detoxification of reactive oxygen species" in the visual cortex of killer whale and long-finned pilot whale compared to the visual cortex of cattle are shown.

Comparative analyses of gene expression in whale and cattle brains

To evaluate lineage-specific adaptations of the whale brain, we compared all available transcriptomes of different whale brains to the brain transcriptomes of cattle. In addition to the transcriptomes of the visual cortex of the killer whale, the visual cortex and the cerebellum of the long-finned pilot whale, which had been generated in this study, we included the transcriptomes of the cerebellum of the bowhead whale and the brain of the minke whale. For the minke whale, no further information on the brain region is given in the annotation. The two transcriptomes of the beluga whale brain were excluded due to large expression differences to the other whale transcriptomes, as evident from PCA (Figure 3). Thus, the final whale dataset included five transcriptomes from four species, the cattle dataset consisted of five transcriptomes (Table 1).

For analyses of the transcripts most highly overrepresented (highest RPKM fold-difference) in the whale brain, we used only transcripts with at least 2 RPKM in all samples. The most highly overrepresented transcript was dehydrogenase/reductase 7 (DHRS7; 14.9-fold), followed by the ribosomal protein S4 y-linked 1 (RPS4Y1; 11.8-fold), the tubulin beta-6 chain (TUBB6; 10.2-fold), which is involved in microtubule cytoskeleton organization, the histone binding

nucleosome assembly protein 1-like 3 (NAP1L3; 9.7-fold) (Park and Luger 2006), and the uronyl 2-sulfotransferase (UST; 9.2-fold) catalyzing the sulfation of uronyl residues (Kobayashi et al. 1999) (Table 5).

Table 5

Most highly overrepresented transcripts in whale vs. cattle brain

Gene	Gene symbol	Function	Whale mean (RPKM)	Cattle mean (RPKM)	Fold difference	*p*-value (Bonferroni)
Dehydrogenase/ Reductase 7	DHRS7	oxidoreductase activity	63.1	4.2	14.9	4.2×10^{-11}
Ribosomal Protein S4 Y-Linked 1	RPS4Y1	translation	237.9	20.1	11.8	1.7×10^{-5}
Tubulin beta-6 chain	TUBB6	microtubule cytoskeleton organization	50.9	5.0	10.2	0.00019
Nucleosome assembly protein 1-like 3	NAP1L3	nucleosome assembly	69.2	7.1	9.7	3.3×10^{-7}
Uronyl 2-sulfotransferase	UST	sulfotransferase activity	51.7	5.6	9.2	0.01368

Transcripts most highly overrepresented in the whale brain (2 × long-finned pilot whale, killer whale, bowhead whale, minke whale) compared to the cattle brain (n=5) are shown

For differential expression analyses, only transcripts that display an at least two-fold difference in normalized unique gene reads, a range (normalized unique gene reads) > 5 and a Bonferroni corrected *p*-value < 0.05 were considered. We found 567 transcripts that were significantly higher expressed in the brain of cattle and 603 transcripts significantly higher expressed in the brain of whales (Additional File 7). No enriched Panther GO terms were found for the genes higher expressed in the cattle brain. For the 603 transcripts higher expressed in whale brain, PANTHER Overrepresentation Test found several terms enriched. In the GO-Slim category Biological Process we found 239 transcripts significantly enriched in ten categories. As previously the highest-fold enrichment was found for the terms "oxidative phosphorylation (GO:0006119)" (5.2-fold, p-value: 0.018), "protein folding (GO:0006457)" (4.04-fold, p-value: 0.0101), "translation (GO:0006412)" (3.8-fold, p-value: 0.0000402), "rRNA metabolic process (GO:0016072)" (3.49-fold, p-value: 0.0124) and "respiratory electron transport chain (GO:0022904)" (3.26-fold, p-value: 0.0339) (Figure 4).

Discussion

The brain in humans accounts for roughly 20 % of the total oxygen consumption at rest (Harris et al. 2012). While the brains of terrestrial mammals are usually highly susceptible to hypoxia, whales repeatedly perform dives of remarkable duration and depth without obvious damage to their brain. The brain of whales must remain functional during diving and whales like the killer whale or the long-finned pilot whale rely on their ability for hunting and communicating during the dives (Baird 1999, Visser et al. 2017). Analyses of gene expression are more reliable with a

high number of replicates (Gierlinski et al. 2015, Manga et al. 2016). In transcriptomic studies, a high number of replicates is usually used to obtain informative results. Because of ethical, legal and practical consideration, sampling of whale brains has to be conducted opportunistically. Therefore, only a limited number of transcriptomes from whale brains is available and different species and brain regions had to be used. We generated transcriptomes of two brain regions of whales, the visual cortex and the cerebellum and of the visual cortex of cattle. For a more robust insight we furthermore included transcriptomes of brain samples from a minke whale, a bowhead whale and four cattle from the SRA database in our analyses (Table 1). Two transcriptomes of the beluga whale brain were excluded, due to general expression differences compared to other whale brain samples (Figure 3). The beluga whale samples were sequenced more detailed than the other samples used in this study, which may explain the observed differences in gene expression. Therefore, we have limited our study to samples that were equally generated, for the further statistical analyses. Our results provide insights into the molecular basis of the hypoxia tolerance of the whale brain. To examine the influence of factors besides hypoxia adaptation, we conducted correlation analyses of gene expression between brain samples of additional terrestrial mammals (mouse, pig, dog, sheep, ferret) and whales. The analyses showed correlation between gene expression patterns and species phylogeny as well as brain region used for sampling (see Additional File 8 Figure S1). From this, we conclude that there is no evidence in our dataset for obvious influence of allometry, different diet or sampling methods, on gene expression patterns. Due to the lack of a non-diving relative is not possible to compare brain transcriptomes of whales to those of closely related non-diving species with a similar lifestyle, diet and under similar sampling conditions. Therefore, differences in gene expression between cattle and whale need not be ultimately linked to diving. However, our findings indicate an adaptation on the molecular level to hypoxia in the whale brain.

Differential gene expression in the visual cortex vs. the cerebellum of the long-finned pilot whale

To examine brain region-specific gene expression, we compared the transcriptomes of the visual cortex and the cerebellum of the long-finned pilot whale (Figure 1, Table 2). We found 2,204 transcripts differentially expressed, with 1,184 genes higher expressed in the visual cortex and 1,020 genes higher expressed in the cerebellum. In the gene set that was more highly expressed in the visual cortex we found enrichment in the expected category "Visual perception" but also in terms and pathways related to axon guidance, neurotransmitter activity and calcium homeostasis (Figure 1). In the cerebellum, fewer categories were found enriched, and these are mainly related to transcriptional regulation (Figure 1). Our results indicate higher synaptic plasticity and activity in the visual cortex than in the cerebellum of the long-finned pilot whale. The visual cortex and the cerebellum seem to make use of different GABA receptor types (McKernan and Whiting 1996). However, GABAeric inhibitory signaling is relevant in both brain regions.

High capacity of aerobic metabolism in the whale brain

Some hypoxia-tolerant animals use extreme hypometabolism to protect their neuronal network. For example, the anoxic freshwater turtle can reduce the most energy demanding processes like excitatory neurotransmitter release, action potentials, and ion flow, thereby entering a coma-like state (Milton and Lutz 1998, Milton et al. 2002, Thompson et al. 2007). However, the whale brain needs to remain functional, at least at some minimum level, for hunting and communicating when submerged. Marine mammals have evolved a number of mechanisms that allow them to tolerate

extended periods of breath-hold diving. Besides elevated hemoglobin and myoglobin concentrations, whales also undergo peripheral vasoconstriction and hypometabolism (Kooyman and Ponganis 1998, Ramirez et al. 2007, Scholander 1940). This results in a redistribution of the blood flow from the peripheral organs towards the more hypoxia-sensitive tissues, like the brain. However, towards the end of long dives the oxygen tension in arterial blood of diving seals drops to 10-20 mmHg(Kerem and Elsner 1973, Meir et al. 2009, Qvist et al. 1986, Williams et al. 1999), corresponding to the threshold for normal brain function in terrestrial mammals (Erecinska and Silver 2001), which implies a high intrinsic hypoxia tolerance of the brain. In vitro studies of brain slices from the hooded seal (*Cystophora cristata*), which can dive for more than 1 h, showed that seal neurons survive much longer under hypoxia than do mouse neurons (Folkow et al. 2008, Ramirez et al. 2011). No such experiments have been conducted in cetaceans, however conformance in physiological adaptations together with similar maximum dive durations in seal and whale species like hooded seal and sperm whale (*Physeter macrocephalus*) imply similar neuronal hypoxia tolerance levels. The balance between metabolic costs and maintaining functional integrity is a major challenge for the brain. In terrestrial mammals acute hypoxia prevents oxidative phosphorylation and anaerobic glycolysis takes over the production of ATP (Kerem and Elsner 1973). The brain of the hooded seal shows a specific hypoxia-adaptation of the brain and a reverse astrocyte-neuron lactate shuttle (ANLS) has been proposed, which predicts that astrocytes work largely aerobically while the neurons rely primarily on anaerobic metabolism (Mitz et al. 2009, Schneuer et al. 2012). Transcriptome analyses using hooded seal brain samples suggested a reduced aerobic energy metabolism, possibly implying the existence of a general energy saving mechanism (Fabrizius et al. 2016). No indication of a similar downward shift in the aerobic metabolism was found in the brain of minke whale and harbour porpoise

(*Phocoena phocoena*) (Schneuer et al. 2012), again suggesting divergent evolutionary strategies in seals and whales.

We compared transcriptomes of the visual cortex as well as of different brain regions of whale and cattle. Both comparisons showed similar results, with enriched gene expression of transcripts involved in oxidative phosphorylation and respiratory electron chain in the whale brains (Figure 2 and 4). An enhanced cerebral aerobic capacity may possibly reflect an adaptation to cope with the reoxygenation event when the whale resurfaces, at which time it will be presented with a high oxygen availability. The enhanced oxidative capacity might, thus, prepare the brain for rapid energy production after the dive. Recently, a similar adaptation in the brain of hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) was found and it was suggested that an increased oxidative capacity of the mitochondria may be needed to meet the high energy requirements during arousal from torpor (Ballinger et al. 2017). The high oxidative capacity in the whale brain might possibly also help neuronal survival under hypoxic conditions by improving the efficiency of ATP production. For example, the high expression of several Cytochrome c oxidase (COX) subunits (Table 4), the final enzyme in the respiratory electron transport chain, might help to improving efficiency of electron transfer when less oxygen is available (Allen et al. 1995). The regulatory mechanisms of COX expression in mammals are not well understood. However, COX subunit IV has been shown to regulate COX activity and oxygen-dependent expression indicates an oxygen sensing role in the brain (Gnaiger et al. 1998, Horvat et al. 2006, Lau et al. 2017, Vijayasarathy et al. 2003). High expression of specific COX subunits in the whale brain might help to improve the efficiency of ATP production under hypoxia, and thereby prevent neuronal damage. Further studies of components of the respiratory electron transport chain in the whale brain are required to test such hypotheses.

High expression of antioxidant enzymes in the whale brain

Enhanced oxidative capacity combined with reoxygenation events during resurfacing are likely to lead to an increased ROS production (Boveris and Chance 1973, Brand 2010, Murphy 2009). Accordingly, we found the Reactome pathway "detoxification of reactive oxygen species" enriched in the transcripts that were more highly expressed in the visual cortex of whale than in the cattle cortex (Figure 2). Three of those transcripts (PRDX6, SOD1 and GSTP1) were also significantly higher expressed in the whale brain when comparing all available whale and cattle transcriptomes (Figure 5). The high expression of antioxidant genes in the whale brain could protect the neuronal cells and minimize damage from ROS and these genes may serve as candidates for functional analyses. These findings are consistent with the recent discovery of cetacean-specific amino acid changes in several antioxidant proteins, as well as of enrichment in the functional category antioxidant activity (GO:0016209) in the minke whale genome (Yim et al. 2014).

Conclusions

Our results provide a first insight into molecular mechanisms of hypoxia tolerance in the whale brain. The focus of this study was the analysis of its baseline gene expression. The enriched gene expression of transcripts involved in oxidative phosphorylation in the whale brain may reflect an adaptation to varying oxygen availability (diving vs resurfacing) and changing energy demands. However, the gene expression changes of the diving whale brain under hypoxic conditions (i.e. during a dive) remain unknown. Transcriptome analyses of whale brain slices subjected to

hypoxia, and functional analysis of candidate genes, could provide further insight into such adaptations.

Declarations

Ethics approval

All whale species are protected in CITES (Appendix II, EU Annex A) and the appropriate permits have been obtained from National (*Bundesamt für Naturschutz*) and foreign authorities (Permit number: E-01456/17). Also, the regulations of the "Convention on Biological Diversity"/Nagoya protocol will be considered, involving documentation of the legal acquisitions of genetic material and the sustainable use of species.

Consent for publication

Not applicable

Availability of data and material

The raw Illumina files of the transcriptomes from the visual cortex of the cattle (SRR8305676) and the killer whale (SRR8305677) and the visual cortex (SRR8305674) and the cerebellum (SRR8305675) of long-finned pilot whale are available from the NCBI SRA database under the Bioproject PRJNA506903. Mammalian brain transcriptomes were retrieved from the NCBI SRA

database (accession numbers SRX790347, SRX313597, SRX211675, SRX211674, SRX196362, SRX196353 SRX2585929, SRX2585928). All other data is provided in the Supporting Information.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Conception and design of the experiments: TB, AF. Analysis and interpretation of data: AK, AF, TB. Drafting of the manuscript: AK, TB. Acquisition of data and material: AF, BM, US. Analysis and interpretation of data: AK, AF, TB. Critical revision of the manuscript for important intellectual content: TB, LPF. Administrative, technical, and material support: AF, BM, US. All authors read and approved the final manuscript.

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Additional Files

Additional File 1: Gene expression (RPKM values) in whale and cattle brain samples. Format: xlsx

Additional File 2: Gene expression (RPKM values) in beluga whale samples. Format: xlsx

Additional File 3: Transcripts used for PANTHER analysis with renamed Ids. Format: xlsx

Additional File 4 Table S1: Most highly expressed genes in the brain of whale (A) and cattle (B). Format: PDF

Additional File 5: Gene expression (RPKM values) of differentially expressed genes in visual cortex and cerebellum samples of the long-finned pilot whale. The cutoff was set to RPKM Fold change > 2 and RPKM > 5 in the respective brain region. Format: xlsx

Additional File 6: Gene expression (RPKM values) of differentially expressed genes in visual cortex samples of the long-finned pilot whale and the killer whale compared to visual cortex samples of cattle. The cutoff was set to Fold change of normalized unique gene reads > 2, Range of normalized unique gene reads ≥ 5, Bonferroni corrected p-value < 0.05. Format: xlsx

Additional File 7: Gene expression (RPKM values) of differentially expressed genes in whale and cattle brain samples. The cutoff was set to Fold change of normalized unique gene reads > 2, Range of normalized unique gene reads ≥ 5, Bonferroni corrected p-value < 0.05. Format: xlsx

Additional File 8 Figure S1: Correlation of gene expression between mammalian brain transcriptomes. The correlation coefficients were converted into distances and visualised by a neighbour-joining tree. Format: PDF

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Figure Captions

Figure 1

Enriched GO-Slim terms in the visual cortex vs. cerebellum of the long-finned pilot whale

Significantly enriched GO-Slim terms for the gene set higher expressed in the visual cortex (black) and the cerebellum (grey) of the long-finned pilot whale for the category Biological Process according to their Fold Enrichment, with corrected *p*-values and the number of genes. (Note: JNK cascade is defined as intracellular protein kinase cascade containing at least a JNK (a MAPK), a JNKK (a MAPKK) and a JUN3K (a MAP3K).)

Figure 2

Enriched GO-Slim terms in the combined average of the visual cortex of killer whale and long finned pilot whale

Significantly enriched GO-Slim terms of the category Biological Process (black) and Reactome pathways (grey) for the gene set significantly higher expressed in the visual cortex of the long-

finned pilot whale and the killer whale compared to the visual cortex of cattle according to their Fold Enrichment, with corrected *p*-values and the number of genes.

Figure 3

Principal component analysis of whale and cattle transcriptomes

Principal component analysis (PCA) of RNA-Seq results (transcript expression) of different species of whale (black dots) and cattle (empty dots). PCA was performed to check for possible differences in global transcript expression between whale and cattle samples and to ensure comparability within groups. Note the beluga whale shows a distinctly different transcript expression and thus was not used for the further analysis of whales versus cattle transcriptomes.

Figure 4

Enriched GO-Slim terms in the whale brain

Significantly enriched GO-Slim terms for the gene set significantly higher expressed in whale brain compared to the brain of cattle for the category Biological Process according to their Fold Enrichment with corrected *p*-values and the number of genes.

Figure 5

Candidate genes for functional analysis

RPKM values of transcripts enriched in the visual cortex of whale in the Reactome pathway "detoxification of reactive oxygen species" (Table 5, Figure 2) are shown for all whale and cattle brain samples. Transcripts of peroxiredoxin-6 (PRDX6), superoxide dismutase (SOD1), neutrophil cytosol factor 2 (NCF2), glutathione peroxidase (GPX2), cytochrome c (CYCS), glutathione S-transferase P (GSTP1) and thioredoxin (TXN2) were significantly enriched in the

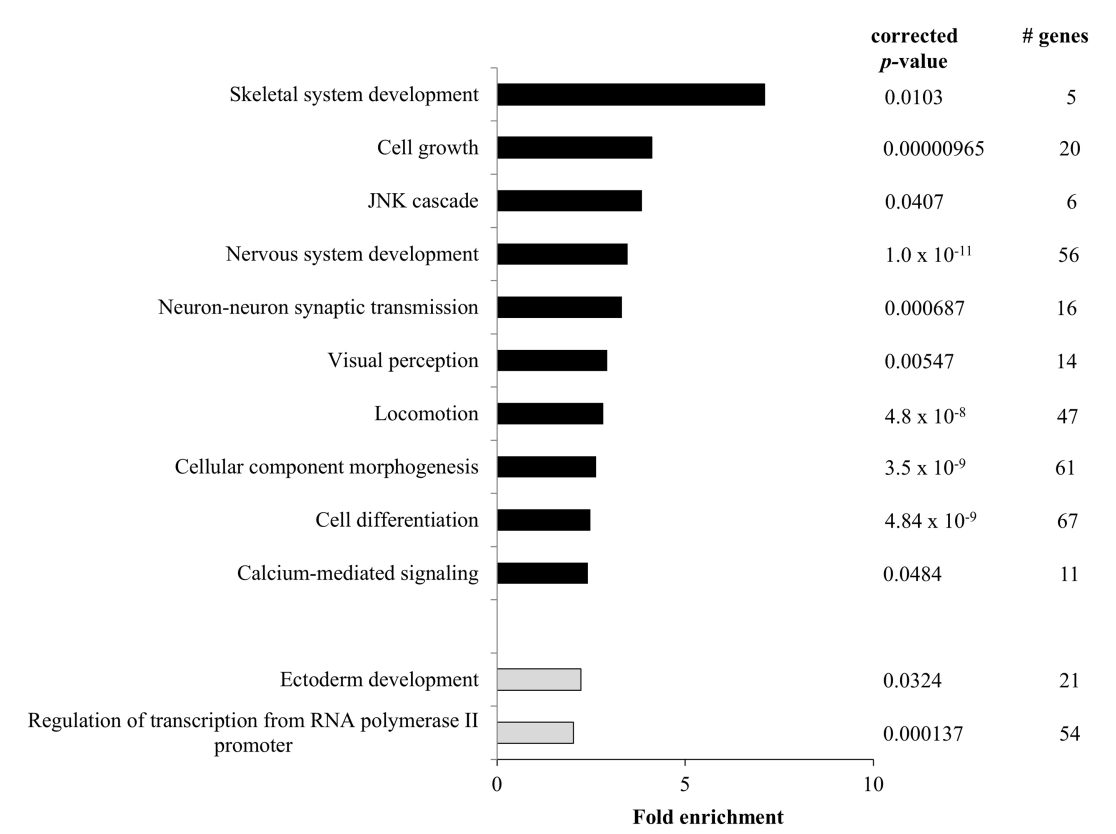
visual cortex of killer whale and long-finned pilot whale. PRDX6, SOD1 and GSTP1 were significantly enriched in all whale samples.

Bta = *Bos taurus*; Bac = *Balaenoptera acutorostrata*; Bmy = *Balaena mysticetus*; Gme = *Globicephala melas*; Gme = *Globicephala melas*; Oor = *Orcinus orca*;; ; ce = cerebellum; fl = frontal lobe; vc = visual cortex;

Highlights

- The brain of diving mammals is repeatedly exposed to low oxygen conditions (hypoxia) that would cause severe damage to most terrestrial mammals.
- An RNA-Seq approach was used to compare the mRNA levels in the brains of several species of whales with those of the cattle.
- Transcriptome analysis revealed a high expression of genes related to oxidative phosphorylation and the respiratory electron chain in the whale brain.
- In the visual cortex of whales, transcripts related to the detoxification of reactive oxygen species were higher expressed than in the visual cortex of cattle.

Graphical abstract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Cell growth
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20
JNK cascade
0.0407
6
Nervous system development
1.0 x 10-11
56
Neuron-neuron synaptic transmission
0.000687
16
Visual perception
0.00547
14
Locomotion
4.8 x 10-8
47
Cellular component morphogenesis
3.5 x 10-9
61
Cell differentiation
4.84 x 10-9
67
Calcium-mediated signaling
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Ectoderm development
0.0324
21
Regulation of transcription from RNA polymerase II promoter
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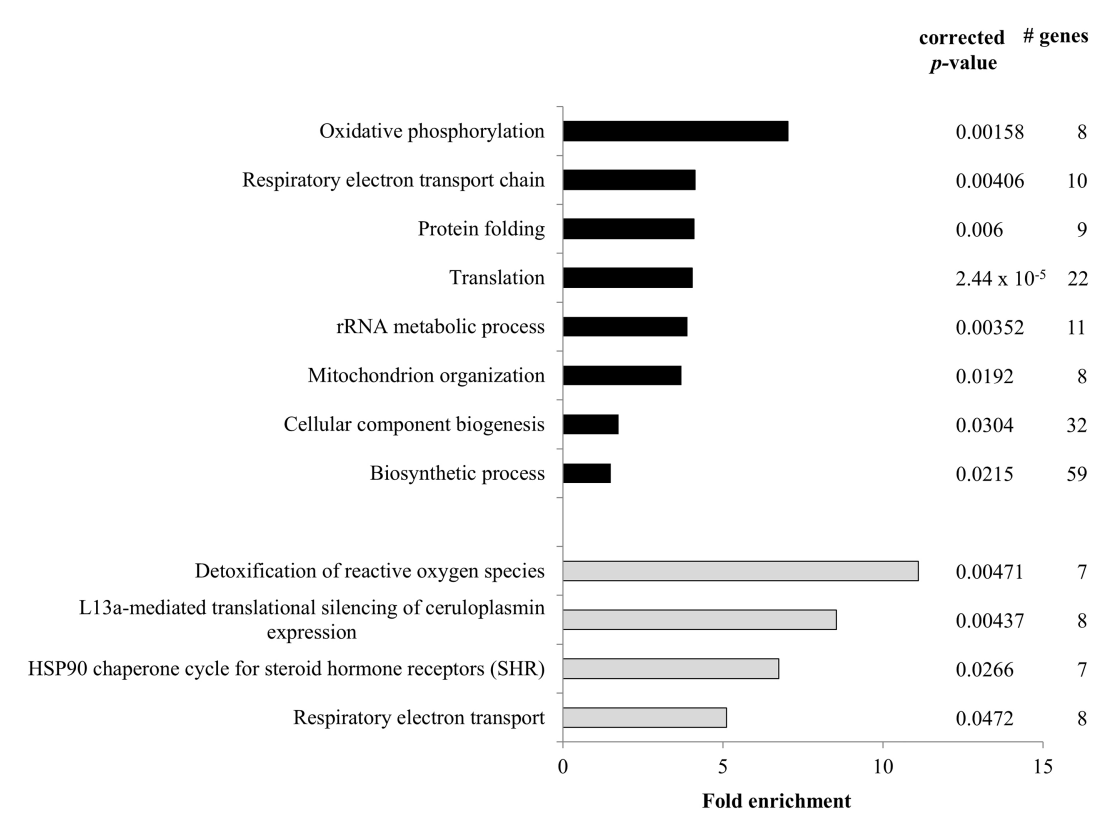
54
0
5
10
Fold enrichment

Figure 1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Respiratory electron transport chain
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10
Protein folding
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9
Translation
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22
rRNA metabolic process
0.00352
11
Mitochondrion organization
0.0192
8
Cellular component biogenesis
0.0304
32
Biosynthetic process
0.0215
59
Detoxification of reactive oxygen species
0.00471
7
L13a-mediated translational silencing of ceruloplasmin expression
0.00437
8
HSP90 chaperone cycle for steroid hormone receptors (SHR)
0.0266
7
Respiratory electron transport
0.0472
8
0
5
10
15
Fold enrichment

Figure 2

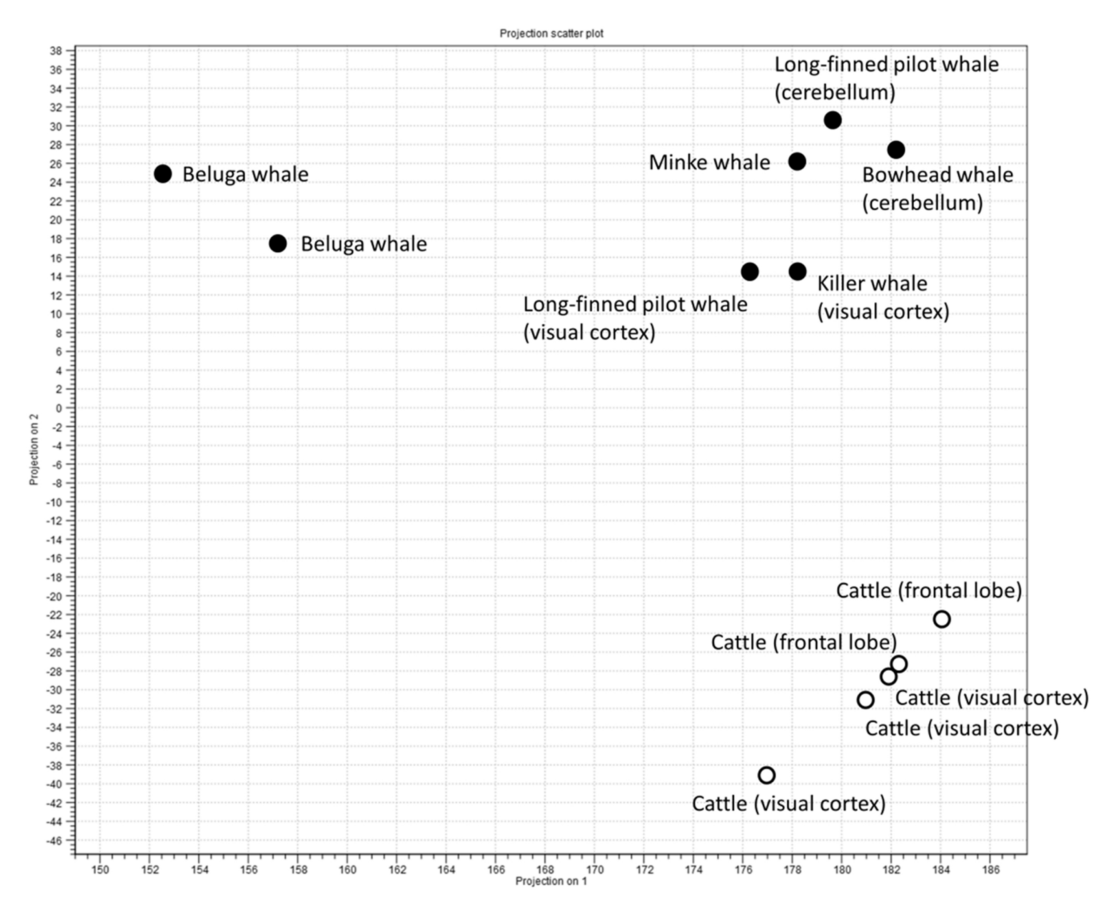
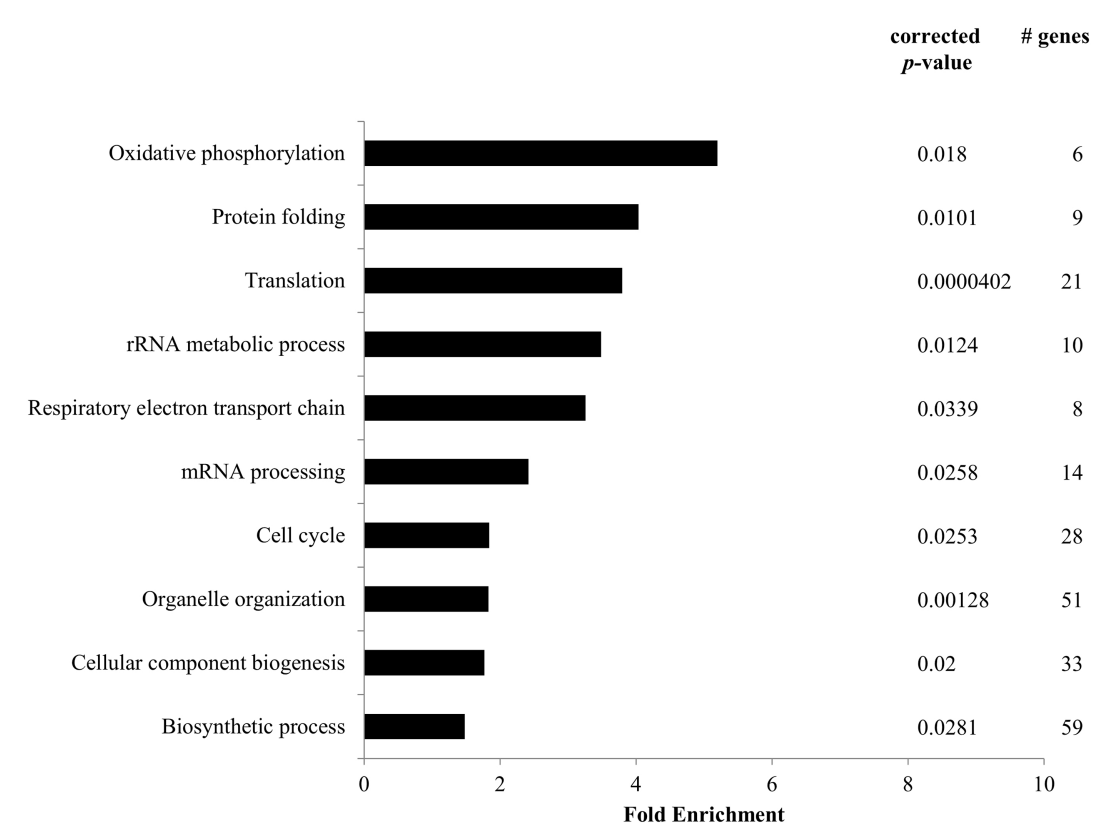

Projection scatter plot
Long-finned pilot whale (cerebellum)
Minke whale
Bowhead whale (cerebellum)
Beluga whale
Beluga whale
Killer whale (visual cortex)
Long-finned pilot whale (visual cortex)
Cattle (frontal lobe)
Cattle (frontal lobe)
Cattle (visual cortex)
Cattle (visual cortex)
Cattle (visual cortex)
Projection on 1
Projection on 2

Figure 3


corrected p-value
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Protein folding
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Translation
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10
Respiratory electron transport chain
0.0339
8
mRNA processing
0.0258
14
Cell cycle
0.0253
28
Organelle organization
0.00128
51
Cellular component biogenesis
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33
Biosynthetic process
0.0281
59
0
2
4
6
8
10
Fold Enrichment

Figure 4

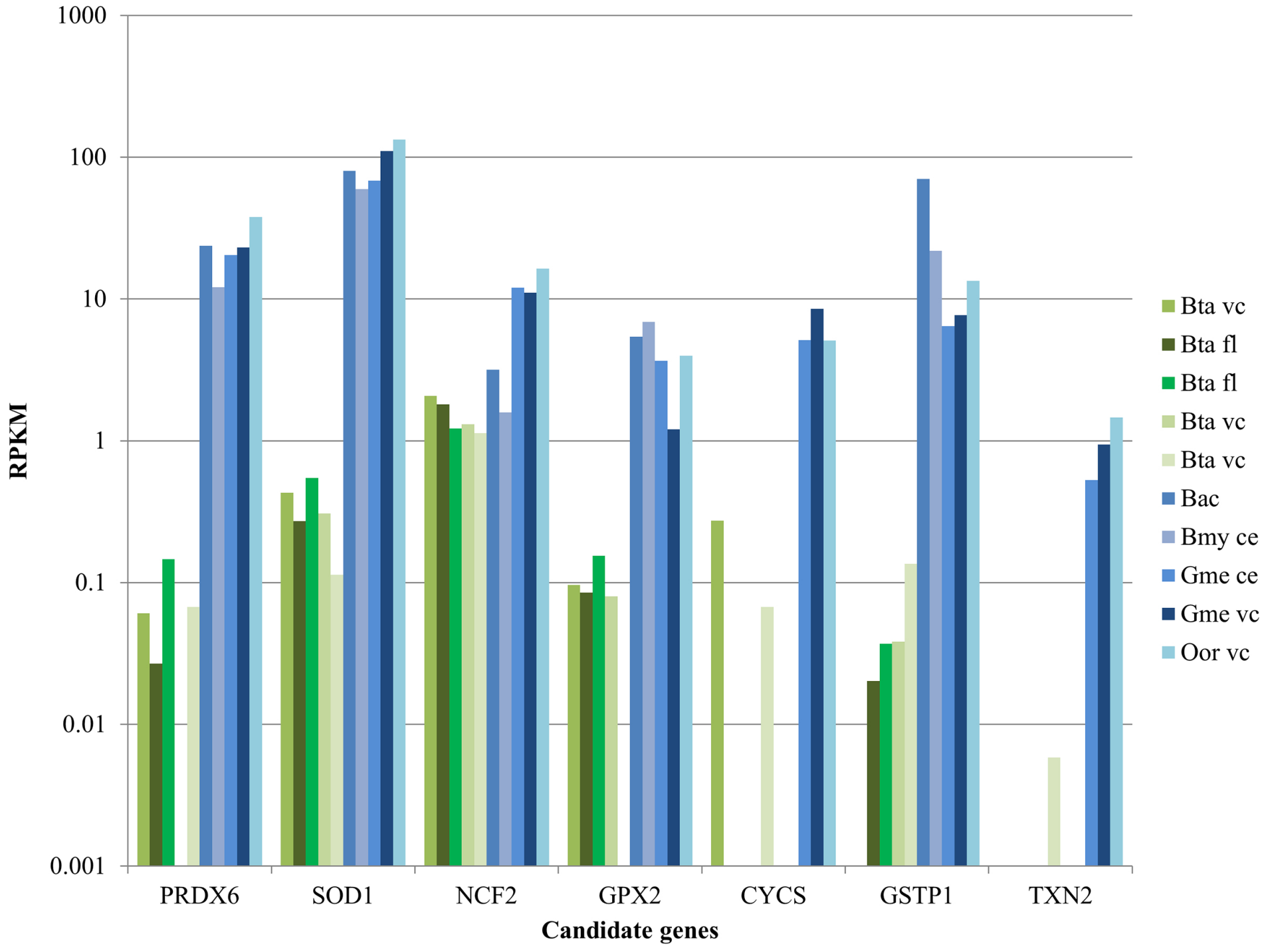
1000
100
10
1
0.1
0.01
0.001
RPKM
PRDX6
SOD1
NCF2
GPX2
CYCS
GSTP1
TXN2
Candidate genes
Bta vc
Bta fl
Bta fl
Bta vc
Bta vc
Bac
Bmy ce
Gme ce
Gme vc
Oor vc

Figure 5