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Hypothalamic transcriptome of tame and aggressive silver foxes (*Vulpes vulpes*) identifies gene expression differences shared across brain regions

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

Abstract

The underlying neurological events accompanying dog domestication remain elusive. To reconstruct the domestication process in an experimental setting, silver foxes (*Vulpes vulpes*) have been deliberately bred for tame *vs.* aggressive behaviors for more than 50 generations at the Institute for Cytology and Genetics in Novosibirsk, Russia. The hypothalamus is an essential part of the hypothalamic-pituitary-adrenal axis and regulates the fight-or-flight response, and thus, we hypothesized that selective breeding for tameness/aggressiveness has shaped the hypothalamic transcriptomic profile. RNA-seq analysis identified 70 differentially expressed genes. Seven of these genes, *DKKL1*, *FBLN7*, *NPL*, *PRIMPOL*, *PTGRN*, *SHCBP1L*, and *SKIV2L*, showed the same direction expression differences in the hypothalamus, basal forebrain, and prefrontal cortex. The genes differentially expressed across the three tissues are involved in cell division, differentiation, adhesion, and carbohydrate processing, suggesting an association of these processes with selective breeding. Additionally, 159 transcripts from the hypothalamus demonstrated differences in abundances of alternative spliced forms between the tame and aggressive foxes. WGCNA analyses also suggested gene modules in hypothalamus were significantly associated with tame *vs.* aggressive behavior. Pathways associated with these modules include signal transduction, interleukin signaling, cytokine-cytokine receptor interaction, and peptide ligand-binding receptors (e.g., G-protein coupled receptor [GPCR] ligand binding). Current studies reveal selection for tameness *vs.* aggressiveness in foxes is associated with unique hypothalamic gene profiles partly shared with other brain regions and highlight differentially expressed genes involved in such biological processes as development, differentiation, and immunological responses. The role of these processes in fox and dog domestication remains to be determined.

Keywords

Domestication; Canine; Evolutionary Selection; Genetics; Breeding; Dog; Brain; Hypothalamus; RNA-seq; Behavior

1 INTRODUCTION

Domestication of various animal species has been occurring historically for at least the last 10,000 years[1]. Domesticated species tend to show enhanced social affiliation but reduced fear and aggression towards humans and individuals within their own species[2–4]. Such changes are likely multifaceted in origin but may be accompanied by physiological and anatomical alterations, collectively referred to as “domestication syndrome”[5,6]. Domesticated dogs (*Canis familiaris*) evolved approximately 15,000 years ago from wolf ancestors (*Canis lupus*)[7,8]. In the process, stress responses became blunted, coat color pellage began to acquire unusual morphs, such as spotting, the tail went from being exclusively straight to sometimes curled, and the cranium became shortened with overall more rounding of the skull[9,10]. Additionally, differences in human-directed behavior of wolf and dog pups are observed. For instance, communication patterns began to diverge relative to ancestral wolves, such that domesticated dog puppies display a greater number of communication signals to stimulate social interactions with human caregivers, including distress vocalizations, tail wagging, and direct gazing at the face of humans[11,12]. While

domestication of diverse species occurred at different times throughout evolutionary history and was presumably driven in each case by unique extrinsic factors, in all species, such alterations were most likely due to changes in gene networks that affected neurobehavioral, hormonal, and structural responses[13]. In support of the idea that common gene changes drive behaviors associated with domestication, the increased human-directed hypersociability demonstrated by dogs but not wolves was shown to be associated with structural variations in *GTF2I* and *GTF2IRD1*[14], the genes implicated in Williams-Beuren Syndrome (WBS) in humans[15,16], which is characterized by hyper-social behavior.

The hypothalamus is an essential organ in orchestrating social and stress responses with the latter being through the hypothalamic-pituitary-adrenal (HPA) axis. Thus, it is one brain region that has been heavily sculpted by domestication in varying species, including domesticated dogs compared to wolf ancestors and coyotes (*Canis latrans*)[17], as well as domesticated chickens (*Gallus gallus domesticus*) relative to ancestral red junglefowl (*Gallus gallus*). Experimental selection of red junglefowl for tameness also leads to changes in hypothalamic gene expression[18–21]. Comparison of wild and laboratory “domesticated” strains of rats (*Rattus rattus*) and mice (*Mus musculus*) reveal that laboratory strains possess higher densities of oxytocin (OXT)- immunoreactive (ir) and vasopressin (AVP)-ir neurons in the medial preoptic area (MPOA) of the hypothalamus[22], consistent with laboratory strains being easier to handle and less aggressive and fearful compared to wild counterparts.

The most longstanding and well-known domestication experiment involves farmed silver foxes (*Vulpes vulpes*) that have been deliberately bred for over 50 generations in an attempt to better understand the molecular and physiological mechanisms contributing to dog domestication[23]. Initially, one group of foxes was selectively bred based on those who exhibited reduced fear of and increased sociableness to humans. At the other end of the spectrum, another group of foxes were selectively bred for increased aggression to humans[13,24]. Comparison of behavioral, biochemical, and molecular responses in the two fox populations representing the extreme ends of the continuum have provided critical insight into how these factors are altered as foxes become more or less tame. Initial studies showed that features of the HPA axis began to diverge between the two groups, as has also been documented to occur in tame vs. aggressive rats and domesticated guinea pigs compared to their wild ancestors[13,24]. Tame foxes show a muted stress response compared to their aggressive counterparts, as evidenced by reductions in ACTH levels, decreased ACTH response to stress, and reduced glucocorticoids in tame foxes[24,25]. However, gene expression of corticotrophin releasing factor (*CRF*) by the hypothalamus did not differ between the two fox groups[25,26]. Differences in serotonergic and catecholaminergic systems have been reported in the hypothalamus of tame compared to aggressive foxes, with the latter having reduced density of serotonin 1A (5-HT1A) receptors in this brain region, but demonstrating greater amounts of serotonin and noradrenaline in the anterior hypothalamus[27–30]. Recently, global transcriptomic changes have been identified in the prefrontal cortex, basal forebrain, and anterior pituitary gland of tame vs. aggressive foxes[26,31]. To determine how selection for behavior affected hypothalamic gene expression differences, RNA-seq analysis was used to examine the transriptomic profile of this brain region and compare it with transriptomic profiles of the prefrontal cortex, basal forebrain and anterior pituitary gland of the same tame and aggressive foxes.

2 Materials and Methods

2.1 Animals, sample collection, and RNA isolation

Foxes were bred at the experimental farm of the Institute of Cytology and Genetics (ICG) in Novosibirsk, Russia. All animal procedures at the ICG were performed in accordance with standards for humane care and use of laboratory animals by foreign institutions. The current study was approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Illinois at Urbana-Champaign. Hypothalamic tissue was dissected from twelve tame and twelve aggressive sexually naive 1.5-yr-old male foxes. The foxes were selected based on their relatedness and behavioral scores[24,32]. All tame foxes used in the study had the highest behavioral score (score 4); the behavior of foxes in tame population is scored on a scale from 1 to 4[32]. The behavior of aggressive foxes ranged from −1.5 to −3, with the later score corresponding to the most aggressive foxes; the behavior of foxes in aggressive population is scored on a scale from −1 to −4[32]. Most of the selected foxes did not have the same parents and grandparents, with the exception being two aggressive foxes that had one common relative (a parent of one fox and a grandparent of another fox), and two pairs of tame foxes, each of which shared one grandparent. All of the selected foxes from the tame population belonged to the “elite” group of domesticated foxes, *i.e.*, had the highest behavioral scores for tameness, while all selected foxes from aggressive population had low (most aggressive) behavioral scores[33,34]. Foxes were euthanized with sodium thiopental. The skull was immediately incised with a saw and the whole brain removed. The brains were cut in the sagittal plane into right and left halves. The left half of brain of each fox was fixed in formalin and used for imaging studies (Supplementary Information Appendix S1: Imaging studies for fox brain samples). The hypothalamus was immediately dissected from the right half of the brain and placed into RNAlater (Life Technologies, Grand Island, NY). The sampling was done with unaided eye and by using a set of scalpels and tissue scissors. The sample collection was video recorded, and anatomical location of the sequenced brain regions is presented in Figure S1–S3. The samples were stored at −70°C. For extraction, samples were minimally defrosted, and ~ 100 mg of hypothalamic tissue was dissected out. In accordance with the manufacture’s protocol, total mRNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA).

2.2 RNA sequencing and quality analysis

One microgram of high quality RNA from each sample was used for sequencing. Stranded RNAseq libraries were prepared using TruSeq SBS Sequencing kit version 3 (Illumina, San Diego, CA). Libraries were barcoded and pooled and sequenced on two lanes on a HiSeq2500. Reads were single-end, stranded, and 100 base pairs in length. Sequencing results were processed by CASAVA 1.8 (Illumina, San Diego, CA). Data quality, including base quality per position across reads, GC content, and distribution of sequence length, was initially assessed with FastQC[35].

2.3 Bioinformatics analyses of RNA-seq data

The raw sequences (FastQ) were checked for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by adapter removal by cutadapt[36] and quality control using windowed adaptive quality trimming by fqtrim (https://

ccb.jhu.edu/software/fqtrim/). The quality trimming was performed for phred score >30 by a sliding window scan for 6 nucleotides. Finally, reads of 30 nucleotides or longer were selected and mapped to the reference genome. In the absence of a complete fox genome assembly[37–39], closely related dog genome assembly (*Canis familiaris 3.1* genome) was used as the reference for mapping. This reference genome was also used in previous studies comparing the prefrontal cortex, basal forebrain, and pituitary gland of tame *vs.* aggressive foxes[26,31]. The reference genome used is available at ftp://ftp.ensembl.org/pub/release-79/fasta/canis_familiaris/dna/. The Hisat2 mapper (https://ccb.jhu.edu/software/hisat2/), which is a fast and sensitive alignment program of next-generation sequencing data[40], was used for alignment of reads to the genome. The program FeatureCounts[41] was then used to quantify read counts that mapped to Ensembl annotation genes (CanFam3.1). The differentially expressed genes (DEGs) between aggressive and tame foxes were determined by edgeR-robust[42] (Supplementary Information Appendix S2: DEGs and all other genes list). A false discovery rate (FDR) < 0.05 was used as a significance threshold.

We also compared the expression pattern of the genes DE of the HYP from the current study with that of the PFC and BFB regions from the previous study[31]. For this analysis, the read count data of a total of 185 genes that were exclusively DE in each of the three regions (PFC, BFB, HYP) were used by PCA. Each gene was labeled to include metadata if it was DE to HYP or BFB or HYP. The PCA was conducted seprately to compare how the gene expression of the 185 genes vary in one brain region relative to the other two regions. We also conducted PCA to compare upregulated vs. downregulated genes within HYP. For this analysis, a subset of genes differentially expressed in HYP was used. This included genes that were DE in HYP but not PFC or BFB, as well as genes that were commonly DE among the three brain regions. Thus, this analysis was conducted to determine the pattern of upregulation and downregulation of genes within HYP which may also be linked to differential regulation of genes in the two regions of the fox brain. The count data included metadata specifying which gene was either upregulated or downregulated in HYP. All the analysis was done using the R package ‘ggfortify.’

2.4 Description of PCA analysis.

The read count data of all identified genes across the 24 tame and aggressive fox samples was used to perform PCA of gene expression. The calculation of the principal components and visualization of the plot was performed in R (package pca3d).

2.5 Functional Analysis

An information approach[43] was adopted to infer gene expression network analysis based on genes identified as DE based on edgeR-robust anlaysis. In this method, mutual information (MI) of expression variation was calculated in pairwise manner between genes across samples. The MI measures the information content between two genes, and determines how much knowing one gene would predict variability of the other. The Maximum Relevance Minimum Redundancy (MRMR) method[44] was then used from the mutual information matrix to infer gene expression networks. Degree centrality and prediction of key players were inferred from the MI networt as described earlier[45].

Functional differences in DEGs were also analyzed with DAVID Bioinformatics Resources 6.8[46] with the Gene Ontology (GO) terms database and the dog genome as the background database.

Finally, the ClueGO app[47] in Cytoscape[48] was used for pathway enrichment analyses based on DEG upregulated in tame foxes (decreased in aggressive foxes) and those upregulated in aggressive foxes. After a gene list is imported into this app, the ones meeting set criteria, GO level, # genes, % genes, are selected. A p value is then determined based on the Fisher exact test. Terms are connected based on shared genes, i.e. a kappa score, which is used to define groups. The edges of the diagram indicate the kappa score with thicker lines delineating the terms have more genes in common than others. Size of the colored nodes indicates the enrichment significance with bigger terms being the most significance ones. Color of each node indicates that proportion of genes from each cluster that are associated with a given term.

2.6 Alternative splicing analysis

Differences in alternative splicing frequencies between tame and aggressive hypothalamic reads were further analyzed by replicate Multivariate Analysis of Transcript Splicing (rMATS)[49] from bam files generated by TopHat. Because rMATS requires reads of equal lengths, post-filtered reads were further filtered using SAMtools to remove all reads < 100 nucleotides in length. Ensembl annotation, version 1.93, was used with rMATS to investigate reads crossing splicing junctions. Transcripts that showed skipped exon differences were further analyzed based on human orthologs and all human encoding protein genes with WebGestalt for functional enrichment in diseases (Disgenet database), phenotype (Human_Phenotype _Orthology), and pathways (KEGG).

2.7 Weighted gene coexpression network analysis

Weighted gene coexpression network analysis (WGCNA) describes network relationships among a collection of genes based on the pattern of gene expression correlations. We utilized the WGCNA package in R[50] to find unsigned weighted gene coexpression modules. The blockwiseModules function was run with a soft thresholding power of 18 to indicate the gene cluster. In order to identify significant gene modules, we calculated the correlation score between genes and trait to rank the gene cluster, which utilized the eigengene network methodology by using Pearson Correlation/Bicor. The trait data include the behavioral score of each sample (−3 to 4), as detailed in[24,32] (Supplementary Information Appendix S3: Behavioral scores for tame and aggressive foxes). For each module, we calculated the MM, which describes the correlation of the modules and genes. We applied the bicor correlation function in MM to calculate the biweight mid-correlations of the ME, expression matrix, and corresponding p-value. Functional enrichment analysis also known as over-representation analysis (ORA) or gene set enrichment analysis for GO Terms was performed by g:Go st on input gene lists. This application maps genes to known functional information sources and detects statistically significantly enriched terms. The statistical domain scope was all hypothalamic genes identified in tame and aggressive foxes, and the significance threshold was Benjamini-Hochberg FDR ≤ 0.05. For those significant gene modules, potential interaction with queried genes in the module and associated genes were identified with

GeneMANIA app[51] in Cytoscape[48]. With this program, black node color represent queried genes, and grey node color indicates associated genes. The size of the nodes for the associated genes indicates the strength of the correlation. For this app, we limited it to searching for co-expressed genes (designated with violet edges) and co-localized genes (designated with blue edges). Finally, the ClueGO app[47] in Cytoscape[48] was used for pathway enrichment analyses. Details of this program and significance of size and color of nodes is detailed above. We use the exportNetworkToCytoscape function in WGCNA package to export the module of interest, midnight blue, into Cytoscape[48]. This function exports the edge and node list files into Cytoscape[48]. In Cytoscape[48], we select the gene with the largest number of connection to other genes in the network as a hub gene.

2.8 qPCR analysis

The quantitative polymerase chain reaction (qPCR) procedure was performed on the Applied Biosystems 7500 Real-Time PCR System (Carlsbad, CA) using the Applied Biosystems PowerUP SYBR Green Master Mix kit (catalogue number A25741). The procedures for this kit were followed. Primer sequences for the candidate genes examined are listed in Table S1, and primers were purchased from IDT (Coralville, IA). All samples were run in duplicate or triplicate. The qPCR conditions employed were 1) 15 minutes at 95°C for polymerase activation 2) 40 cycles of: denaturation, 40 seconds at 94°C; annealing, 40 seconds at 56°C; and extension, 1.50 minutes at 72°C 3) dissociation melt curve analysis from 60°C to 90°C. Gene expression for each of the test genes was normalized to the expression pattern of the housekeeping gene, succinate dehydrogenase complex, subunit A *(SDHA*), a gene that previously has not shown DE between tame and aggressive foxes in the anterior pituitary gland[26] nor in the current studies for the hypothalamus. Mean ΔCt values for each test gene relative to *SDHA* expression were analyzed by using SAS (version 9.4, SAS, Cary, NC) and ANOVA analyses with treatment as main effects and individual fox as statistical unit. Graphs for these data represent $2^{-\Delta\Delta Ct}$ values where mean gene expression values of aggressive foxes was set to 1 (by subtracting mean ΔCT of aggressive fox group from all individual tame ΔCT values to obtain ΔΔCT values, and then calculating the $2^{-\Delta\Delta Ct}$ values). Thus, the expression pattern (fold changes of individual genes) in the tame group of foxes was considered relative to those of the results obtained for the aggressive foxes.

3 Results

3.1 General Characterizations of Fox Hypothalamus Transcriptome

To identify gene expression differences in brains of tame *vs.* aggressive foxes, we performed hypothalamic RNA-seq analysis with 12 replicates in each group and compared hypothalamic transcriptome with previously analysed transcriptomes of pre-frontal cortex and basal forebrain of same foxes[31]. The anatomical location and connectivity of each sampled region are shown in Figure S1–S3. Table S2 summarizes the alignment of hypothalamic RNA-seq reads to the *Canis familiaris 3.1* reference genome. For tame and aggressive foxes analyzed together, the average number of QC reads was ~30 million, average number of mapped reads was ~23 million, and average mapping rate was 75%. In both groups of foxes, 14,976 genes were identified altogether (Supplementary Information Appendix S2: DEGs and all other genes list). Some genes were highly expressed in both

tame and aggressive foxes, though the expression level of the top 10 most abundant genes in tame foxes was different in rank order than those in the aggressive foxes (Table 1). Principal component analysis (PCA) of hypothalamic transcripts did not show clear separation between tame and aggressive individuals, which might be due to heterogeneity within hypothalamic samples (Figure 1).

PCA analysis and hierarchical cluster analysis show that the gene expression profile in the fox hypothalamus (HYP) is distinct from that previously identified in the basal forebrain (BFB) and prefrontal cortex (PFC)[31] of the same foxes examined in the current study (Figures 2A and B, respectively).

3.2 Gene Expression Differences

Comparison of hypothalamic gene expression between tame and aggressive foxes identified 70 differentially expressed genes (DEGs) at an FDR q-value of 0.05 (Figure 3A; Supplementary Information Appendix S2: DEGs and all other genes list). Predictably, a hierarchical clustering heatmap based on the DEGs in this analysis revealed relatively distinct separation between tame vs. aggressive foxes (Figure 3B), however, as with the PCA analysis, heterogeneity of hypothalamic samples within the tame and aggressive groups was observed.

Among the 70 DEGs, 33 genes showed increased expression in HYP of tame foxes with $\log_2$ fold changes ranging from −4.6 to −0.31 (mean −1.17; SD 1.18) and 37 showed increased expression in HYP of aggressive foxes with $\log_2$ fold changes ranging from 0.31 to 2.96 (mean 0.93; SD 0.71) (Figure 3A). The genes up- and down-regulated in tame foxes vs aggressive foxes at FDR ≤0.01 are presented in Tables 2 and 3, respectively.

While a different number of genes were differentially expressed (DE) among HYP, PFC[31] , and BFB[31], as shown in the Venn diagram (Figure 4A), there were selected genes that were shared between one or more brain regions (Figures 4B and C). Overall, out of 70 DE genes identified in the HYP, 7 genes (10 %) showed the same pattern of differential expression in the HYP, PFC and BFB[31] (Figure 4D; Table S3). Two of these genes (*PRIMPOL* and *SHCBP1L*) were also DE in anterior pituitary[26] (Table S3). In all three brain regions (HYP, PFC, and BFB), *DKKL1*, *FBLN7*, *PTGFRN*, and *SKIV2L* were upregulated in tame foxes, whereas *NPL*, *PRIMPOL*, and *SHCBP1L* were upregulated in aggressive foxes.

We also compared expression patterns of DEGs that were DE in each of the three brain regions (PFC, BFB, HYP) and accounted in total for 185 genes. The read counts of those 185 genes extracted from the current study (for HYP) and the previous study[31] (for PFC and BFB) were analyzed by PCA (Figure S4). The dataset included samples of the three brain regions from both tame and aggressive animals (total 70 samples, this number is due to the fact that two samples were dropped from the analysis of BFB[31]). In addition, the dataset included a metadata information column specifying which gene was DE in which brain region. Each dot in the PCA plot represents a brain sample, and the samples representing the three brain regions have been color coded. Thus, in each plot within this figure, a comparison was made of specific brain region with the other two regions. This analysis revealed that although these 185 genes show distinct groupings in expression in different

regions, PC1 explains a lower level of variance (85.4%) in the hypothalamus (HYP) compared to that of the PFC (96.79%) and BFB (93.88%). Thus, based on the difference in variance for PC1 for the HYP , it is possible that these 185 genes are differentially regulated in the hypothalamus than in PFC and BFB, although additional studies would be needed to confirm this potential differential regulation. In addition to comparing brain regions, we also compared how the DE genes (upregulated vs. downregulated) varied within HYP (Figure S5). For this purpose, genes were selected if they were DE in HYP but not PFC or BFB (n = 45) along with genes that were commonly DE in all three brain regions (n =7). The purpose of using this subset of genes was to identify the differential expression pattern of genes in HYP whose regulation may be linked to differential regulation of genes in PFC and BFB. The count data included metadata specifying whether each gene was up-regulated or downregulated in HYP. This analysis showed that the PC1 explains a relatively higher level of variance in the BFB (90.81%) compared to that of the PFC (88.23%) and HYP (88.8%). Thus, based on this variance within PC1, these genes may be regulated in the HYP in a similar manner to PFC but regulation of these genes in both of these brain regions differ from the potential regulation in the BFB.

Comparison of 70 hypothalamic DEGs with genomic regions associated with selection for behavior[37] identified 10 DEGs located in such regions (*CDKL2*, *CLEC7A*, *FBLN7*, *PSD4*, *PTGFRN*, *STAC*, *THNSL2*, *TRIM52*, *WASHC1*, and *ENSCAFG00000009852*), three of which were also DEGs in PFC (*THNSL2*), BFB (*WASHC1*) or both PFC and BFB (*FBLN7* and *PTGFRN*).

3.3 Functional Annotation

3.3.1 GO and Pathway Analysis—Functional analysis for GO terms and pathways (KEGG and Reactome) was performed with ClueGO[47], a Cytoscape app[48]. Groups of genes analyzed included genes upregulated in tame foxes and, separately, genes upregulated in aggressive foxes, as determined by edgeR-robust analysis. No significant GO terms were identified in these groups, but both sets of DEGs were associated with functional enrichment for KEGG/Reactome pathways (Figure 5). Primary pathways enriched in upregulated genes in tame foxes included *phase I functionalization of compounds* (including *biological oxidations* and *metabolism*), *neutrophil degranulation* (including *innate immune system*), *metabolism of proteins*, and *extracellular matrix organization* (Figure 5A). Those pathways enriched in upregulated genes in aggressive foxes included *mTOR signaling pathway*, *innate immune system*, *endocytosis*, and *cilium assembly* (Figure 5B).

3.3.2 Network Analysis and Prediction of Key Players—Gene expression network analysis of 70 hypothalamic DEGs predicted several transcriptional interactions between genes that are downregulated in aggressive foxes (those listed in blue on Figure 6A) and those upregulated in aggressive foxes (those listed in red on Figure 6A). This network analysis, specifically the degree centrality of the interacting genes (as detailed in the Methods) was also used to identify the top 10 genes that are considered key players (Table 4). In this list, most are upregulated in aggressive foxes and involved in a variety of processes. The only key player gene that was downregulated in aggressive foxes is *ITGA8* (the most significant upregulated gene in tame foxes), which mediates varied cellular

processes, including cell adhesion, cytoskeleton rearrangement, and activation of cell signaling pathways. The other 9 key player genes are upregulated in aggressive foxes: *CD302*, *CEP104*, *FUZ*, *PRIMPOL*, *NPL*, *PRKDC*, *S100A6*, *SMPDL3A*, and *TRIM52*. Using the GTEx Portal (a human based database located at https://gtexportal.org/home/), a heatmap was generated based on key player genes that were DE in tame vs. aggressive foxes. This heatmap revealed that *S100A6* and *FUZ* are the two most abundant genes in the hypothalamus and other brain regions of humans (Figure 6B). The expression levels of these two genes in foxes are *S100A6:* 221.54 and 125.71 RPKM values in aggressive and tame foxes, respectively (FDR = 0.00525), and *FUZ:* 11.30 and 8.95 RPKM values in aggressive and tame foxes, respectively (FDR = 0.04605). Two of these genes (*PRIMPOL* and *NPL*) are also DE in PFC and BFB[31] and one gene (*CD302*) is DE in PFC[31]; all three genes showed differential expression in these bran regions in the same direction as in hypothalamus samples of tame and aggressive foxes.

The hypothalamus, similar to other brain regions, includes a heterogenous mix of cells, and thus, we searched each of these 10 genes with the Brain RNA-Seq database (https://www.brainrnaseq.org/)[52] to define the potential neuronal cells expressing these genes in mouse brain. As shown in Figure 7, *Cd302* and *Itga8* are expressed in highest amounts in oligodendrocyte precursor cells (OPC). Oligodendrocytes myelinate axons within the CNS. Microglia (resident macrophages), astrocytes (support glial cells), and endothelial cells (lining blood vessels within the CNS) also expressed high amounts of *Cd302*. *Fuz* and *Primpol* are expressed in relatively similar amounts in all nervous tissue cells screened, including astrocytes, neurons, OPC, newly formed oligodendrocytes, myelinating oligodendrocytes, microglia, and endothelial cells. *Prkdc* and *Smpdl3a* are abundantly expressed in astrocytes with the latter also widely prevalent in endothelial cells. *S100a6* expression is primarily confined to various stages of oligodendrocytes (OPC, newly formed oligodendrocytes, and myelinating oligodendrocytes). Taken together, the 10 key player genes likely originate from a variety of nervous tissue cells, including microglial or immune cells.

3.4 qPCR validation

Validation by qPCR analyses revealed that NADH:Ubiquinone Oxidoreductase Subunit A6 (*NDUFA6*), neurensin 1 (*NRSN1*), integrin subunit α 8 (*ITGA8*), cyclin dependent kinase like 2 (*CDKL2*), and olfactory receptor family 51 Subfamily E Member 2 (*OR51E2)* were significantly upregulated in tame *vs.* aggressive foxes; whereas protein kinase, DNA-activated, catalytic polypeptide (*PRKDC)*, and DNA directed primase/polymerase protein (*PRIMPOL*) were strongly upregulated in aggressive *vs.* tame foxes ($p < 0.05$, Figure 8). These qPCR results are consistent with the RNA-seq data. Based on the importance of OXT and AVP in modulating social behaviors and the potential linkage of these neuropeptides with dog and other species' domestication[53–56], their expression pattern was also examined with qPCR. However, no differences in expression were detected for these two genes, again consistent with our RNA-seq results (Figure S6). Expression results for other genes that did not differ significantly based on qPCR analyses between tame and aggressive foxes are shown in Table S4.

3.5 Alternative Splicing and Pathway Differences

rMATS[49] was used to identify 159 different genes with exons skipped at different frequencies in tame *vs*. aggressive fox hypothalamic reads at FDR < 0.05 (Supplementary Information Appendix S4: rMATS splice form analysis) and 25 transcripts at FDR < 0.0001 (Table 5). Six of these genes (*FBRSL1*, *MFAP5*, *MTHFSD*, *SPICE1*, *TRIM65, UNC5D*) were also found among genes with skipped exons in anterior pituitary samples of tame and aggressive foxes[26]. WebGestalt[57] was then used to examine human-associated diseases, human phenotype ontology, and KEGG pathways associated with transcript variants between tame and aggressive foxes (Figure 9). Many of these relate to central nervous system disorders, including dementia, neurodegenerative disease, learning and memory disorders, anxiety disorders, Alzheimer's disease, repetitive compulsive behavior, dyskinesia, agitation, restlessness, stereotypy (repetitive behaviors), which may also be influenced by the fact that nervous tissue is being screened. The KEGG pathways affected include fatty acid biosynthesis, fatty acid metabolism, glutathione metabolism, glycerolipid metabolism, arachidonic acid metabolism, the glucagon signaling pathway, drug metabolism (cytochrome P450), the calcium signaling pathway, tight junction, and cell adhesion molecules.

3.6 Integrative Correlation Analyses

All genes identified in tame and aggressive foxes (14,976 genes) were further analyzed with a Weighted Gene Correlation Network Analysis (WGCNA[58]) to identify modules of co-expressed genes. Individual modules were represented by different colors (Figure S7) and module eigengenes (ME) were correlated to quantitative measures of tameness *vs*. aggressiveness. This approach identified 34 distinct color modules. Significant correlations were found for four modules: dark orange, midnight blue, dark turquoise, and pale turquoise (Figure 10). All of the modules were negatively associated with tameness, indicating the genes within these modules showed reduced expression in tame foxes. However, within the significant modules, only one gene (*BPHL*) in the midnight blue module overlapped with DE genes (Supplementary Information Appendix S5: gene module membership for those that are significantly correlated with tameness vs. aggressiveness). We further analyzed the hub genes in the significant modules listed above. Of these, only midnight blue contained a significant hub gene, which was *BPHL*. As shown in Figure S8, 135 different nodes came off the hub gene, *BPHL*. Of these, 70 have identified gene names and corresponding symbols. Supplementary Information Appendix S6: distribution of the other 69 DEG within the various color modules provides information on the distribution of the other 69 DEG within the various color modules. The two modules that contained most of the DEG were Grey (n = 34) and Turquoise (n = 18). The grey module correponds to sets of genes that do not cluster in any of the other modules. In other words, the grey module is reserved for genes that are not part of a co-expressed module. Since it is a random collection of genes, we have not included further details on the grey module. The rest of the genes within the Turquoise module are listed in Supplementary Information Appendix S7: DEG and other genes within the Turquoise module.

As determined by the GeneMANIA app[51] in Cytoscape[48], extensive interactions that included co-expressed and co-localized genes within each of these four significant modules

were evident (Figures S9 and S10). Pathway analysis with ClueGO[47] reveals that signal transduction, immune system, Cho transports from the extracellular space to the cytosol, developmental biology, signaling by interleukins, cytokine-cytokine receptor interaction, and peptide ligand-binding receptors (e.g., G-protein coupled receptor [GPCR] ligand binding) were enriched in pale turquoise and midnight blue modules, Figure 11). Overall, these modules highlighted the importance of cell membrane processes associated with immune response and development, which were also identified in the KEGG/Reactome pathway analysis of hypothalamic DEGs (Figure 5). The novel finding included choline (Cho) transports pathway. Choline is implicated in the synthesis of the phospholipid components of the cell membrane, serves as a methyl-group donor in methionine metabolism, and it is a precursor of the neurotransmitter acetylcholine[59]. Choline is essential for brain development and the loss of cholinergic neurons is associated with neurodegenerative disorders[60,61] further suggesting the relevance of this pathway to brain processes. The dark turquoise and dark orange modules were also enriched for pathways related to cell membrane processes and signal transduction: cGMP-PKG signaling pathway, regulation of gene expression, cellular response to cytokine stimulus, and cell surface receptor signaling pathway (Figure S11). The GO terms significantly enriched for module genes (Supplementary Information Appendix S8: GO terms associated with genes in modules that are significantly correlated with tameness vs. aggressiveness) further highlighted processes associated with acetylcholine, neurotransmitter transporter activity, cognition, learning and memory, vocalization behavior, forebrain neuron fate commitment, and immune responses in the pale turquoise module; extracellular region, chemotaxis, and immune response in the midnight blue module; hormones, receptor ligand/regulator activity and neuropeptide receptor binding in the dark turquoise module; and thyroid hormone generation, NAD(P)H oxidase activity, and metabolic processes in the dark orange module.

4 DISCUSSION

Changes in complex neurological processes presumably accompanied the domestication of dogs and other species. As the hypothalamus is a key brain region involved in regulation of fear and aggression[62,63], we sought to compare the transcriptome profiles in tame *vs.* aggressive silver foxes, hypothesizing that differences in gene expression between the fox strains may provide insight into genes and pathways associated with domestication. We identified 70 DE genes in the hypothalamus of tame and aggressive foxes. Of these, 10 DEGs with the highest number of connections to other DEGs were considered to be key players (Table 4; Figure 6B). The *ITGA8* gene, encoding the alpha8 subunit of the integrin alpha8beta1, is a key player upregulated in tame foxes and the most significant DEG (Table 2; Supplementary Information Appendix S2: DEGs and all other genes list). Integrins are heterodimeric transmembrane receptors which mediate binding extracellular matrix proteins and interactions between cells[64,65]. The GO term *extracellular matrix organization* was also found to be enriched for genes upregulated in tame foxes (Figure 5A). Although none of the integrin subunits is neural specific, the alpha8 subunit is expressed in several brain regions,[66] and it is implicated in neuronal development, differentiation, migration, and synaptic plasticity[67–69]. Mouse mutants for alpha8 were found to exhibit a specific impairment of long term potentiation (LTP) at CA1 synapses in hippocampus[70] and beta1 conditional

knockouts show deficiencies in the cortical neuron layer formation and the development and function of central excitatory synapses[71]. Integrins are likely to contribute to imbalanced synaptic function in neurological diseases[72] and a missense mutation in the *ITGA8* gene was found to be protective against schizophrenia in Japanese female patients[73]. Although *ITGA8* functions in hypothalamus remain to be investigated, its importance for brain development, neuron outgrowth, and signal transduction suggests that differences in expression of *ITGA8* may be implicated in behavioral differences between the tame and aggressive foxes.

The remaining nine key player genes are all upregulated in aggressive foxes. These genes are involved in diverse processes in the body, but their functions in hypothalamus remain to be investigated. *S100A6* belongs to the family of low-molecular-weight calcium-binding proteins[74], and it is implicated in cell proliferation, differentiation, migration, cytoskeletal dynamics, and ubiquitination of β-catenin[75], which acts as a signal transducer in the Wnt signaling pathway. It is also suggested that *S100A6* transduction through integrin beta1 can increase adhesion and inhibit cell proliferation[76]. In the mouse brain, the level of S100A6 decreases in response to chronic mild stress, suggesting that this protein may modify stress responses[77]. Two of the key player genes: *CEP104* (Centrosomal Protein 104) and *FUZ* (homolog of *Fuzzy* in Drosophila) are involved in ciliogenesis. Cilia function as sensors of extracellular cues and represent a critical part of signaling pathways such as Hedgehog (Hh), PDGF and Wnt,[78,79] pathways that are critical in neuronal development. In humans, mutations in *CEP104* cause Joubert syndrome, a developmental disorder characterized by a distinctive mid-hindbrain and cerebellar malformation[80]. *FUZ* plays a crucial role in embryonic development and it is involved in planar cell polarity, ciliogenesis and directional cell movement[81]. *FUZ* is implicated in both Hh and Wnt/b-catenin signaling[82] and mutations in this gene are associated with neural tube defects in humans[83].

Two key player genes, *NPL* (N-Acetylneuraminate Pyruvate Lyase) and *CD302* (C-type lectin receptor) are associated with carbohydrate modification and carbohydrate binding. *NPL* regulates cellular concentrations of N-acetyl-neuraminic acid (sialic acid) which typically occupy the terminal position of glycan chains[84]. The brain has the highest concentration of N-acetylneuraminic acid, which serves as an integral part of ganglioside structure in synaptogenesis and neural transmission[85]. *CD302* binds specific types of carbohydrates, but a ligand for *CD302* has yet to be identified. CD302 is colocalized with F-actin–rich filopodia and lamellopodia, suggesting a potential role of this gene in cell adhesion and cell migration[86]. In addition to the key player genes, four other hypothalamus DEGs (*EIF4E1B*, *IDNK*, *KHK*, *MGAM*) are involved in carbohydrate/glucose metabolism[87]. The differences in glucose level were observed between strains of tame and aggressive rats (*Rattus norvegicus*) with higher glucose levels in the blood of aggressive rats[88]. Differences in brain expression of genes involved in glucose metabolism were also shown in populations of honey bees with different levels of aggression[89,90]. Taking into account the role of hypothalamus in glucose homeostasis maintenance[91], it is possible to suggest that changes in glucose matabolism were associated with selection of foxes for tame vs. aggressive behavior.

Four other key player genes display significant differences between tame and aggressive foxes, but there is no clear mechanism that connects these genes and their functions to the

behavioral differences we see in the foxes. Two key player genes are associated with DNA replication and DNA double strand break repair and recombination. *PRIMPOL* (Primase and DNA Directed Polymerase) catalyzes the synthesis of short RNA primers that serve as starting points for DNA synthesis and DNA polymerase activity, and facilitates DNA damage tolerance[92,93]. *PRKDC* (Protein Kinase, DNA-Activated, Catalytic Subunit) acts as a molecular sensor for DNA damage. It functions with the Ku70/Ku80 heterodimer protein in DNA double strand break repair and recombination[94]. Another key player gene, *SMPDL3A* (Sphingomyelin Phosphodiesterase Acid Like 3A) hydrolyzes nucleotide tri- and diphosphates and their derivatives[95]. Finally, the *TRIM52* (Tripartite Motif Containing 52) is a novel noncanonical antiviral TRIM gene which is involved in NF-κB pathway activation and plays an important role in antiviral innate immunity[96,97].

The functions of key player genes are in line with the GO terms identified in the enrichment analysis of hypothalamus DE genes: *cilium assembly*, *innate immune system* and *extracellular matrix organization* (Figure 5). Overall, the functions of the key player genes and GO terms identified in the enrichment analysis of hypothalamus DEGs correspond to a broad spectrum of biological processes, indicating that differential expression in hypothalamus of tame *vs.* aggressive foxes involves development, cell differentiation, cell interaction, and migration, rather than specific neurotransmitter pathways.

Comparison of expression patterns of DEGs that were DE in each of the three brain regions (PFC, BFB, HYP) reveales that selected genes are differentially regulated in each of these three brain regions (Figures S4 and S5). These findings highlight that expression of certain genes is important in regulating specific brain regions. On the other hand, the comparison of DEGs in the three brain regions highlighted seven genes which showed differential expression in the same direction in all three tissues (Figure 4). Two of these genes (*NPL* and *PRIMPOL*) are also the key player genes in hypothalamus. Little is known about the functions of the remaining five genes. *DKKL1* (Dickkopf Like Acrosomal Protein 1) is one of the least investigated members of the Dickkopf family[98]. It is abundantly expressed in testis and likely involved in testicular development and spermatogenesis. In mouse brain, *DKKL1* was shown to be expressed in embryonic dorsal root ganglia neurons[99] and in cortical neurons of the adult brain[100]. Previously, we demonstrated that *DKKL1* is one of the most significant DEGs in PFC of tame *vs.* aggressive foxes[31]. *DKKL1* is most closely related to *DKK3*, which acts as antagonist of Wnt signaling[101] and is involved in many cellular processes including cell proliferation, differentiation, adhesion, and apoptosis[102]. *FBLN7* (Fibulin 7) is a member of the fibulin protein family, cell-secreted glycoproteins that function as cell adhesion molecules and interact with other extracellular matrix proteins as well as cell receptors[103]. First identified as an extracellular matrix molecule in developing teeth[104], *FBLN7* was found to be expressed in a variety of tissues and upregulated in several cancers, with the highest expression in glioblastomas[105]. *PTGFRN* (Prostaglandin F2 Receptor Inhibitor) is a glycosylated type 1 integral membrane protein with immunoglobulin domains that interacts with tetraspanins CD9 and CD81[106], integral membrane proteins present on the plasma membrane of many cells[107,108]. Tetraspanins are involved in regulation of cell interaction and cell migration and implicated in numerous physiological processes including immune response, reproduction, development, angiogenesis, and tumorogenesis[109–112]. It was also shown that expression of *PTGFRN* in COS-1 cells inhibits

the binding of prostaglandin F2-alpha (PGF2-alpha) to its specific FP receptor[113]. The functions in brain tissues of the two other genes from Figure 4D in brain tissues are largely unknown. *SHCBP1L* (SHC Binding And Spindle Associated 1 Like) maintains stability of the spindle integrity during meiosis and implicated in spermatogenesis[114]. *SKIV2L* (Ski2 Like RNA Helicase) encodes a DEAD box protein which shares homology with yeast exosome components. Based on this similarity it was suggested that *SKIV2L* functions as an RNA helicase[115,116]. *SKIV2L* has a protective effect against the age-related macular degeneration[117].

Analysis of the genes which show gene expression differences between tame and aggressive foxes in three brain regions showed consistent differences in expression of genes involved in broad biological processes rather than in processes specific to CNS. It would be interesting to test if expression of these genes is also altered in other tissues of tame and aggressive foxes and particularly in neural crest cells. Given the importance of these genes for such processes as cell division, differentiation, and adhesion they may potentially have an effect on neural crest cell migration and differentiation, which was proposed to be involved in the domestication syndrome[6].

The functions of both key player genes and genes from Figure 4D are in agreement with GO terms identified in the current study as well as in the analysis of genomic regions which differentiate fox populations[37]. Comparison of genomic regions which differentiate tame and aggressive foxes also identified GO terms related to *damaged DNA binding*, *carbohydrate binding*, and immunity (*interleukin-1 receptor binding* and *cytokine activity)*[37]. Tame and aggressive foxes are maintained under the same farm conditions, and thus, it is more likely that differences in genomic regions containing immune genes are directly or indirectly associated with selection for behavior. The link between behavior and immunity has been demonstrated in a large body of literature (reviewed in[118–120]).

Although finding consistent gene expression differences across brain tissues increases confidence that our experimental and analytical methods detect DEGs between sampled foxes, we cannot conclude that all identified DEGs genes are implicated in behavioral differences between tame and aggressive fox strains. While sufficient replicates were used in the current study, it is possible that some alleles could drift in frequency in these outbred populations sufficiently to give the observed changes in expression by chance alone. Further studies including different sets of tame and aggressive foxes and comparisons with transcriptomic differences between pairs of domesticated *vs.* wild species need to be performed to pinpoint genes whose expression affects behavior of these foxes.

WGCNA identified four eigengene modules with significant gene expression differences between tame and aggressive foxes (Figure 10). Integrative correlation analyses revealed that all four modules that were significantly different were negatively associated with tameness. Pathway analyses of these modules identified biological processes similar to the process identified in the enrichment analysis of hypothalamus DEG. However, the enrichment analysis of significant WGCNA modules also highlighted GO terms more specific to neural system such as acetylcholine, neurotransmitter transporter activity cognition, learning and memory, and forebrain neuron fate commitment (Supplementary Information Appendix S8:

GO terms associated with genes in modules that are significantly correlated with tameness vs. aggressiveness). We found only one DEG (*BPHL*) in the significant WGCNA modules.This gene is also the only hub gene identified in all four significant modules, with connections to 135 other genes in this significant module (Midnight Blue). As a hub gene, *BPHL*, might regulateother genes connected to it. Some of the genes directly connected to it include keratin 36 (*KRT36*), *AVP*, myosin light chain 3 (*MYL3*), solute carrier family 5 member 1 (*SLC5A1*), tissue factor pathway inhibitor 2 (*TFPI2*), growth factor independent 1B transcriptional repressor (*GFI1B*), T cell receptor beta variable 3–1 (*TRBV3–1*), and vasoactive intestinal peptide (*VIP*). Of these, we did not detect any differences in *AVP* mRNA expression with RNA-seq (Supplementary Information Appendix S2: DEGs and all other genes list) or qPCR analysis (Figure S6).

Finding GO terms more specific to neural system in WGCNA open the possibility that differences in expression of genes related to brain-specific pathways take place in particular cell types or nuclei in hypothalamus. Because our hypothalamus samples include heterogeneous populations of cells and nuclei, differences in expression of these genes between tame and aggressive samples can be masked and may not reach statistical significance.

Analysis of alternative splicing identified 159 genes with differentially skipped exons in reads from tame and aggressive fox hypothalamus. Six of these genes were also differentially spliced in anterior pituitary samples of tame and aggressive foxes[26]. Comparison of DEGs (Supplementary Information Appendix S1: DEGs and all other genes list) with genes with differentially skipped exons (Supplementary Information Appendix S3: rMATS splice form analysis) identified only a small overlap, three genes (*CDKL2*, *CES2*, and *SLC38A9*) were found to be significant in both datasets. These results indicate that differential expression is not the only mechanism leading to transcriptomic differences and detailed analysis of gene transcription and processing is needed to identify a more complete picture of transcriptomic differences between samples. Functional enrichment analysis of skipped exon data based on rMATS analysis suggests that primary KEGG pathways affected include those regulating fatty acid biosynthesis, fatty acid metabolism, glutathione metabolism, glycerolipid metabolism, and glucagon signaling pathways, providing evidence that selection pressures as foxes were bred for tameness also acted on the individual transcript level for these metabolic pathways. Further screening of this database also revealed differential expression of transcripts associated with various neurobehavioral disorders in humans, including dementia, neurodegenerative disorders, Alzheimer's disease, learning and memory dysfunction, anxiety disorders, and repetitive behaviors. As foxes were selected for tameness, they showed reduced anxiety and cognitive alterations[24,25,121].

We hypothesized that primary differences that would emerge between tame and aggressive foxes would be found in neuropeptides, such as *OXT* and *AVP*, and other genes associated with social affiliation. These neuropeptides and their receptors have been proposed as facilitators of domestication in the dog and other species[53–56]. However, neither *OXT*, *AVP*, nor any of their splice forms were altered with RNA-seq or qPCR analyses. This could be due to the area of the hypothalamus sampled, as we did not selectively screen the paraventricular (PVN) and supraoptic (SON) nuclei. Alternatively, differences might emerge

in tame and aggressive foxes in the release of these neuropeptides from the posterior pituitary gland. Previous work only examined the anterior pituitary gland from these fox populations[26].

We compared DEGs and GO terms identified in hypothalamus of tame and aggressive foxes with genes and pathways identified in transcriptomes of domesticated and wild species. A study by Albert et al.,[122] used frontal cortex tissues obtained from dogs and wolves, pigs and wild boars, domesticated and wild guinea pigs, and domesticated and wild rabbits and identified a large number of GO terms differentiating wild and domestic species. Similar to our findings, the identified GO terms largely belonged to a broad spectrum of biological processes such as immunity, development, reproduction, and metabolism; as well as cellular processes such as cell adhesion, cell-cell interaction, motility, and signal transduction. Comparably, the immune-related processes were enriched in the comparison of frontal cortex transcriptomes of wild boar and domestic pig in a study by Long et al.[123] and in a study of blood transcriptomes between wolves and dogs[124]. The comparative studies of transcriptomes from thalamus/hypothalamus of wild Red Junglefowl and Red Junglefowl selected for tameness for five generations largely identified genes related to spermatogenesis and immunity[21], but the comparative analysis of cerebral hemisphere transcriptomes in these populations suggested an enrichment for genes associated with behavioral processes[125]. Furthermore, genes that were DE between the two groups of foxes include those regulating the metabolism of fats and carbohydrates, such as *MGAM*, *KHK*, *IDNK*, *FABP7*, and *CES2*. Differences in fat metabolism have also been identified in the adrenal glands of these two fox populations (Hekman et al., In Preparation). A previous study on dog domestication also found enrichment in genes for fat and other metabolism, e.g., *CCRN4L*, *SCP2D1*, and *PDXC1*[126]. The findings led the authors to speculate that domestication impacted dietary selection as proto-dogs hunted and fed alongside hunter-gatherers. Overall, the results of transcriptomic studies between domesticated species and their wild progenitors indicate importance of genes whose functions in adult brain are not well understood. These findings may suggest that genes involved in neural functions are less important for domestication than genes involved in immunity and development, e.g. affecting sizes of brain regions implicated in fear processing[127]; alternatively, these results may indicate current limitations of transcriptomic studies of domestication. Changes in expression of genes with neurological functions may be specific to particular cell types and therefore not found in the heterogeneous tissue samples used in conventional RNA-seq experiments.

Comparison of hypothalamus DEGs with the gene content of genomic regions differentiating fox populations[37] identified 10 genes in common. Two genes highlighted in our study, *PTGRFN* and *FBLN7*, are both located on fox chromosome 8 in genomic regions (regions 50 and 52, respectively) which show increased divergence between tame and aggressive fox populations[37]. Because *PTGRFN* and *FBLN7* are two out of many genes located in these regions, it is impossible to conclude without additional experiments whether *PTGRFN* and *FBLN7* are genes targeted by selection for behavior or their differential expression in the two fox strains is a result of a tight linkage of these genes with genes under selection. However, finding DEGs within genomic regions targeted by selection allows us to prioritize positional candidate genes for screening. A hypothalamus DEG, *STAC* (SH3 And Cysteine Rich Domain), is located in a genomic region (region 61) which includes only

seven genes[32]. In neurons, *STAC* modulates Ca^{2+} entry via l-type, but not via non-l-type, Ca^{2+} channels. Expression of the major neuronal isoform (*STAC2*) is increased in postnatal forebrain and cerebellum, which could provide developmental regulation of l-type channel Ca^{2+} signaling in these brain regions. In skeletal muscle, STAC protein is essential for proper trafficking and function of the l-type Ca^{2+} channel and mutations in *STAC* cause severe myopathy[128]. *STAC* will be prioritized for screening as a positional candidate gene in region 61.

One limitation of the current study is that there was likely cellular heterogeneity in the hypothalamus tissue samples, as shown in the PCA analysis (Figure 1) and hierarchical heatmap clustering analysis (Figure 2B). In foxes, as in dogs, the hypothalamus is a substantial brain region, and since we used only a small amount of dissected tissue for RNA extraction, we inevitably introduced heterogeneity across sequenced samples. However, in these initial studies, the initial aim was to broadly analyze the hypothalamus in these two fox populations with the idea that further work can examine discrete nuclei, including the PVN and SON. Future studies will employ single cell RNA-seq to pinpoint which cells have been affected to the greatest extent by the selective breeding scheme as evidenced by the cell-specific transcript changes. Our abilities to collect cell-specific information in undisturbed landscapes in solid tissues is limited but growing arsenal of omics techniques[129] and precise brain mapping approaches[130] provide a framework for these future studies. Revealing brain cell-specific activity is critical for understanding complex processes underlying cognitive and behavioral functions.

Domesticated dogs demonstrate increased sociability to humans and a greater ability to use human directed cues relative to their wolf ancestors, traits that appear to have been selected for during the domestication process[131–136]. Besides showing reduced fear and aggression towards humans, similar socio-cognitive traits have evolved in this silver fox population bred for tameness[121], providing robust evidence that these foxes have potential to provide valuable insight into the underlying neurological events promoting canine domestication. The current studies reveal that selection for tameness *vs.* aggressiveness in foxes is associated with unique hypothalamic gene and transcript signature profiles. Functional analyses of genes altered between these two groups reveals that processes affected by this selective breeding include those regulating development, immunity, lipid and carbohydrate metabolism, and genes involved in extracellular matrix organization, cell interaction, and several signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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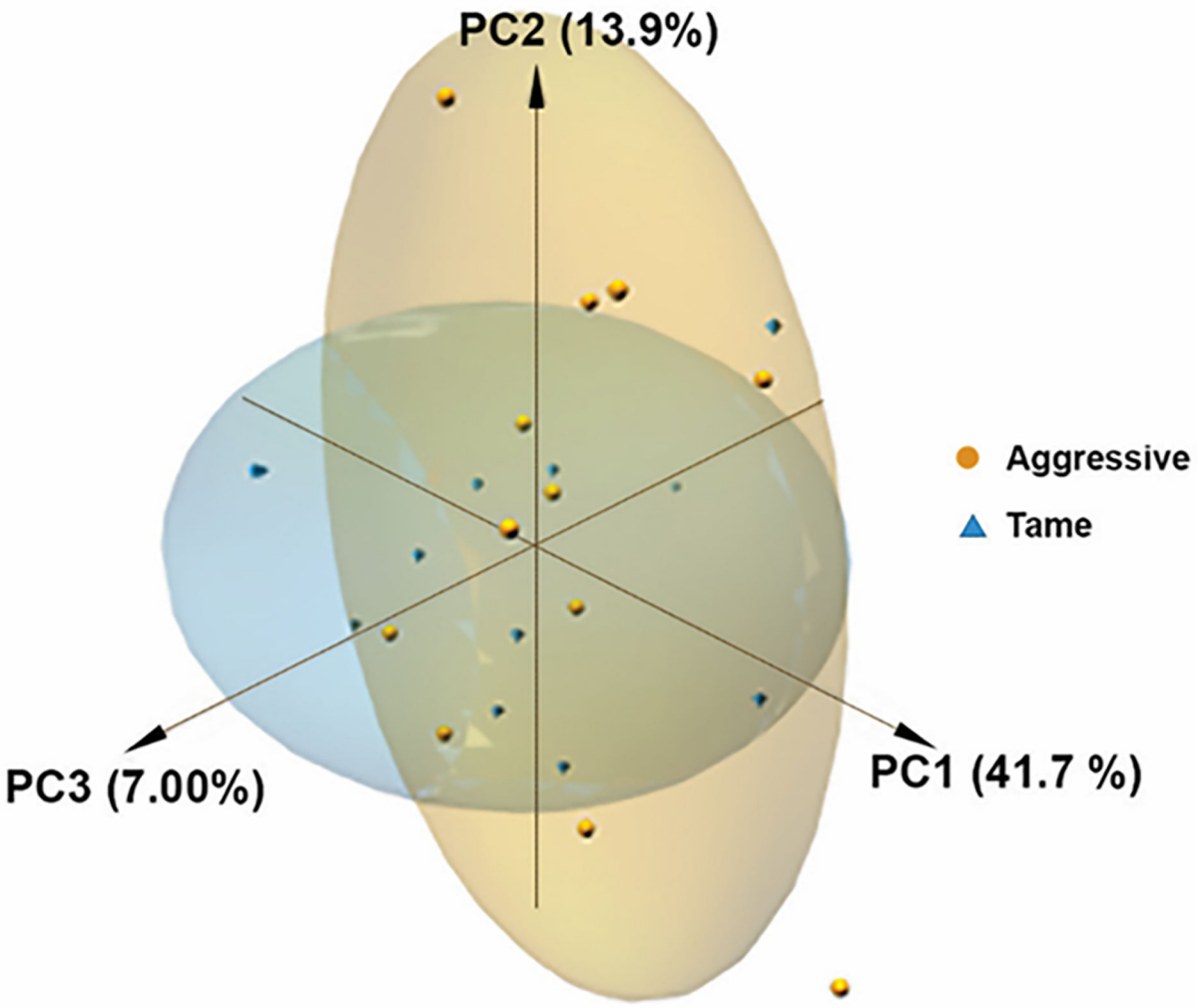

Figure 1.
PCA analysis of all genes. A) No clear clustering is evident in the hypothalamic transcriptome results between tame vs. aggressive foxes.

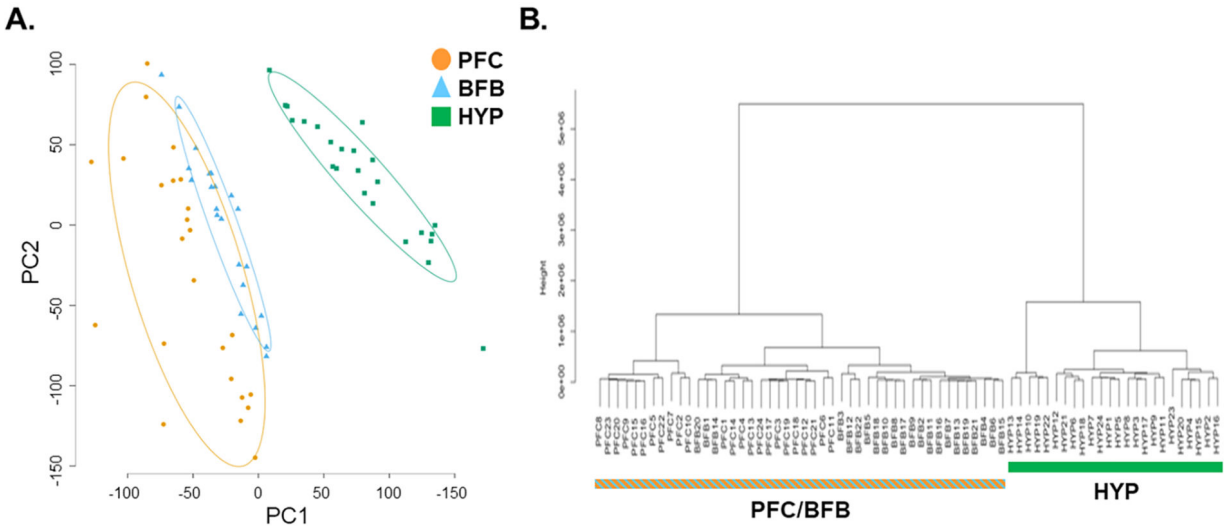

Figure 2.

PCA plot and hierarchical clustering of current hypothalamic results and previous basal forebrain (BFB) and prefrontal cortex (PFC) gene expression results. A) The PCA plot using expression of all genes shows that the fox hypothalamic transcriptome is distinct from that of PFC (cortex) and BFB (forebrain). B) Similarly, hierarchical cluster analysis using expression of all genes also shows that the fox hypothalamus transcriptome is distinct from that of cortex and forebrain.

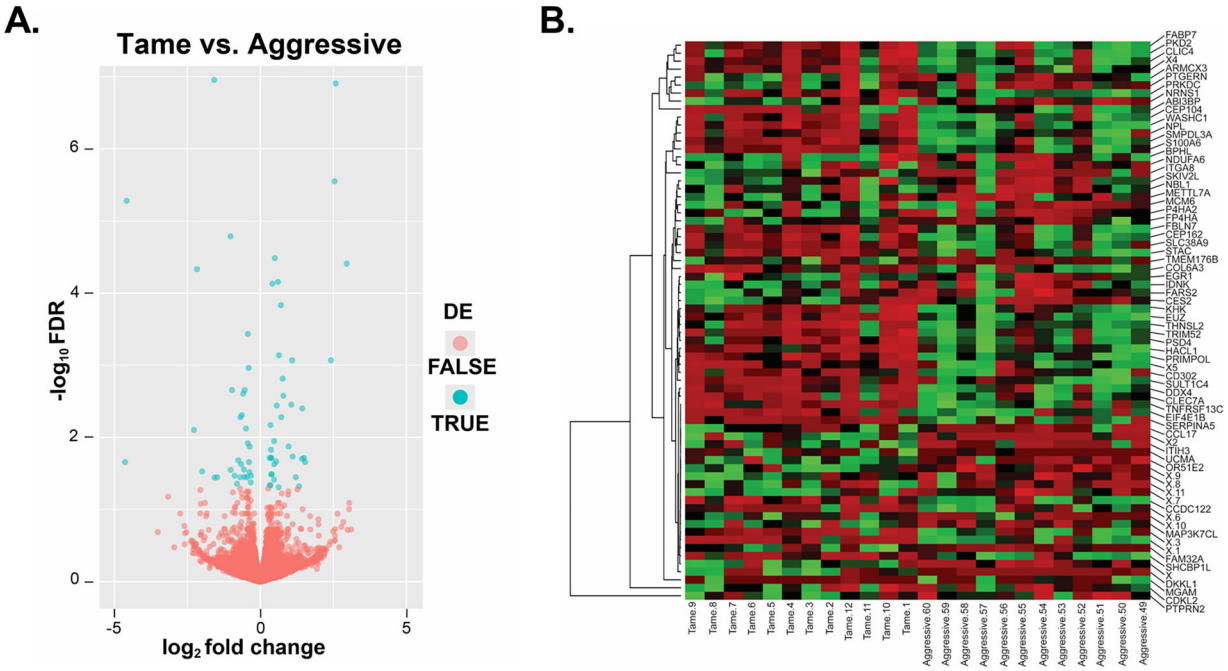

Figure 3.

Volcano plot and hierarchical clustering heatmap analyses of genes differentially expressed in tame vs. aggressive foxes. A) The volcano plot represents the relationship of each gene's $\log_2$ fold change vs $-\log_{10}$ FDR. Red points represent those genes that are not differentially expressed in the hypothalamus of tame vs. aggressive foxes; whereas those in blue are significantly different (FDR < 0.05, and $\log_2$ fold change > 0 or $\log_2$ fold change < 0). B) Consideration of only those hypothalamic genes that were DE based on EdgeR analyses revealed overall separate clustering between tame vs. aggressive foxes with heatmap analysis. The heatmap was generated with http://heatmapper.ca/[137].

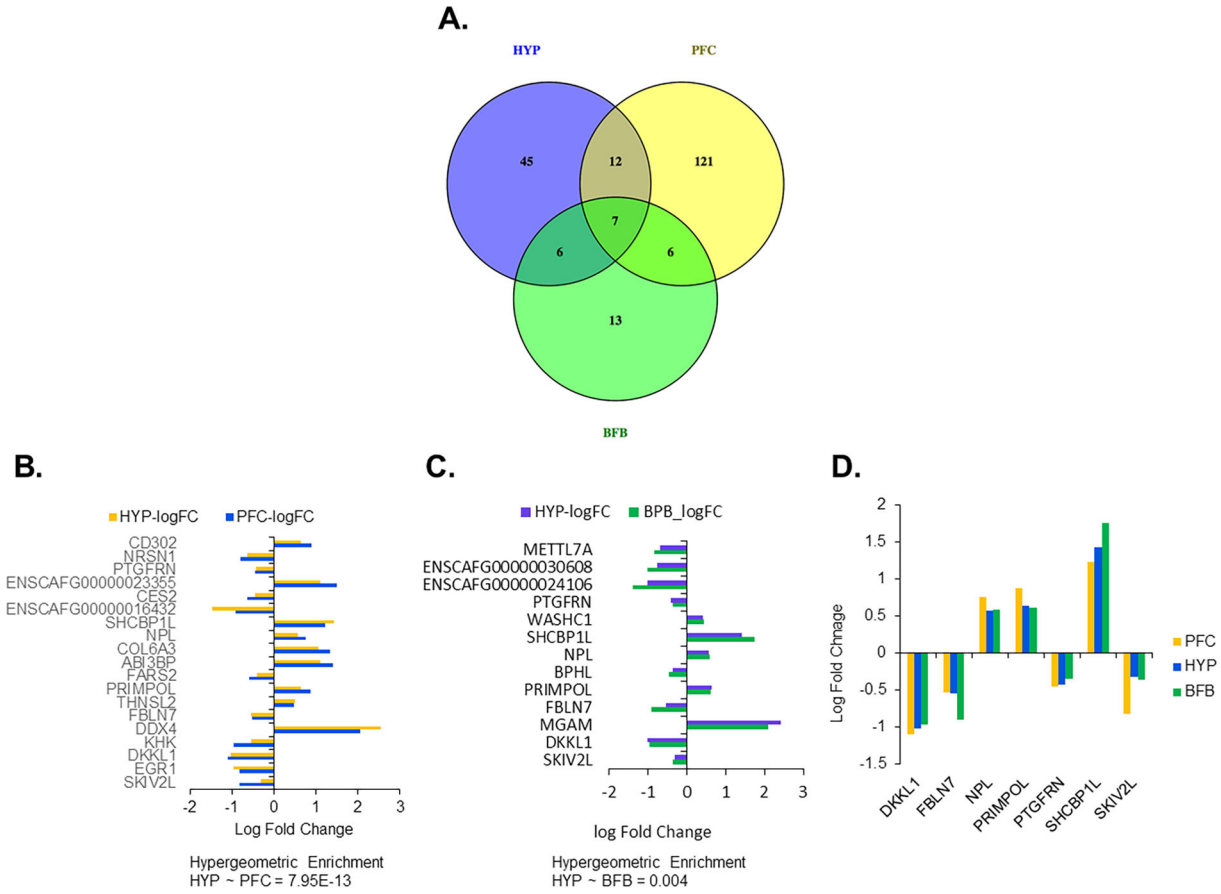

Figure 4.

Comparison of DEG identified in hypothalamus compared to those previously identified in the prefrontal cortex (PFC) and basal forebrain (BFB). A) Venn diagram showing number of DEG common or specific to hypothalamus, frontal cortex or forebrain. B and C) The expression (log fold changes between tame and aggressive) of the common genes between hypothalamus and PFC (B) and between hypothalamus and BFB (C). The enrichment was determined by calculating p-value of hypergeometric test from the number of common genes, specific genes to each and the total number of genes for each comparison. D) A column graph showing the expression (log fold changes between tame and aggressive) of the common genes among hypothalamus, PFC and BFB.

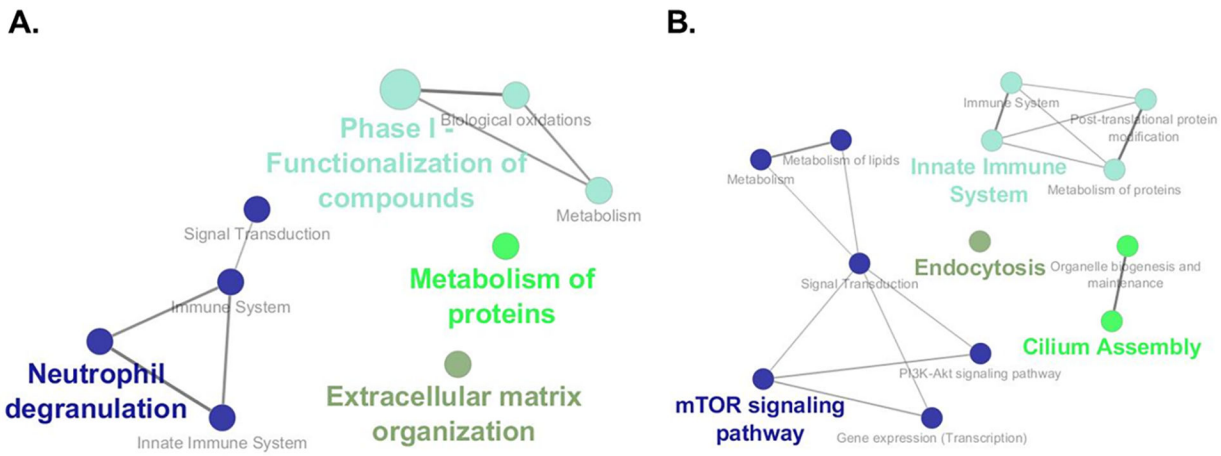

Figure 5.

Pathways enriched based on upregulated genes in tame and those upregulated in aggressive foxes. ClueGo App[47] was used to examine for enriched KEGG and Reactome pathways based on DEG upregulated in tame (A) and those upregulated in aggressive (B). Primary pathways enriched based on upregulated genes in tame foxes included phase I functionalization of compounds, including biological oxidations and metabolism, neutrophil degranulation, including innate immune system, metabolism of proteins, and extracellular matrix organization. Those pathways enriched based on upregulated genes in aggressive foxes include mTOR signaling pathway, innate immune system, endocytosis, and cilium assembly.

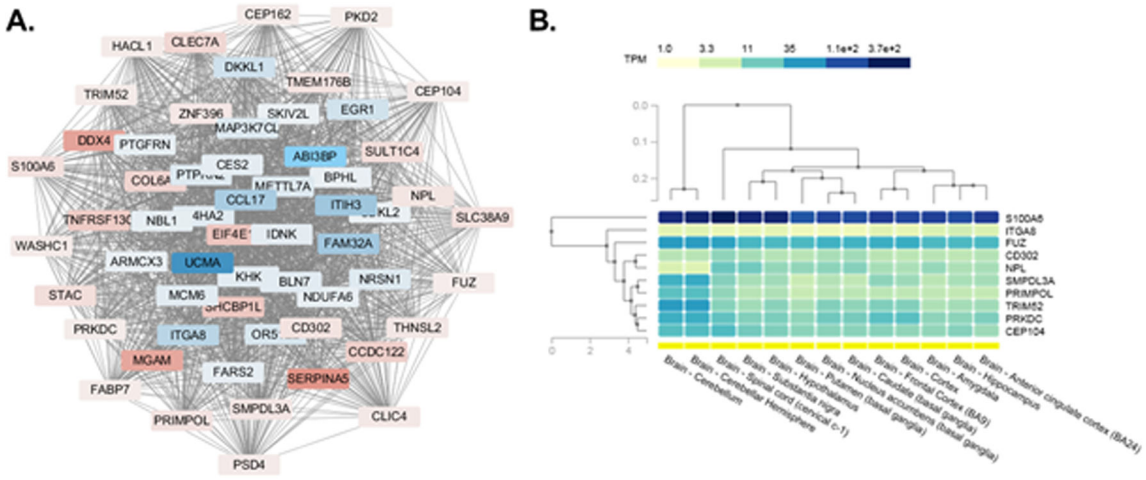

Figure 6.

Gene expression network to identify ten hub or “key player” genes and their expression pattern in human brain regions. A) The diagram shows the inter-relationships of the gene to be DE based on edgeR robust. As shown, there are connections between almost all 70 genes that are DE. This network analysis was used to identify ten key player genes within the 70 DEG. Those in blue represent genes that are downregulated in aggressive relative to tame foxes; whereas, those in red indicate genes that are upregulated in aggressive relative to tame foxes. B). Analysis of the expression pattern for the ten key player genes in various human brain regions. The GTEx Portal site (https://gtexportal.org/home/) was screened to determine the expression pattern of the identified ten key player genes that were DE in tame vs. aggressive foxes. As shown, *S100A6* followed by *FUZ* has greatest expression in all human brain regions, including the hypothalamus.

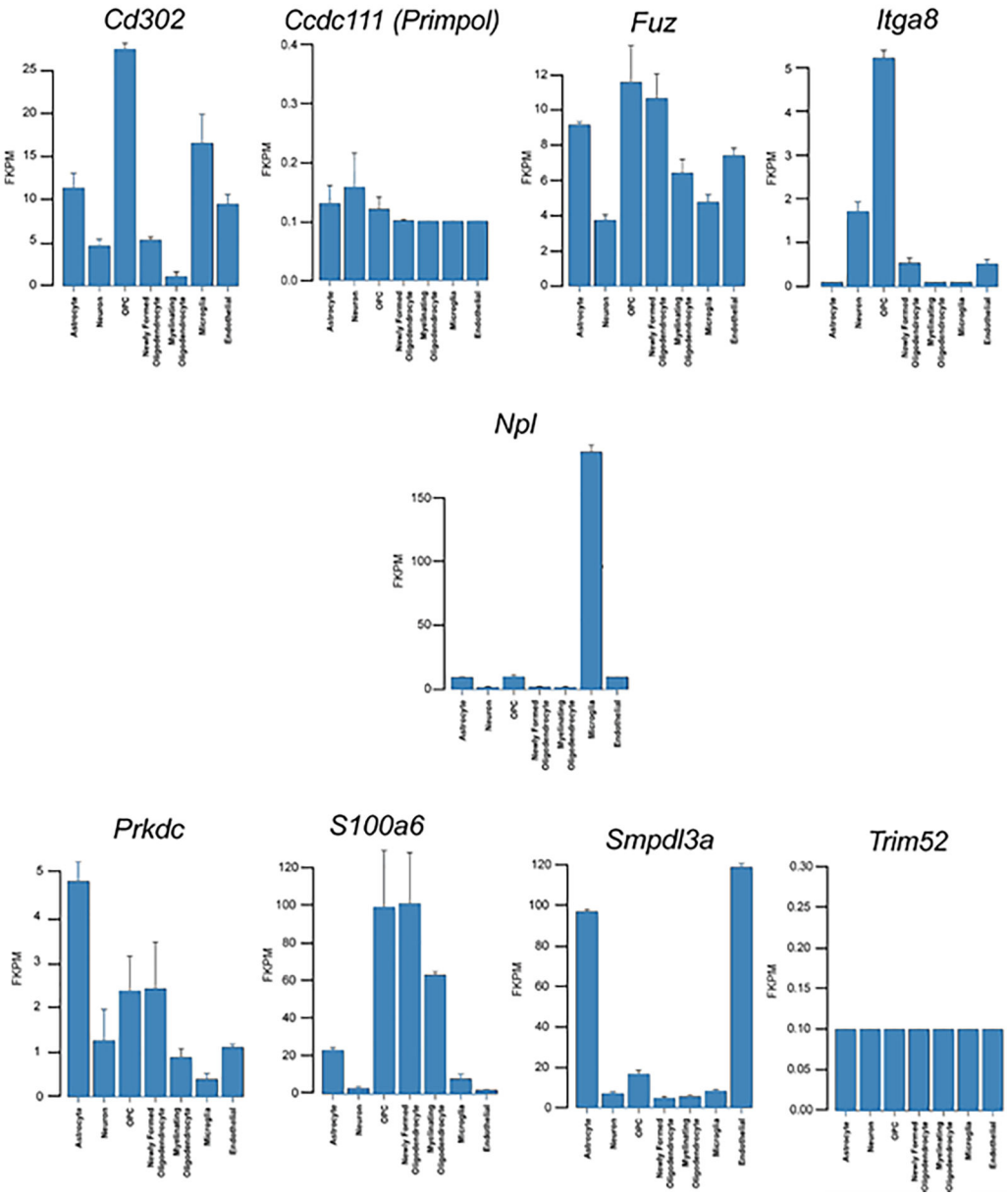

Figure 7.

Neuronal cellular analysis of the ten key player genes. To pinpoint which neuronal cells might be contributing to the difference in expression for nine of the ten key player genes, were analyzed with the Brain RNA-Seq (http://www.brainrnaseq.org/) mouse database. No information was available for *Cep104*. As shown, differences in DEG might arise from various neuronal cell type and depends upon the gene. More details on individual genes are provided in the Results section.

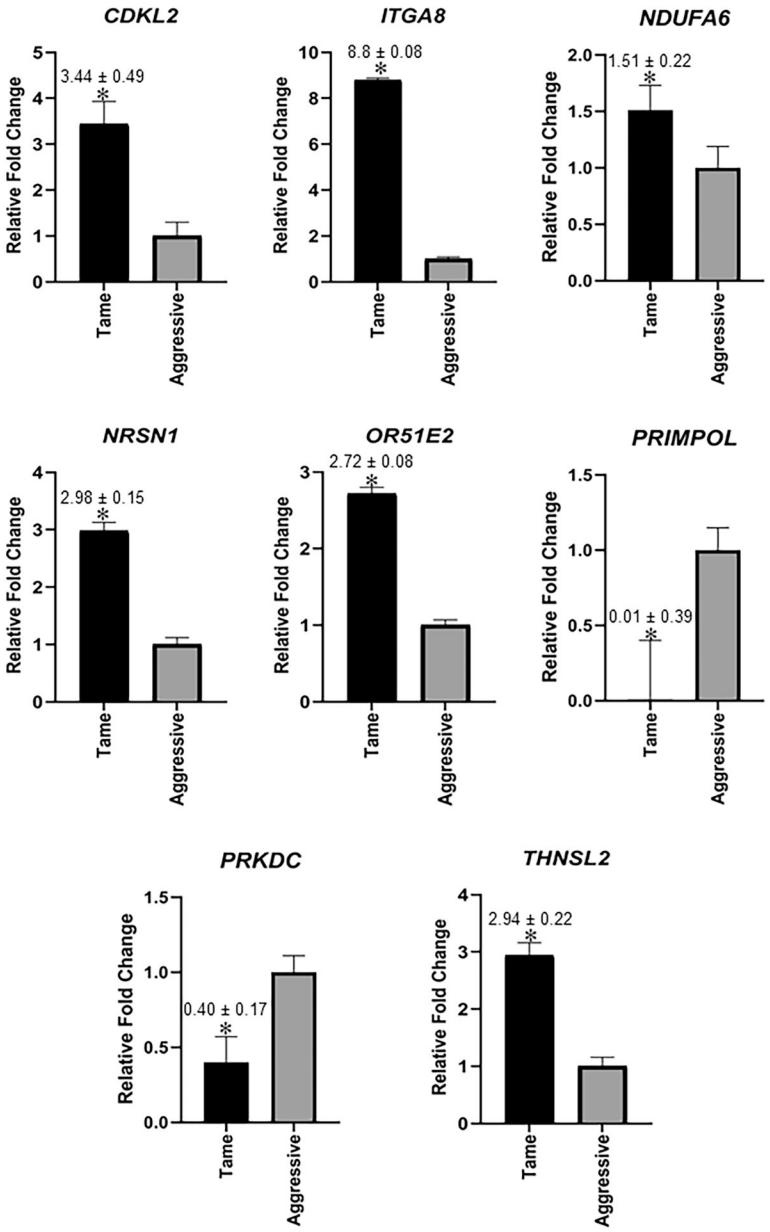

Figure 8.

qPCR validation of select hypothalamic genes that were shown to be DE based on RNA-seq results. Graphs represent average $2^{-\Delta\Delta CT}$ ± SEM with aggressive foxes used as the reference and mean value for this group set to 1. As detailed, statistical analyses were based on ΔCT values. N= 12 individuals for tame and 12 individuals for aggressive foxes, i.e. same samples used for RNA-seq were used for qPCR analysis.

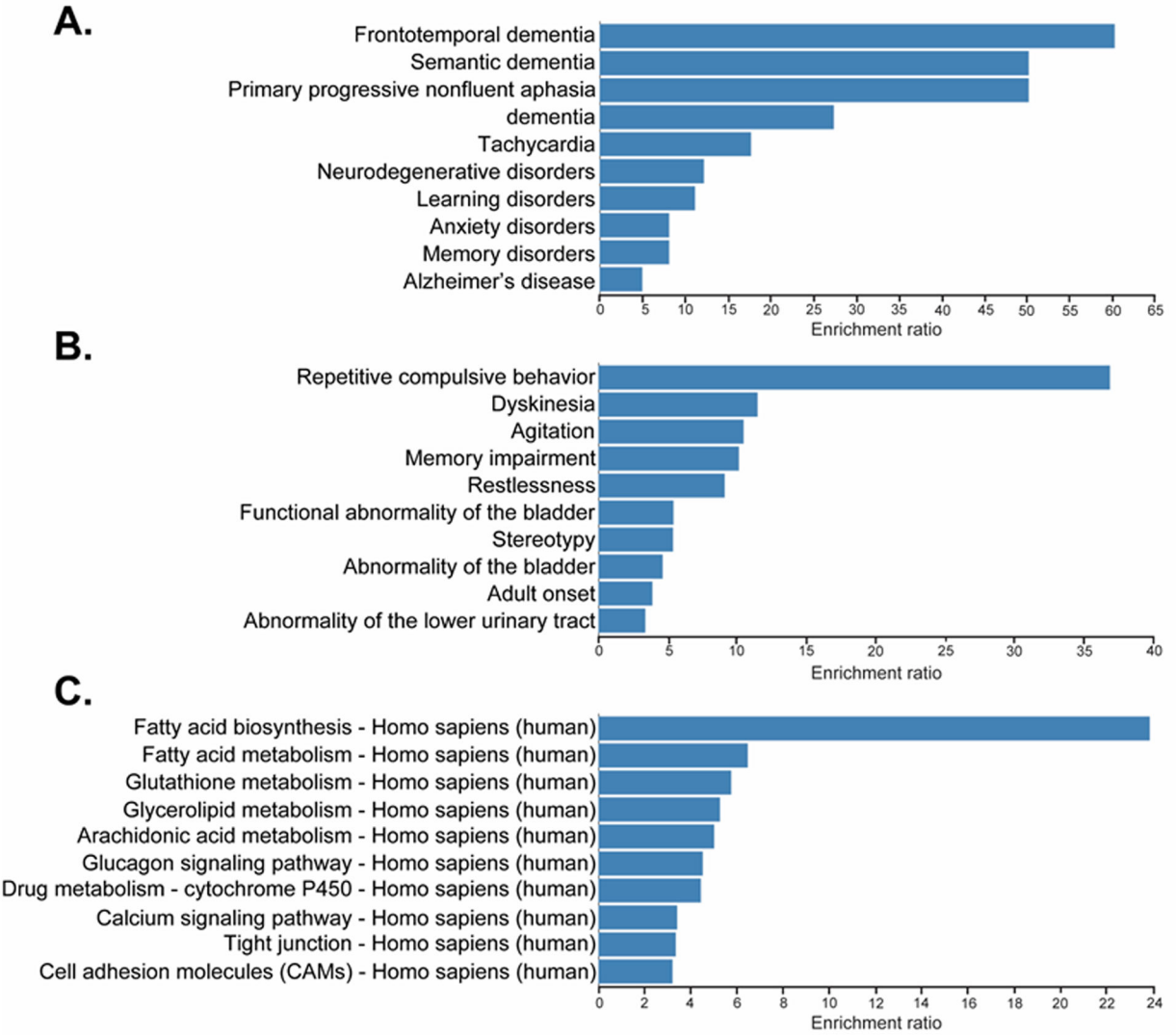

Figure 9.

Functional analyses based on splice variant data. Transcript isoforms shown to be differentially expressed in the hypothalamus of tame vs. aggressive foxes was analyzed with WEB-based Gene SeT AnaLysis Toolkit (WebGestalt, http://www.webgestalt.org/option.php)[57]. To perform this analyses, the canine genes were converted to their human orthologs, and three functional analyses were then considered: A) Diseases based on Disgenet database, which reveals that several human neurological diseases, including neurodegenerative, anxiety, and learning and memory disorders, and Alzheimer Disease; B) Phenotype based on Human_Phenotype _Orthology. This functional analysis revealed the splice form differences between the two groups of foxes are associated with human phenotypic conditions, including repetitive compulsive behavior, memory impairment, restlessness, and stereotypy. C) KEGG pathway functional analysis showed that these transcript differences are linked to various types of carbohydrate and lipid biosynthesis and metabolism, glucagon signaling pathway, drug metabolism, calcium signaling pathway, and cell adhesion molecules (CAM).

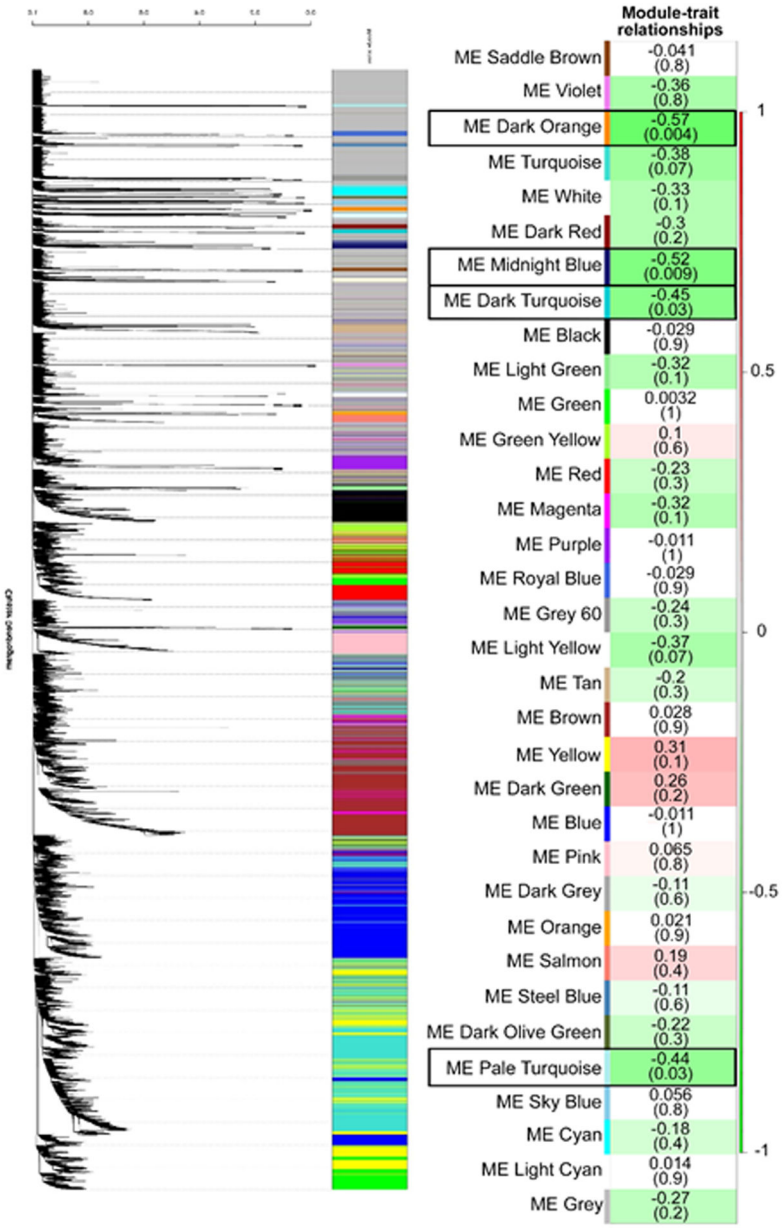

Figure 10.

Relationship of WGCNA results and tameness vs. aggressiveness. The modules identified in Figure S7 were then correlated with tameness vs. aggressiveness for the same foxes whose hypothalamus was analyzed with RNA-seq. As detailed in[33,34], the foxes were assigned a tameness score of −3 to 4 with 4 being the highest degree of tameness. Each row corresponds to a Module Eigengene (ME) and colors represent the correlation coefficient between the ME and tameness vs aggressiveness. There are two numbers on the right of each row, the number at the top of each row represents the degree of correlation, and those values with a negative integer indicate an inverse correlation with degree of tameness, numbers on the bottom, in parentheses represent the p value associated with the ME~tameness correlation. There are black boxes around the four ME that were significantly

associated with degree of tameness: ME dark orange, ME midnight blue, ME dark turquoise, ME pale turquoise ($p \leq 0.05$). All four ME were negatively correlated with this behavioral trait.

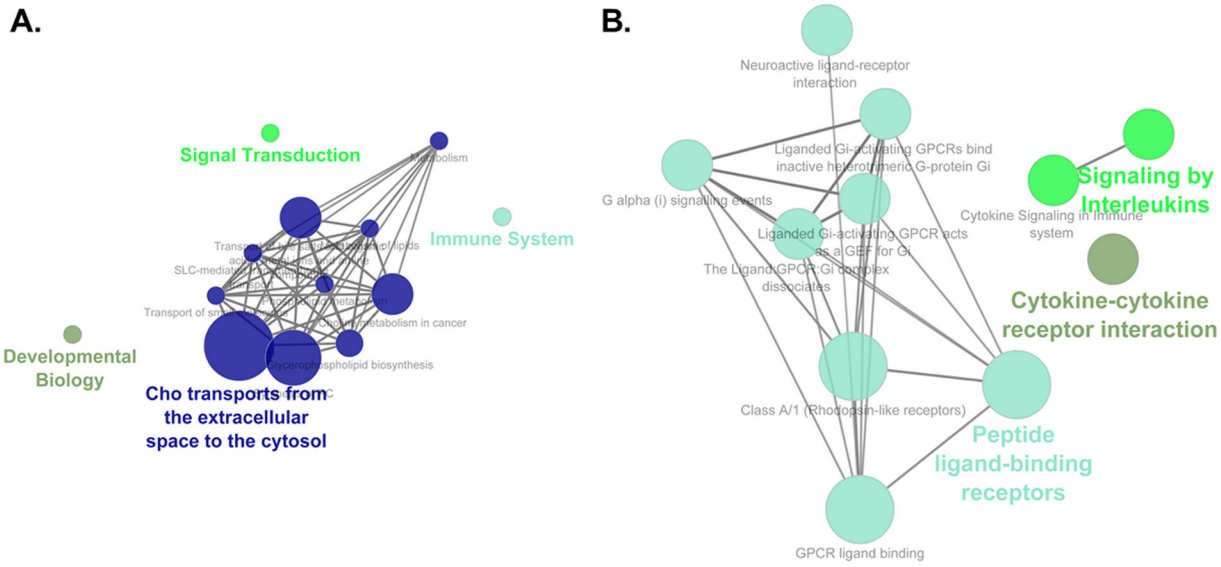

Figure 11.

Functional enrichment pathway analyses for the pale turquoise and the midnight blue modules, as determined by the ClueGo App[47] in Cytoscape[48]. A) For the pale turquoise module, signal transduction, immune system, Cho transports from the extracellular space to the cytosol, and developmental biology were enriched. B) In the midnight blue module, signaling by interleukins, cytokine-cytokine receptor interaction, and peptide ligand-binding receptors were enriched.

Table 1.

The top expressed genes in the hypothalamus of tame and aggressive foxes. The gene expression rank is shown as (GER#). The mean RPKM values are indicated in parentheses. The top 10 GER are italicized or bolded for aggressive and tame foxes, respectively.

Gene Symbol	Gene Name	Aggressive Foxes	Tame Foxes
MBP	Myelin basic protein	*GER# 1 (5467.567)*	**GER# 1 (4576.599)**
PLP1	Proteolipid protein 1	*GER# 2 (4581.421)*	**GER# 2 (3556.126)**
PTGDS	Prostaglandin D2 synthase	*GER# 8 (1204.963)*	**GER# 3 (1476.062)**
SPARC	Secreted protein acidic and cysteine rich	*GER# 4 (1538.644)*	**GER# 4 (1440.66)**
APOE	Apolipoprotein E	*GER# 6 (1453.506)*	**GER# 5 (1385.807)**
ENSCAFG00000005106		*GER# 3 (1982.721)*	**GER# 6 (1302.408)**
SPARCL1	SPARC like 1	*GER# 7 (1392.475)*	**GER# 7 (1299.786)**
GFAP	Glial fibrillary acidic protein	*GER# 5 (1500.543)*	**GER# 8 (1232)**
CLU	Clusterin	GER# 15 (965.258)	**GER# 9 (1189.477)**
MT3	Metallothionein 3	GER# 12 (1015.945)	**GER# 10 (1124.97)**
CPE	Carboxypeptidase E	*GER# 10 (1057.1)*	GER# 11 (1080.682)
FTH1	Ferritin Heavy Chain 1	*GER# 9 (1108.135)*	GER# 17 (868.742)

Table 2.

Genes downregulated in aggressive vs. tame foxes with a FDR ≤ 0.01.

GeneID (CanFam3.1)	Gene Symbol	Gene Name	q-Value	Log2 Fold Change (aggressive vs. tame)	Fold Change (aggressive vs. tame)
ENSCAFG00000004656	*ITGA8*	integrin subunit alpha 8	$1.11e^{-7}$	−1.58	0.33
ENSCAFG00000003681	*DKKL1*	dickkopf like acrosomal protein 1	$1.63e^{-5}$	−1.02	0.49
ENSCAFG00000002070	*FAM32A*	family with sequence similarity 32 member A	$4.66e^{-5}$	−2.17	0.22
ENSCAFG00000023837	*PTGFRN*	prostaglandin F2 receptor inhibitor	0.0004	−0.42	0.74
ENSCAFG00000009483	*FARS2*	phenylalanyl-tRNA synthetase 2, mitochondrial	0.001	−0.40	0.76
ENSCAFG00000004673	*KHK*	ketohexokinase	0.002	−0.53	0.69
ENSCAFG00000001254	*EGR1*	early growth response 1	0.002	−0.97	0.51
ENSCAFG00000000824	*P4HA2*	prolyl 4-hydroxylase subunit alpha 2	0.002	−0.58	0.67
ENSCAFG00000028566	*NRSN1*	neurensin 1	0.005	−0.63	0.65
ENSCAFG00000032583	*METTL7A*	methyltransferase like 7A	0.005	−0.67	0.62
ENSCAFG00000029948	*IDNK*	IDNK, gluconokinase	0.007	−0.48	0.71
ENSCAFG00000015068	*ITIH3*	inter-alpha-trypsin inhibitor heavy chain H3	0.008	−2.27	0.21
ENSCAFG00000015137	*NBL1*	neuroblastoma suppressor of tumorigenicity 1	0.01	−0.42	0.74
ENSCAFG00000017704	*ARMCX3*	armadillo repeat containing X-linked 3	0.01	−0.36	0.78

Table 3.

Genes upregulated in aggressive vs. tame foxes with a FDR ≤ 0.01.

GeneID (CanFam3.1)	Gene Symbol	Gene Name	q-Value	Log2 Fold Change (aggressive vs. tame)	Fold Change (aggressive vs. tame)
ENSCAFG00000006875	*DDX4*	DEAD-box helicase 4	$2.81e^{-6}$	2.54	5.82
ENSCAFG00000007408	*THNSL2*	threonine synthase like 2	$3.28e^{-5}$	0.50	1.41
ENSCAFG00000029000	*SERPINA5*	plasma serine protease inhibitor	$3.91e^{-5}$	2.96	7.78
ENSCAFG00000004585	*TMEM176B*	transmembrane protein 176B	$7.01e^{-5}$	0.60	1.52
ENSCAFG00000015743	*WASHC1*	WASH complex subunit 1	$7.42e^{-5}$	0.41	1.33
ENSCAFG00000006851	*SLC38A9*	solute carrier family 38 member 9	0.0001	0.71	1.63
ENSCAFG00000007746	*PRIMPOL*	primase and DNA directed polymerase	0.0007	0.64	1.56
ENSCAFG00000003841	*MGAM*	maltase-glucoamylase	0.0008	2.42	5.35
ENSCAFG00000009506	*ABI3BP*	ABI family member 3 binding protein	0.0008	1.09	2.13
ENSCAFG00000004768	*STAC*	SH3 and cysteine rich domain	0.001	0.77	1.70
ENSCAFG00000023655	*SULT1C4*	sulfotransferase family 1C member 4	0.002	0.79	1.73
ENSCAFG00000012226	*COL6A3*	collagen type VI alpha 3 chain	0.003	1.06	2.09
ENSCAFG00000013160	*NPL*	N-acetylneuraminate pyruvate lyase	0.004	0.56	1.48
ENSCAFG00000017553	*S100A6*	S100 calcium binding protein A6	0.005	0.72	1.64
ENSCAFG00000006555	*PRKDC*	protein kinase, DNA-activated, catalytic polypeptide	0.007	0.35	1.28
ENSCAFG00000016848		acyl-coenzyme A thioesterase 1	0.01	0.47	1.39
ENSCAFG00000013504	*CLEC7A*	C-type lectin domain containing 7A	0.01	0.97	1.96
ENSCAFG00000019498	*CEP104*	centrosomal protein 104	0.01	0.37	1.29

Table 4.

The top 10 DEGs that are considered to be key players in aggressive vs. tame foxes.

GeneID (CanFam3.1)	Gene Symbol	Gene Name	q-Value	Log2 Fold Change (aggressive to tame)	Directionality in terms of expression in tame foxes
ENSCAFG00000030080	*CD302*	CD302 molecule	0.04	0.62	↓
ENSCAFG00000019498	*CEP104*	centrosomal protein 104	0.01	0.36	↓
ENSCAFG00000003502	*FUZ*	fuzzy planar cell polarity protein	0.05	0.33	↓
ENSCAFG00000004656	***ITGA8***	**integrin subunit alpha 8**	**$1.11e^{-7}$**	**−1.58**	↑
ENSCAFG00000007746	*PRIMPOL*	primase and DNA directed polymerase	0.0007	0.64	↓
ENSCAFG00000013160	*NPL*	N-acetylneuraminate pyruvate lyase	0.003	0.56	↓
ENSCAFG00000006555	*PRKDC*	protein kinase, DNA-activated, catalytic polypeptide	0.007	0.35	↓
ENSCAFG00000017553	*S100A6*	S100 calcium binding protein A6	0.005	0.72	↓
ENSCAFG00000001002	*SMPDL3A*	sphingomyelin phosphodiesterase acid like 3A	0.02	0.54	↓
ENSCAFG00000012697	*TRIM52*	tripartite motif containing 52	0.03	0.38	↓

Table 5.

Genes containing exons skipped at different frequencies in aggressive vs. tame fox hypothalamic reads (FDR < 0.0001), including conserved domains overlapping the skipped exon.

Gene	Exon Location	Percent Reads Skipping Exon (Tame)	Percent Reads Skipping Exon (Aggressive)	FDR	Skipped in Isoforms*	In Conserved Domains
GSTA4	chr12: 20433452–20433585	0.253	1.773	0	X1, X2, X3	Thioredoxin_like super family
GSTA4	chr12: 20435194–20435246	1.162	6.964	0	X1, X2, X3	None
GSTM4	chr6: 42206292–42206393	0.391	4.652	0	X1, X2, X3, X4 (*Canis* forms)	GST_C_family super family
GSTM4	chr6: 42206493–42206575	0.562	5.977	0	X1, X2, X3, X4 (*Canis* forms)	Thioredoxin_like super family
TMA16	chr15: 59743149–59743234	0.204	5.519	4.92E-11	X1, X2, X3 (*Canis* forms)	Tma16 super family
MTHFSD	chr5: 66312701–66312815	0.000	0.929	6.00E-11	None	None
NA	Chr23: 27215997–27216139	4.983	0.715	5.33E-10	X5, X6, X7	None
CDKL2	chr32: 168420–168527	3.841	14.218	5.62E-10	X1, X2, X5	None
DNAJC15	chr22: 7795378–7795430	3.804	4.762	1.89E-08	X1, X2 (*Canis* forms)	None
LRP12	chr13: 5710069–5710126	14.973	28.571	2.53E-08	X1	None
MFAP5	chr27: 37034668–37034704	8.654	0.921	4.55E-08	X1	None
MIOS	chr14: 23205051–23205181	4.888	4.211	8.67E-08	X1, X2, X3	RING_Ubox super family
UNC5D	chr16: 29308689–29308857	12.844	0.895	7.93E-08	None	TSP1
BCAS1	chr24: 39703194–39703265	2.025	18.421	1.32E-07	X1, X2, X3, X4, X5, X6	None
FAM71E1	chr1: 106261638–106261757	37.838	7.264	2.04E-07	X1, X2	None
DNAJC15	chr22: 7792280–7792354	1.429	5.000	1.05E-06	X1, X2 (*Canis* forms)	None
MPZL1	chr7: 30684041–30684144	1.124	0.413	3.62E-06	None	None
PTGDS	chr9: 48649471–48649611	0.724	10.638	7.45E-06	None	Lipocalin
FBRSL1	chr26: 542234–542303	2.769	2.542	8.98E-06	X1, X2, X3, X4, X7, X8, X9, X12, X13, X15, X16, X17, X18, X19, X20, X21	None
KIAA0556	chr6: 19001226–19001494	5.252	10.294	1.70E-05	X1, X2, X3	None
TRIM65	chr9: 4716736–4716911	19.811	4.594	2.92E-05	X1, X2	None
PECAM1	chr9: 12181163–12181220	21.071	2.933	3.50E-05	X1	None

Gene	Exon Location	Percent Reads Skipping Exon (Tame)	Percent Reads Skipping Exon (Aggressive)	FDR	Skipped in Isoforms*	In Conserved Domains
TADA2A	chr9: 37232686–37232758	7.849	2.616	3.96E-05	X1, X2	None
NA	chr17: 48250060–48250102	0.880	12.000	6.17E-05	X1, X2, X3, X4, X5, X6, X7	None
EPB41L5	chr19: 29940406–29940471	27.778	3.826	0.000136	X1, X2, X3, X4, X5	None

*If not specified, the listed isoforms represent those in *Vulpes vulpes*.