RESEARCH ARTICLE

RUNNING HEAD: Captivity effect on shrew brain gene expression.

Large captivity effect based on gene expression comparisons between captive and wild shrew brains

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ABSTRACT

Compared to their free-ranging counterparts, wild animals in captivity are subject to different conditions with lasting effects on their physiology and behavior. Alterations in gene expression in response to environmental changes occur upstream of physiological and behavioral phenotypes, but there are no experiments analyzing differential gene expression in captive vs. free-ranging mammals. We assessed gene expression profiles of three brain regions (cortex, olfactory bulb, and hippocampus) of wild juvenile shrews (*Sorex araneus*) in comparison to shrews kept in captivity for two months. We found hundreds of differentially expressed genes in all three brain regions, suggesting a large and uniform captivity effect. Many of the downregulated genes in captive shrews significantly enrich pathways associated with neurodegenerative disease ($p<0.001$), oxidative phosphorylation ($p<0.001$), and genes encoding ribosomal proteins ($p<0.001$). Transcriptomic changes associated with captivity in the shrew resemble responses identified in several human pathologies, such as major depressive disorder and neurodegeneration. Thus, not only does captivity impact brain function and expression, but captivity effects may also confound analyses of natural physiological processes in wild individuals under captive conditions.

NEW & NOTEWORTHY

To our knowledge, this is the first study that identifies a captivity effect on brain transcriptomic profiles in a mammalian species, identifying 4,094 differentially expressed genes ($p<0.05$) in at least one brain region with implications for experimental comparisons.

Keywords: Captivity, brain, shrew, transcriptomics.

INTRODUCTION

Wild animals placed in captivity are subject to a variety of changed conditions in comparison to their free-ranging counterparts. Environmental conditions such as shelter (1), movement space, diet, climate, lighting (1–3), social factors (4), and the presence of humans (1) vary drastically between captive and wild. Organisms can rapidly respond to these changes through behavioral, physiological, biochemical, and molecular mechanisms, however, many of these responses may occur at different time scales (5). For example, even short-term habituation to captive conditions can affect brain morphology and function, endocrine status (6), immune function (3), and reproductive pathways (7). But even when animals are unharmed (2) long-term habituation to captivity can have negative consequences, including the activation of multiple stress responses (1, 3, 6, 8). Symptoms of chronic stress can also appear because of frequent exposure to perceived threats (2) that together with reduced environmental stimulation can lead to depressive-like states in captive animals (9). Thus, despite the perception of a comfortable and controlled environment associated with captivity, these conditions create a myriad of responses, some of which appear to be detrimental to the now captive individual.

Altered gene expression likely induces phenotypic responses to captivity, as it is one of the fastest responses to environmental change (5), upstream of most biochemical, physiological, and behavioral phenotypes (10, 11). Long-term environmental changes also lead to lasting changes in gene expression, playing a central role in cellular adaptation with extensive transcriptional and post-transcriptional regulation (12). Although some studies in mammals have addressed the effects of captivity in a wide range of these downstream phenotypes, including morphology (13, 14), longevity (15), hematology (16), gut microbiome (17, 18), torpor pattern (19), adrenal function, and stress (20, 21), to our knowledge, there are no experiments analyzing gene expression divergence in captive mammals. This is of particular interest to modern research, as many ecological and clinical genetic experiments are performed on mammals in laboratory settings and may yield different results from those obtained in the field (6, 22). Thus, research on the effects of captivity on mammalian gene expression is important, as failing to acknowledge these effects could confound the effects of captivity with those of phenotypes of interest.

Sorex araneus, the common shrew, is an ideal candidate for elucidating the potential effects of captivity on gene expression. While *S. araneus* has been an organismal model for speciation and chromosomal rearrangements over the past 50 years, it has also gained relevance in neuroscience for its seasonal reversible change in brain size, or Dehnel's phenomenon (23). These research lines have helped build many resources and methods to identify captivity effects. First, the common shrew was one of the first species of mammals selected for a complete genome sequence and more detailed genomic coverage as well as studies on functional genome components by the Broad Institute and the ENCODE project (24). Additionally, there is now a shrew brain atlas with region-specific gene expression profiles (25), further facilitating research. Finally, natural seasonal changes in shrew brain and skull size can be modified by environmental conditions, with shrews exposed to constant temperature in captivity ceasing to express

Dehnel's phenomenon, showing a steady decline in skull size and lack of regrowth (26). This environmentally induced plasticity indicates the vast potential for captivity associated changes, making the shrew an ideal candidate for discovering transcriptomic brain changes stemming from captivity.

We assessed the brain gene expression profiles of wild fall juvenile shrews (*Sorex araneus*) in comparison to same age shrews held captive for two months. This allowed us to determine if there is a captivity effect that should be considered in future studies on genes underlying physiological phenomena. We outline strong differences in brain gene expression between wild and captive shrews detected using differential gene expression analysis of mRNA. Our results support a captivity effect in the shrew brain, and we highlight similar responses found in studies of metabolism, neurodegenerative diseases, and depression.

MATERIALS AND METHODS

Shrew Collection

All experimental procedures were carried out according to guidelines for the care and use of animals approved by the Regierungspräsidium Freiburg, Baden-Württemberg (captive: 35-9185.81/G-19/131, wild: 5-9185.81/G-20/24). Shrews were caught in Liggeringen, Germany, using wooden live traps and mealworms as bait, as described previously by Lázaro et al. (2018) (27) . Captive juvenile shrews (n=4, all male) were caught in September of 2020 and housed individually for two months at the Max Planck Institute of Animal Behavior before samples were taken. Captive shrews were allowed to move freely between two connected cages, the first containing a running wheel, bowls of food and water, a nesting pot, with the second connected cage consisting of several layers of soil and hay (detail on housing in Lázaro et al., 2019 (26)). Captive shrews were kept at ambient temperature and provided a daily diet of 5g of raw meat and 2g of mealworm. Wild juvenile shrews (n=4, two female and three male) were caught in November and sampled the same day of capture.

Tissue Sampling, RNA Extraction, and Sequencing

Shrews were anesthetized and euthanized under deep anesthesia by perfusion of the vascular system with PAXgene Tissue Fixative (perfusion procedure described in Lázaro et al., 2018 (27)). The brain was extracted immediately, dissected into individual regions, and submerged in cold fixative. In this experiment, three brain regions were compared between captive and wild individuals: cortex, olfactory bulb, and hippocampus. These brain regions were held in fixative within 24 hours, then switched to PAXgene Tissue Stabilizer and frozen for long term preservation in liquid nitrogen. RNA was extracted from each region using a modified Qiagen Micro RNAeasy protocol designed to retrieve RNA from small sensory tissues (28). Quality control, library preparation, and sequencing were conducted by Genewiz. RNA integrity numbers (RIN) were measured with RNA ScreenTape and samples below a RIN of 6 were removed from the experiment. After library preparation, mRNA were selected by polyA enrichment followed by paired-end sequencing with Illumina HiSeq, with a targeted depth between 15-25 million reads per sample.

Bioinformatics

Before analyzing differential expression, sequencing results were assessed for both quantity and quality. Samples with low sequencing depths (<20 million reads) were removed from the experiment. Adapters were trimmed and low-quality reads removed with fastp version 0.20.1 (29). Reads were mapped by

pseudo-alignment to the *S. araneus* reference transcriptome (GCF_000181275.1_SorAra2.0_rna) using Kallisto version 0.46.1 (30). Samples with mapping percentage below 45% were removed. Read counts were then normalized for library size and content using the median of ratios from DESeq2 version 1.36.0 (31). To visualize the overall effect of captivity within brain regions and to verify how samples grouped, we ran a principal component analysis (PCA) of the 500 most varying genes for each brain region using variance stabilizing transformed (VST) read counts (32). Differential gene expression was analyzed and visualized in R (version 4.2.0) (33) with DESeq2, which tested for differential expression between captive and wild individuals using negative binomial generalized linear models (32) (Love et al., 2023).

Brain region comparisons between captive and wild individuals were normalized and tested for differential gene expression separately. Statistical significance for each region comparison was determined by conducting a Wald test and adjusting p-values for multiple testing using the Benjamini and Hochberg method (34). All genes with an adjusted $p < 0.05$ were judged to be differentially expressed, regardless of log-fold change. Genes identified as differentially expressed in all three brain regions were separated by direction (upregulated and downregulated). Pathway enrichment analysis was carried out for both directional gene lists using Database for Annotation, Visualization and Integrated Discovery (DAVID) (35, 36), with *Mus musculus* as the reference species and selecting results for KEGG pathways. Gene enrichment according to the KEGG annotations were measured using a Modified Fisher's Exact test.

RESULTS

We analyzed 24 samples, three brain regions each from four captive and four wild juvenile *S. araneus*. RNA extraction and sequencing data were determined to be of satisfactory quantity and quality to study potential captivity effects on regional brain expression. RINs ranged from 6.6-8.6 in all brain regions; cortex (6.7-8.6, mean: 7.6), hippocampus (7.1-8.4, mean: 7.9), and olfactory bulb (6.6-8.5, mean: 8.0). This RNA was sufficient in quality to produce high quality libraries, which produced 26.5-79.7 million reads per sample; cortex (38.4-79.7, 49.0 mean), olfactory bulb (26.5-58.6, 41.3 mean), and hippocampus (32.1-54.7, 41.3 mean). To reduce the number of lower quality reads in the dataset, we filtered reads based on fastp default parameters. Between 228,172 and 2,353,398 reads were removed per sample, with an average of 979,721. This increased the quality and reliability of our data, such that all reads had 87% of bases with Qscores ≥ 30. These reads were mapped to the *S. araneus* reference transcriptome, with 48.63-60.45% of the processed reads pseudo-aligned to the reference transcriptome. Both quality scores and mapping rates indicate sequences produced by the 24 samples are of sufficient quality, granting confidence in subsequent comparisons.

We identified divergences in the expression profiles of all three brain regions associated with a potential captivity effect. For each brain region, we used the 500 genes with the most variation to run a PCA, which produced two principal component axes that accounted for most of the variation from these genes (Figure 1). This analysis separated each brain region into two distinct clusters along the first principal component, with divergence between each cluster apparently associated with captivity. The first principal component separated wild from captive individuals, accounting for 42% of the variation in gene expression in the hippocampus and 47% in the olfactory bulb. Percent variation expressed was even greater in the cortex, where the first principal component accounted for 63% of the variation. These results suggest captivity may have major functional impacts across the brain, especially in the cortex, which appeared to be the most perturbed by captivity.

Differential expression analysis confirmed transcriptomic differences in the brain of captive and wild shrews, further emphasizing the captivity effect on neural gene expression (Figure 1). Of the 19,296 genes analyzed, we identified 4,094 differentially expressed genes (DEGs; $p<0.05$) in at least one brain region (Figure 2). In the cortex, we found a total of 3,037 DEGs; 1,436 were upregulated in captive shrews and 1601 downregulated in captive shrews. Compared to the olfactory bulb and hippocampus, the cortex had the most DEGs, validating PCA results that suggested a larger effect of captivity in cortex. The olfactory bulb had 1,892 divergent DEGs, with 910 upregulated and 982 downregulated in captive animals. In the hippocampus, we identified 1,702 DEGs, with 704 upregulated and 998 downregulated in captive animals. We compared lists of DEGs between brain regions and found 803 genes that were differentially expressed across all regions, with another 931 overlapping in two of three regions (Figure 2). These results suggest both a large captivity effect across the shrew brain, with further validation of greater changes in the cortex, and parallel effects may also exist among the cortex, hippocampus, and olfactory bulb.

Pathway enrichment analysis revealed the captive effect on gene expression is highly linked to ribosomal and mitochondrial function, with potential associations to neurodegenerative processes (Figure 3A). We identified 27 biological pathways significantly enriched (modified Fisher's Exact Test) in the 686 annotated overlapping differentially expressed genes, with the largest enrichment in the Ribosome pathway (p value: 1.7×10^{-46}, n= 58 genes). As the core function of ribosomes is to translate messenger RNA during protein formation, this pathway has many potential cascading effects. For example, 49 of the genes found in this pathway were also found in the second most enriched pathway, COVID-19 (p value: 3.8×10^{-30}, n= 51 genes), corroborating the vast effects ribosomal DEGs can induce. Within enriched pathways, we also identified captivity-associated expression changes that likely affect mitochondrial function in the brain. Strikingly, 22 genes encoding mitochondrial complex I NADH:ubiquinone oxidoreductase subunit proteins (the largest enzyme of the respiratory chain), were found in 12 out of the 27 enriched pathways suggesting a captivity induced change in shrew energy metabolism (Figure 3B). Six of the pathways containing NADH:ubiquinone oxidoreductase subunit proteins were associated with neurodegenerative diseases, including Parkinson's disease (p value: 1.5×10^{-13}, n= 34 genes), amyotrophic lateral sclerosis (p value: 4.2×10^{-12}, n= 38 genes), pathways of neurodegeneration-multiple diseases (p value: 9.7×10^{-11}, n= 41 genes), prion disease (p value: 1.3×10^{-12}, n= 33 genes), Huntington's disease (p value: 4.5×10^{-14}, n=37 genes), and Alzheimer's disease (p value: 4.8×10^{-8}, n= 32 genes). This suggests captivity induced changes to mitochondrial function and metabolism in the brain that resemble processes underlying neurodegeneration.

DISCUSSION

Faced with environmental change, organisms react through behavioral, physiological, biochemical, and molecular responses (5), including changes in gene expression (37). In this study, we assessed the molecular responses to a major shift in environment, captivity, and found drastic expression changes in the brain. We identified thousands of differentially expressed genes in the cortex, olfactory bulb, and hippocampus, and although the cortex experienced the most significant divergence, similar shifts in expression between regions suggest parallel responses to captivity. Parallel responses include the downregulation of genes associated with ribosome production and function, as well as 22 genes encoding for mitochondrial complex I NADH:ubiquinone oxidoreductase subunit proteins. Changes in expression of mitochondrial complex I genes, which play an important role in energy production (38), have been linked to depression and anxiety-related behavior mice (39–41), suggesting a similar phenotype for shrews in

captivity conditions potentially related to a dysregulation of energy metabolism. Overall, these findings indicate a significant captivity effect in the brain, highlighting the importance of research on physiological processes in wild individuals, especially when linking results to neurodegenerative diseases.

To our knowledge, despite vast interest in studying diverse wild species outside of standard laboratory environments in neuroscience (22, 42–48) we conducted the first study that identifies a captivity effect on brain transcriptomic profiles in a mammalian species (Figure 1). This captive effect in shrew brain transcription corroborates previously identified gene expression responses in a limited, yet diverse number of organisms and tissues, including the brains of cane toad and house sparrows (7, 49) freshwater mussel gill (50), zooplankton (51), and the liver of an anoline lizard (52). While some studies have identified domestication-associated neural changes in subsets of mammals (53–55) many have focused on other sources of environmental change such as hypoxia (56, 57), metabolism (56, 58, 59), temperature, feeding behavior (60), stress (61), photoperiod (62, 63), and activity (64). And yet, no experiment analyzing expression divergence has been conducted on captive mammal brains, despite their use in clinical and ecological experimentation. Although captivity is routine in many experiments, effects on expression may parallel or directly counter many of the underlying responses to other forms of induced or natural environmental change. Thus, our identification of large expression changes in the brain after a brief stint in captivity highlights the importance of working with both wild and captive individuals to avoid confounding gene expression changes from phenotypes of interest with those associated with captivity.

Of the three brain regions tested, the cortex had the most differentially expressed genes in captivity, indicating an increased propensity for plastic responses to environmental change (Figure 1). Nearly twice as many differentially expressed genes between conditions were identified in the cortex (n=3037) compared to both the hippocampus (n=1,702) and olfactory bulb (n=1,892). The six-layered mammalian cortex, whose size, complexity, and circuitry are correlated with information processing and "intelligent behavior" (65–67), is a uniquely arranged mosaic of conserved and recently evolved neurons (68, 69). Greater captivity-associated differential expression of the shrew cortex suggests a shift in processing power, perhaps responding to the changes in stressors and stimulation of captive environments. Differences in stimulation may also suppress other region-specific functions, such as spatial memory or smell, resulting in the changes observed in the hippocampus and olfactory bulb. Despite many fewer DEGs in the hippocampus and olfactory bulb, 803 genes, ~22% of all DEGs identified in these two regions, overlapped with cortex DEGs. While the cortex appears to be most affected by captivity, similar changes in expression are occurring across all three brain regions.

We discovered downregulation of ribosomal protein genes in all three brain regions of the captive shrews, suggesting a potential for vast downstream effects. Ribosomes compose the cell's protein synthesis machinery, and their own synthesis, ribosomal biogenesis, is a highly regulated dynamic process that requires the assembly of subunits consisting of rRNA and ribosomal proteins (RP) (70, 71). In the captive shrew brain, parallel downregulation of 57 RPs enriching ribosomal pathways suggests impaired regulation of ribosomal biogenesis and subsequent functions. Ribosomal biogenesis constrains animal growth and development (60, 72, 73) by determining translational capacity of proliferating cells (74). Thus, dysregulation of ribosomal biogenesis is implicated in numerous phenotypes including neurite survival and synaptic function associated with human neurodegeneration (70). These effects appear to be mirrored in captive shrews, as we found enrichment of downregulated genes for pathways associated with neurodegeneration. Although neurodegeneration has been linked to changes in stress (75, 76), it is

important to consider that such transcriptomic phenotypes, or shifts in processes such as reduced synaptic efficiency, might be occurring because of captivity and not a disease.

Differential expression of ribosomal protein-coding genes in the brains of captive shrews may also have drastic effects on behavior. Many of differentially expressed ribosomal protein genes, a large gene family, have been highlighted in previous experiments for their regulatory changes associated with behavioral phenotypes including domestication and social defeat stress (55, 61). For example, comparisons between domestic rabbits and wild counterparts identified 612 differentially expressed genes, which also enriched ribosomal pathways, across four brain regions (55). Five of these genes (*RPS14*, *RPL23*, *RPL29*, *RPL32* and *RPL36*) overlapped ribosomal protein genes downregulated in captive shrews, indicating the underpinnings of both domestication and captivity may be similar. Changes in expression of ribosomal genes have also been associated with decreased proliferation in the hippocampus of mice under chronic social defeat stress, which has been linked to states like anxiety and depression (61). Captivity-associated differential expression of ribosomal proteins in the shrew brain may thus indicate a depressed neural state, especially as studies have argued that animals in captive environments often experience repeated stressors of the same kind used to induce depressive-like states in animal models (9). Overall, captivity effects appear to show similar patterns of brain expression to organisms in domesticated and in depressive-like states, as they all have been taken out of their natural environment and exposed to the conditions and stressors of confinement.

As with potential behavioral responses, captivity conditions such as altered diet and physical activity may also influence shrew energy metabolism. In captive shrews, we identified the downregulation of mitochondrial complex I NADH:ubiquinone oxidoreductase subunit proteins, which is essential for energy production. Complex I is the first and largest enzyme complex of the electron transport chain (38, 77), it contributes to the transmembrane proton gradient used to synthesize ATP (38), and is also involved with the formation of reactive oxygen species (78). Impairments to complex I function are linked to different pathophysiological states, genetic diseases (77, 79–81), and related to several neurodegenerative disorders, leading to decline of energy production and increased production of reactive oxygen species (82–85). Downregulation of 22 genes encoding for mitochondrial complex I subunit proteins in the three shrew brain regions studied suggests a dysfunction of the oxidative phosphorylation (OXPHOS) system and dysregulation of energy metabolism as a parallel response in captivity. Notably, these genes have also been shown to be downregulated in the brains of patients with autism (86), and neurodegenerative diseases such as Parkinson's (85, 87), Huntington's (88), and Alzheimer's (89). Indeed, the effects of certain conditions found in captivity, such as reduced stimulation, could also modify OXPHOS subunit gene expression in association with neurodegeneration, as normal brains exposed to chronic sensory deprivation exhibit a downregulation expression pattern for OXPHOS subunits similar to downregulation patterns found in Alzheimer's brains (90).

As with differential ribosomal protein expression, mitochondrial dysfunction and changes in expression of complex I subunits have also been linked to depression phenotypes and stress-related behaviors. For example, mice transplanted with gut microbiota from patients with major depressive disorder (MDD) showed specific complex I subunit inhibition (40), including 5 genes (*NDUFA5, NDUFA6, NDUFB3, NDUFS4,* and *NDUFV3*) that were also downregulated in the three brain regions of captive shrews, suggesting similar underlying mechanisms related to a depressive state. Additionally, female mice bred for low activity (an anxiety-like behavior genetic model) showed upregulation of complex I subunit genes (39), including 13 genes downregulated in captive shrews (*NDUFA2, NDUFA4, NDUFA6, NDUFA7, NDUFA12,*

NDUFA13, NDUFB5, NDUFB6, NDUFB7, NDUFB9, NDUFC1, NDUFC2, and *NDUFS5*). These genes exhibit opposite expression patterns between low activity mice and captive shrews. However, other studies have found the directionality of changes in mitochondrial gene expression related to anxiety-like behavior and after stress may depend on stressor duration, genetic background, and strain of the studied model. Nevertheless, such shifts still point to mitochondrial pathway dysregulation as a major brain stress response (41). Thus, the differential expression of complex I subunit genes observed further supports a phenotype associated with depression and stress in captive shrews.

Conclusion

Despite attempts to limit differential stressors and optimize holding conditions, our results reveal transcriptomic changes in the brain of juvenile shrews after being placed in a novel captive environment. These changes could have profound effects on phenotype, including shifts in energy metabolism, synaptic function, cell survival, and behavior. Captivity-associated changes in the shrew resemble responses that have been identified in several human diseases, most importantly, neurodegeneration. These changes are unlikely to be shrew-specific, as similar changes in expression have been found in other organisms experiencing changes in their natural environments. Thus, when investigating the underlying molecular mechanisms of disease using model organisms, captivity can clearly confound analyses of natural physiological processes. There are vast benefits to using captive organisms in disease research, but our results indicate future studies should make use of natural environments to illuminate brain function and responses to environmental change.

DATA AVAILABILITY

Sequencing archive link will be submitted and provided based on acceptance.

SUPPLEMENTAL MATERIAL

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Images were created with a licensed version of BioRender.com.

GRANTS

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DISCLOSURES

AUTHOR CONTRIBUTIONS

Conceived and designed research: William R. Thomas, Liliana M. Dávalos

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FIGURE LEGENDS

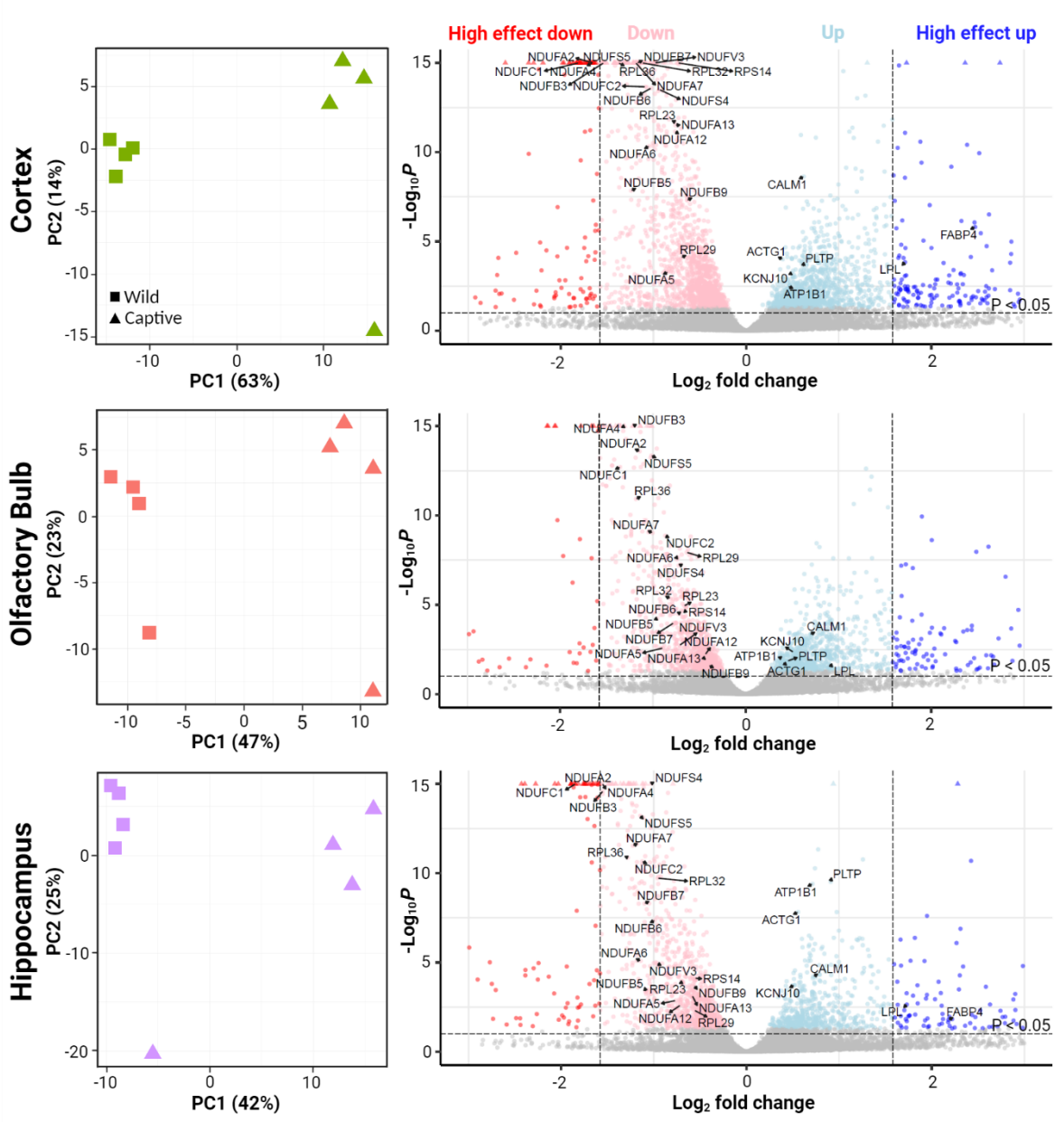

Figure 1.

Differential gene expression between wild (n=4) and captive (n=4) shrews in the cortex, olfactory bulb, and hippocampus. *Left:* principal component analysis of the top 500 varying. Axes show which percentage of the variance each component accounts for. *Right*: Volcano plots of the cortex, olfactory bulb, and hippocampus gene expression. Overlapping significant (p<0.05) DEGs are labeled. Labeled downregulated genes encode for ribosomal proteins and mitochondrial complex I subunit proteins, which have also been differentially expressed in previous studies. Labeled upregulated genes correspond to those associated with metabolism from pathway enrichment analysis, from Gastric acid secretion and PPAR pathways. Total number of genes: 19,296.

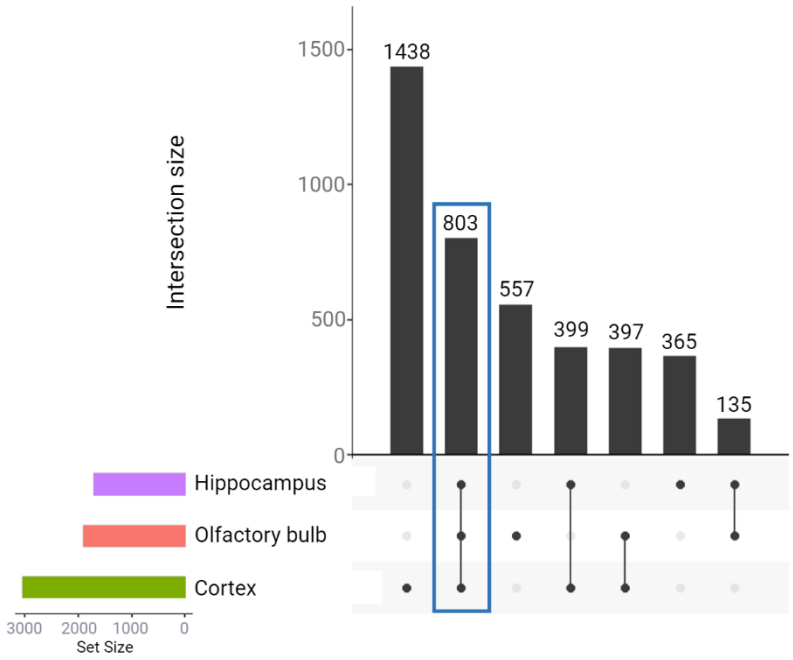

Figure 2.

Overlapping differentially expressed genes between the three brain regions. The side histogram corresponds to the number of differentially expressed genes for each brain region (set size), and the top histogram corresponds to intersections between the sets of DEGs across the three brain regions. All the 803 differentially expressed genes that overlap between the three brain regions (highlighted in blue) follow the same direction of gene expression (555 downregulated, 248 upregulated).

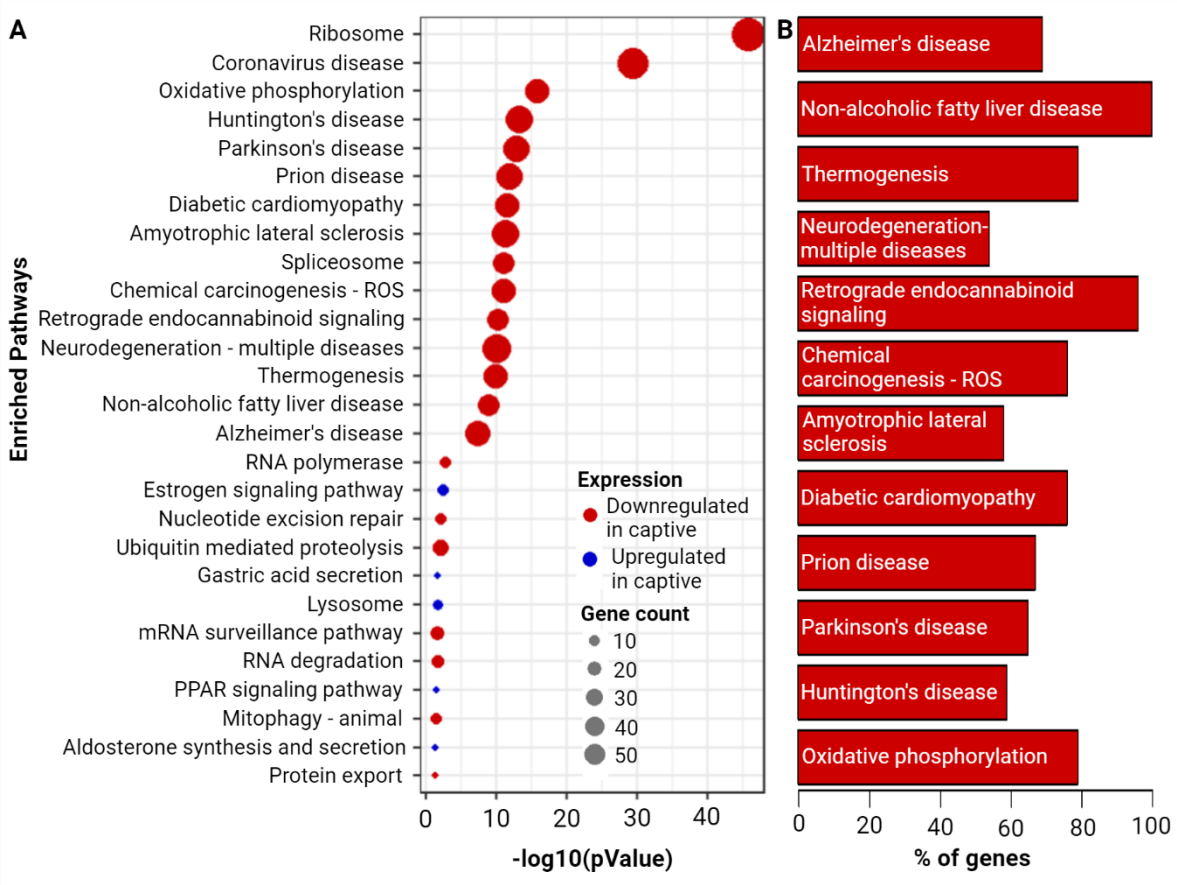

Figure 3.

Pathway analysis for the cortex, olfactory bulb, and hippocampus. *A*. Enriched pathways for overlapping DEG. *B*. Percentage of complex I NADH:ubiquinone oxidoreductase subunit genes in 12 of the enriched pathways.