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Social status mediated variation in hypothalamic transcriptional profiles of male mice☆

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ABSTRACT

Animals of different social status exhibit variation in aggression, territorial and reproductive behavior as well as activity patterns, feeding, drinking and status signaling. This behavioral and physiological plasticity is coordinated by underlying changes in brain gene transcription. Using Tag-based RNA sequencing (Tag-seq), we explore RNA transcriptomes from the medial preoptic area (mPOA) and ventral hypothalamus (vHYP) of male mice of different social ranks in a dominance hierarchy and detect candidate genes and cellular pathways that underlie status-related plasticity. Within the mPOA, oxytocin (*Oxt*) and vasopressin (*Avp*) are more highly expressed in subdominant mice compared to other ranks, while nitric oxide synthase (*Nos1*) has lower expression in subdominant mice. Within the vHYP, we find that both orexigenic and anorexigenic genes involved in feeding behavior, including agouti-related peptide (*Agrp*), neuropeptide-Y (*Npy*), galanin (*Gal*), proopiomelanocortin (*Pomc*), and Cocaine- and Amphetamine-Regulated Transcript Protein prepropeptide (*Cartpt*), are less expressed in dominant animals compared to more subordinate ranks. We suggest that this may represent a reshaping of feeding circuits in dominant compared to subdominant and subordinate animals. Furthermore, we determine several genes that are positively and negatively associated with the level of despotism (aggression) in dominant males. Ultimately, we identify hypothalamic genes controlling feeding and social behaviors that are differentially transcribed across animals of varying social status. These changes in brain transcriptomics likely support phenotypic variation that enable animals to adapt to their current social status.

1. Introduction

Social dominance hierarchies are a common form of social organization found throughout the animal kingdom. They emerge when individuals living in a group compete for resources such as territory, food and mates (Chase, 1982; Drews, 1993). As relationships within a hierarchy are established, individuals shift multiple phenotypes as a function of their status (Milewski et al., 2022). Dominant animals will often increase their status signaling, reproduction, aggression, food intake and metabolic rate, while subordinate animals typically inhibit these traits (Biro and Stamps, 2010; Ellis, 1995; Emery Thompson et al., 2009; Lee et al., 2018).

Across taxa, this phenotypic plasticity relies on underlying changes in gene expression. A striking example is found in the eusocial caste system of female paper wasps. Whole-body and central variation in metabolic function, stress response, and foraging genes have been identified between reproductive queens and non-reproductive workers (Sumner et al., 2005; Toth et al., 2010). In male African cichlid fish, social rank ascension is linked to rapid changes in body color and reproductive activity. These shifts in status-related phenotypes are associated with elevated sex steroid receptor gene expression in sex organs as well as the brain (Burmeister et al., 2007; Maruska and Fernald, 2011). In particular, androgen receptors α and β additively regulate testes growth, coloration, reproduction and aggression in male cichlids (Alward et al., 2020, 2019). Primate studies have also thoroughly investigated the link between shifts in social status and genomic expression. Immune gene expression profiles associated with acquisition of social status have been identified in male baboons and female macaques (Lea et al., 2018; Snyder-Mackler et al., 2016; Tung et al., 2012). Recent work by our lab also suggests that differential immune gene

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expression in hepatic and splenic tissues also exists among hierarchically organized mice (Lee et al., 2022; Williamson et al., 2016b).

We have previously demonstrated that dominant male mice in social hierarchies are more active, aggressive, urinate more frequently and consume more food and water compared to subordinate males (Lee et al., 2018, 2017; Williamson et al., 2016b). We have also found that dominant male mice exhibit elevated expression of hepatic genes that promote fatty acid catabolism and energy production and lower expression of genes involved in fatty acid synthesis compared to subordinate males, which may be indicative of status-related differences in energy usage (Lee et al., 2022) Additionally, dominant males exhibit elevated hepatic expression of major urinary proteins (MUPs) genes that are used to attract mates and mark territory (Lee et al., 2017). Others have identified *Crh* expression in the pontine micturition center (PMC) as a key regulator of urination in dominant and subordinate mice (Hou et al., 2016). Using a candidate gene approach, we have also found that dominant male mice have elevated expression of glucocorticoid receptor, *Crh*, *Gnrh1*, and *Bdnf* mRNA and reduced expression of DNA methyltransferases (*DNMTs*) in various brain regions including the amygdala, hippocampus and medial preoptic area (mPOA) (So et al., 2015; Williamson et al., 2017b, 2016a). Despite these findings, there is still much that is unknown about differential gene expression in the brains of dominant and subordinate animals. In the present study, we use a global transcriptomic approach to identify potential gene targets that underlie divergent social status phenotypes. Although we chose male subjects to build on our previous findings, future studies will investigate transcriptomic differences in female hierarchies.

We collected mPOA and ventral hypothalamus (vHYP) tissue from the most dominant (alpha), the second-most dominant (subdominant) and the most subordinate (subordinate) male mice living in stable linear social dominance hierarchies of 10 mice. The mPOA is known to mediate a variety of social behaviors in mammals including sexual behavior, scent marking and aggression (Albers, 2012; Chen and Hong, 2018). Significantly elevated immediate early gene expression is observed in the mPOA during agonistic resident-intruder interaction (Motta et al., 2009) and activity in this region guides rank-dependent micturition patterns via projections to the PMC (Hou et al., 2016). Additionally, our previous candidate gene approach demonstrated transcriptional differences between dominant and subordinate mice in this region (So et al., 2015; Williamson et al., 2017b). The vHYP includes both ventromedial hypothalamus (VMH) and arcuate nucleus (ARC). The VMH, particularly the subpopulation of neurons expressing estrogen receptor 1, is engaged during aggressive, mating and defensive social behaviors and, along with the ARC, is implicated as a crucial node in feeding and drinking regulation (Kohno and Yada, 2012; Pinto et al., 2004).

Total mRNA from each mPOA and vHYP sample was extracted, and we used a tag-based RNA sequencing (Tag-seq) method to obtain transcriptome profiles (Lohman et al., 2016; Meyer et al., 2011). Tag-seq is an efficient and cost-effective approach for this initial exploration of differential gene expression in dominance hierarchies. It requires very few sequencing reads and is resilient to variation in sample integrity (Stark et al., 2019). While it does not yield complete RNA sequences including splice variants (i.e. coding and non-coding RNA), it is suitable for identifying coding genes that are differentially expressed. With these transcriptome profiles, we identified differentially expressed genes (DEG) among alpha, subdominant, and subordinate mice. We focused our analysis on genes previously reported to regulate neuroendocrine status and social and feeding behavior, but also identified several novel DEGs that merit future investigation. We also explored gene networks using weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) to identify modules of co-expressed genes that differ by status.

2. Materials and methods

2.1. Animals and housing

A total of 110 male outbred Crl:CD1 (ICR) mice aged 7 weeks old were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in the animal facility at the University of Texas at Austin. The animal facility was kept on 12/12 light/dark cycle, with white light on 2300 h and red lights (dark cycle) on at 1100 h with constant temperature (21–24 °C) and humidity (30–50%). Upon arrival, mice were housed in pairs in standard cages (27 × 17 × 12 cm) with pine shaving bedding (Nepco, Warrensburg, NY, USA). After 72 h of habituation to the facility, all mice were marked according to their unique IDs with nontoxic animal markers (Stoelting Co., Wood Dale, IL, USA). At Zeitgeber Time (ZT) 00 on the 11th day since arrival, groups of 10 mice were weighed and placed into large, structurally complex, custom built vivaria (Supplemental Fig. S1; 150 × 80 × 220 cm^3; Mid-Atlantic, Hagerstown, MD). Each vivarium consists of an upper level with three floors and a lower section with five standard cages covered by a red transparent curtain, mimicking a burrow system and creating one-way visibility for experimenters to observe mice while they are shielded from light. In total, each vivarium provides approximately 62,000 cm^2 total surface area. We provided standard chow and water ad libitum at the top of vivaria. Trained observers conducted live behavioral observation for 2 weeks only during the dark cycle, recording the winner, the loser, and specific agonistic behavior observed in all aggressive instances (see Supplemental Table S1 for ethogram). Data were collected and instantly uploaded to Google Drive with timestamps. We conducted all procedures with approval from the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC protocol: AUP-2018-00119) and in accordance with National Health Institute (NIH; Bethesda, MD, USA) animal care guidelines.

2.2. Tissue collection and preparation

At Zeitgeber Time (ZT) 06 on the 15th day of group housing, mice were weighed and euthanized via decapitation. Trunk blood samples were collected into EDTA-coated Vacutainer tubes (Becton Dickinson) and gently inverted a few times and stored on ice for 45–60 min prior to subsequent procedures. 200 μl of blood aliquots were centrifuged to collect plasma and stored at −80 °C until corticosterone assay. Remaining blood was used for immune cell profiling (see Lee et al., 2022). Brains were collected, flash frozen in hexane and stored at −80 °C until dissection. We selected brains from the alpha, subdominant and the most subordinate or the second most subordinate individuals based on Glicko rating (see below), then dissected two brain regions separately, mPOA (bregma +0.14 mm) vHYP (bregma −1.46 mm, containing ventromedial hypothalamus (VMHvl) and arcuate nucleus (ARC)), using Stoelting 0.51 mm tissue punch (Stoelting, Wood Dale, IL, Cat. No. 57401) and homogenized in 100 μl lysis buffer (Thermo Fisher Scientific, Waltham, MA; MagMax Total RNA isolation kit, Cat. No. A27828) with 0.7% beta-mercaptoethanol by vortexing at 3000 rpm speed for 15–20 s. Lysates were incubated at room temperature for 5 min and stored at −80 °C until RNA extraction. Once dissection of all individuals was completed, we proceed to RNA extraction using the KingFisher Flex (Thermo Fisher Scientific, Cat. No. 5400630l) with an additional DNase step added according to the manufacturer's protocol. Samples from each brain region of all animals were extracted in the same batch. RNA quality was determined using RNA 6000 Nano Assay with BioAnalyzer (Agilent Technologies, Santa Clara, CA) and RNA concentration was determined with Quant-it RNA High Sensitivity assay kit (Thermo Fisher Scientific, Cat. No. Q33140). RNA samples were normalized to 100 ng/μl and stored at −80 °C before sequencing.

2.3. Analysis of agonistic behavior data

An extensive characterization of the behavior of subjects used in this study is presented elsewhere (Lee et al., 2022). Briefly, we aggregated win-loss data for each social group to calculate Landau's modified h′ value and triangle transitivity to determine whether and how quickly each social group formed a significantly linear social hierarchy (see Williamson et al., 2016b for details) via compete R package (Curley, 2016). Glicko ratings were used to identify the social rank of each mouse in each social group. Briefly, all individuals start with the same initial rating (set as 2200) and gain or lose points after each aggressive interaction. The amount of points gained or lost is weighed based on the rating differences between two individuals engaging in aggressive interactions. A mouse with the highest Glicko rating in each social group at the end of group housing period is considered as an alpha male, the ones with Glicko ratings higher than initial points (2200) are subdominant males (Lee et al., 2018). We used the PlayerRating R package (Stephenson and Sonas, 2020) to calculate the Glicko ratings. We also calculated the degree of despotism in alpha males in each hierarchy and assessed how this measurement varied with brain transcriptomes. The degree of despotism of each alpha male is defined as the proportion of wins attributed to the alpha males to all aggressive interactions occurring during the group housing period (Williamson et al., 2016b). All calculations and statistical analysis were conducted in R v 4.1.0 (R Core Team, 2021).

2.4. Bioinformatics data analysis

Extracted RNA samples (N = 33/region, N = 11/status/region (alpha, subdominant, subordinate)) were submitted to the Genome Sequence and Analysis Facility at the University of Texas at Austin for Tag-based RNA sequencing. This method is a cost-effective approach specifically designed to measure abundances of polyadenylated transcripts yielding highly reliable data for differential gene expression analysis in well annotated genomes (Lohman et al., 2016; Meyer et al., 2011). All 66 samples were processed in the same batch throughout the process. Libraries were constructed with a protocol modified from Meyer et al. (2011) and Lohman et al. (2016). Reads were sequenced on the NovaSeq 6000 SR100 with minimum reads of 4 million and the target reads per sample of 5 million. Raw reads were processed to obtain gene count data by following the TagSeq data processing pipeline provided based on Meyer et al. (2011) and Lohman et al. (2016). Briefly, customized perl script utilizing FASTX-Toolkits and CUTADAPT 2.8 (Martin, 2011) was used to remove reads with a homo-polymer run of "A" $\geq$ 8 bases and retain reads with minimum 20 bases and removal PCR duplicates. Processed reads were then mapped to *Mus musculus* reference genome (Ensembl release 99) using Bowtie2 (Langmead and Salzberg, 2012).

Differential gene expression analysis was conducted using Bioconductor package limma (Smyth et al., 2021). With 33 samples (N = 11/status) for each brain region, we conducted principal component analysis filtered gene counts data (filtered out genes with less than 10 counts for each sample) to visually inspect for outliers. No outliers were detected for both regions. Filtered read counts were then normalized to account for different library sizes among samples with a voom transform. Our preliminary analysis showed that expression profiles of immediate early genes are associated with the time between mice being taken out of their housing and brain tissues being collected. The median time for removal and extraction was 12 min (range 1–50 min). Therefore, to avoid false positive discovery, we eliminated immediate early genes from the analysis. Differentially expressed genes were identified for three comparisons: alpha vs. subdominant, alpha vs. subordinate, and subdominant vs. subordinate. We adjusted raw p-value via empirical false discovery rate (eFDR) (Storey and Tibshirani, 2003) (eFDR). To estimate eFDR, we permuted sample IDs for 5000 times and obtained a null distribution of p-values. The significance threshold for differentially expressed genes (DEGs) was set as 15% of change in the absolute values of log 2-fold change at the eFDR of 5%. With the same normalization procedure and the same p-value adjustment method, we identified genes associated with the degree of despotism using mPOA and vHYP transcriptomes of 11 alpha males. We performed gene ontology (GO) enrichment analysis to explore functional differences among identified DEGs for each comparison using clusterProfiler R package (Wu et al., 2021). We tested for an association between corticosterone levels and regulatory feeding genes in the vHYP using a Bayesian hierarchical linear regression model, with corticosterone level and social status as fixed factors and social group ID as a random factor via the brms R package (Bürkner, 2020). The estimated associations were considered statistically significant if their 95% highest density interval (HDI) did not overlap with zero.

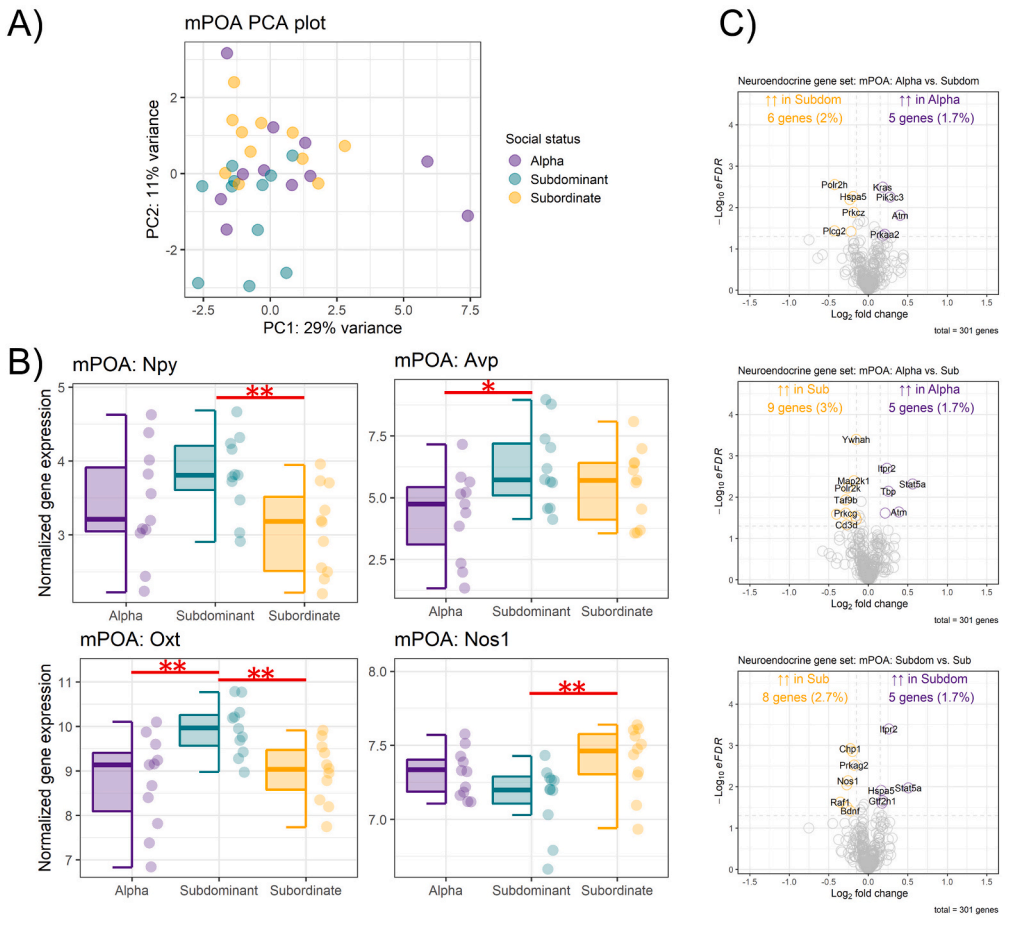
Weighted gene co-expression network analysis was performed using the WGCNA R package (Langfelder and Horvath, 2008). WGCNA provides advantages in navigating groups of genes associated with experimental factors by constructing Pearson correlation matrices and clustering highly co-expressed genes into several modules. WGCNA then examines the association between given experimental factors and module eigengenes (MEs) which is the first component from the principal component analysis (PCA) for each module. WGCNA further calculates module membership for each gene. Module membership is measured as the Pearson correlation between the gene expression level and the module eigengene and the absolute value of module membership close to 1 indicates that the gene is highly connected to other genes in the module. As we collected 11 samples per social status (alpha, subdominant and subordinate) and the use of WGCNA analysis is recommended to use with a total sample number greater than 20, we aggregated count data across all three social status groups (total of 33 samples) for each brain region and constructed gene co-expression network for each brain region. Gene counts were normalized with the limma package and genes that had low expression counts (<10) in more than 90% of samples were filtered out prior to network construction. Each WGCNA model was constructed in signed hybrid mode with power of 4 and minimum module size set as 50 genes. We tested whether each module eigengene is significantly different across social status using linear regression models with social group ID as a random factor. We used brms R package (Bürkner, 2020) with student family combined with identity link for robust linear regression to avoid artifacts influenced by outliners. We considered the estimated differences are statistically significant when their 95% highest density interval (HDI) of the estimated effect value did not overlap with zero.

3. Results

3.1. Differentially expressed genes analysis

All 11 cohorts of 10 males formed linear and stable social hierarchies as measured by modified Landau's h′ (median, (interquartile range, IQR) = 0.87 (0.81–0.92), all $p < 0.001$) and triangle transitivity (0.91 (0.80–0.94), all $p < 0.001$). More extensive characterization of the behavior of these subjects is presented elsewhere (Lee et al., 2022). Mice occupied unique social ranks 1 to 10 in each hierarchy and we selected rank 1 (alpha), rank 2 (subdominant) and rank 10 (subordinate) mice to investigate their brain transcriptomes. Using the limma R package (Smyth et al., 2021) we identified differentially expressed genes in three comparisons: a) alpha vs. subdominant, b) alpha vs. subordinate, and c) subdominant vs. subordinate.

In the mPOA, 2002 out of 15,655 genes, 12.8% of genes were differentially expressed in at least one of the three comparisons. The numbers of DEGs, genes with the highest log 2-fold changes, and corresponding gene ontology terms for each comparison are summarized in Fig. 1. PCA of mPOA transcriptomes showed a greater distinction between subdominants and subordinates relative to other comparisons (Fig. 1A). Interestingly, we observed that subdominant males either



D)

	# of DEG	Top genes	GO terms
↑ in alpha vs. subdom	367	*Samd3* *Cryba4* *Clec3b* *Spp1* *Col3a1*	No GO terms identified
↓ in alpha vs. subdom	405	*Avp* *Padi4* *Oxt* *Sst* *Omp*	No GO terms identified
↑ in alpha vs. sub	431	*Sema3b* *Myh6* *Jhy* *Tmem52* *Fgl2*	response to zinc ion alpha-beta T cell proliferation mucosa-associated lymphoid tissue develop. Peyer's patch development
↓ in alpha vs. sub	327	*Rpl21* *Nfe2* *Itga1* *Olfr17* *Myoz2*	reg. of small molecule metabolic process neg. reg. of small molecule metabolic process reg. of purine nucleotide metabolic process reg. of nucleotide biosynthetic process reg.of purine nucleotide biosynthetic process
↑ in subdom vs. sub	533	*Padi4* *Cdh23* *Omp* *Msantd1* *Acsbg2*	No GO terms identified
↓ in subdom vs. sub	415	*Rps24-ps3* *Ttr* *Cdhr1* *Smoc2* *Slc25a21*	regulation of small molecule metabolism cellular ketone metabolic process regulation of axonogenesis neuron recognition regulation of collateral sprouting

Fig. 1. Results of differential gene expression (DGE) analysis in the mPOA. (A) Principal component analysis (PCA) of RNA-seq data in the mPOA. (B) Box plots show normalized expression levels of genes implicated in social behavior across social status. *eFDR<0.05, **eFDR<0.01. (C) Volcano plots show significance (eFDR) for differentially expressed genes (DEGs) for each pairwise comparison of social status in neuroendocrine gene sets. DEGs met criteria if the absolute values of log2 fold change were greater than 15% at the empirical false discovery rate (eFDR) of 5%. (D) Summary of DGE analysis by socials status and Gene Ontology Biological Process (GO-BP) analysis for upregulated or downregulated DEGs for each status comparison. Top genes include five genes with the highest absolute value of log2 fold change in gene expression. Alpha = alpha male, Subdom = subdominant male, Sub = subordinate male.

have the highest or lowest gene expression levels in several genes associated with social behavior. For instance, *Oxt* (oxytocin) expression (Fig. 1B) was significantly greater in subdominant mice compared to both alpha (eFDR = 0.008) and subordinate mice (eFDR = 0.006). Subdominant males also showed greater *Avp* (vasopressin) expression compared to alpha mice (eFDR = 0.017), while subordinate mice did not (Fig. 1B). *Npy* (neuropeptide-Y) expression was also significantly greater in subdominant mice compared to subordinate (eFDR = 0.005). In contrast, *Nos1* (nitric oxide synthase 1) was more highly expressed in subordinate compared to subdominant mice (eFDR = 0.007) but not alphas. Further, we examined transcription profiles of genes known to be involved in neuroendocrine functions as previously curated by (van Donkelaar et al., 2020) (Fig. 1C). Across all three comparisons, the number of up- and down-regulated genes did not differ significantly (Fig. 1C & D).

In vHYP, 2317 out of 15,396 genes (15%) were differentially expressed in at least one of the three comparisons. Social status did not critically account for separation of the PCA plot of vHYP, as shown in Fig. 2A. Unsurprisingly, given the inclusion of ARC in the vHYP, genes associated with the regulation of food intake and energy expenditure were differentially expressed across status, such as *Npy*, *Agrp* (agouti-related peptide), *Pomc* (proopiomelanocortin) and *Gal* (galanin) (Fig. 2B & D). These four genes had lower expression in alpha males compared to subdominant and subordinate mice. We further investigated expression patterns of other vHYP genes involved in food intake and energy metabolism (Fischer and O'Connell, 2017) (Supplemental Fig. S2). *Cartpt* (Cocaine- and Amphetamine-Regulated Transcript Protein prepropeptide) was also significantly less expressed in the vHYP of alpha males compared to subdominant males (eFDR = 0.045) and moderately less expressed compared to subordinate males (eFDR = 0.058). Conversely, *Avp* was expressed at higher levels in alpha males compared to subdominant males (eFDR = 0.048). There were no differences in *Hcrt* (hypocretin), *Oxt*, *Crh* (corticotropin releasing hormone), or *Pmch* (pro-melanin concentrating hormone) expression levels across status.

We additionally examined the differential expression of steroid hormone receptors. We found no evidence in either brain region of differential expression of glucocorticoid receptor (*Nr3c1*), mineralocorticoid receptor (*Nr3c2*), progesterone receptor (*Pgr*), estrogen receptor alpha (*Esr1*) or estrogen receptor beta (*Esr2*). In the vHYP, androgen receptor (*Ar*) was moderately significantly more highly expressed in subdominants compared to alphas in the vHYP (eFDR = 0.014) (Supplemental Fig. S2). Also in the vHYP, estrogen-related receptor alpha (*Esrra*) and estrogen-related receptor gamma (*Esrrg*) were significantly more highly expressed in alphas compared to subdominants (eFDR < 0.003). Subdominants also had significantly lower levels of *Errra* compared to subordinates (eFDR = 0.043).

While we did not observe differential expression of *Crh* in the vHYP, we did find that levels of circulating corticosterone were higher in subordinate mice, and even more so in mice living in highly despotic hierarchies. The methods and findings are described in a previous publication using the same experimental subjects (see Lee et al., 2022). We further investigated a possible link between corticosterone and feeding genes (Supplemental Fig. S3). However, we did not find any significant positive associations between corticosterone levels and gene expression for *Agrp*, *Npy*, *Gal*, *Pomc* or *Cartpt* after accounting for status.

3.2. Weighted gene expression analysis (WGCNA)

WGCNA identified 24 modules in mPOA and 12 modules in vHYP. The module eigengenes (MEs) of eight mPOA modules (blue, brown, green, red, pink, magenta, greenyellow, lightcyan, and royalblue) showed significant differences in at least one of the three comparisons across social status (Fig. 3A). Fig. 3C summarizes genes with the highest module membership (MM; genes that are closely connected with other genes in the module) and GO terms associated with the genes in each module. Of particular note, genes in the greenyellow module were significantly more highly expressed in subordinate males compared to both alphas and subdominants. These genes included those involved in the protein catabolism and the regulation of the intracellular estrogen receptor signaling pathway. Supplemental Fig. S4 summarizes this information for other social-status-relevant modules that showed significant differences by social status.

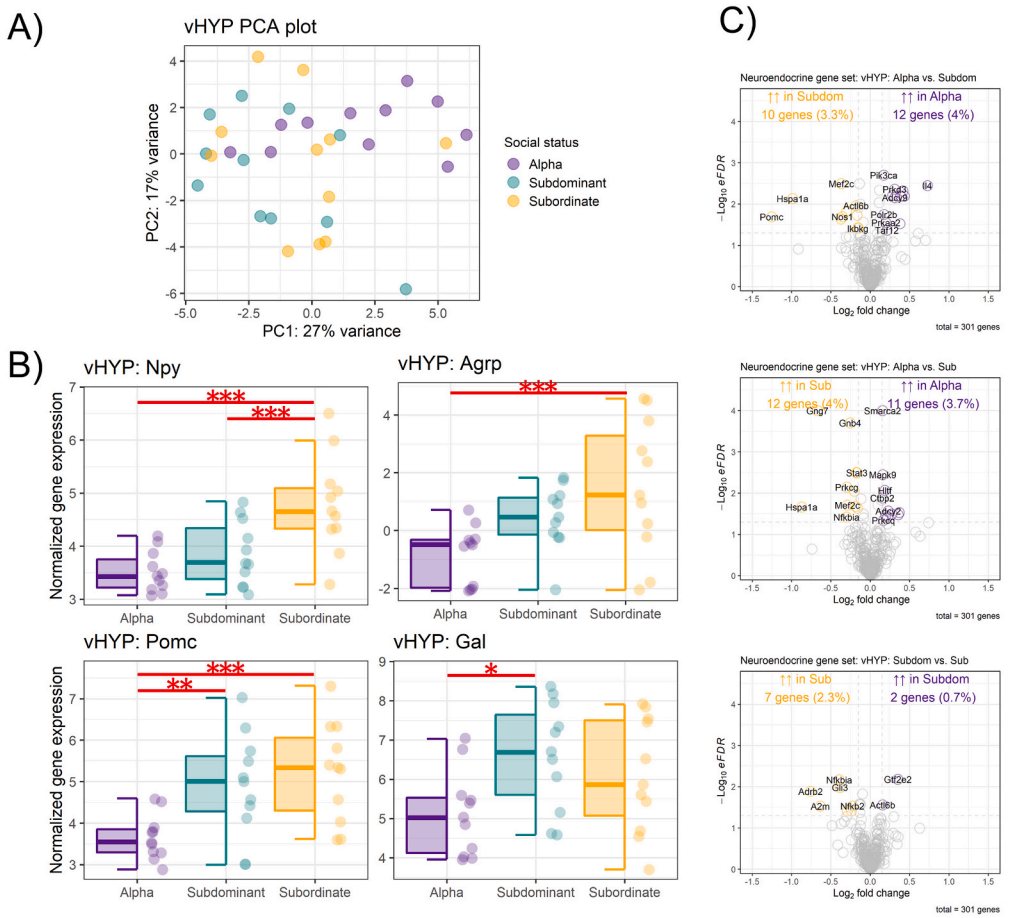
In vHYP, the MEs of three modules (blue, green, black) showed differences between alpha males and other mice (Fig. 3B). Genes with high module membership (MM) and GO terms of these modules are summarized in Fig. 3C. and Supplemental Fig. S4. The black module had significantly higher ME expression in alpha males compared to subordinate males. This module included genes related to histone and chromatin modification including the imprinted gene *Peg3*. The blue module had significantly higher ME expression in alphas compared to both subordinate and subdominant males. The blue module included genes involved in cellular respiration for generating energy. The green module exhibited significantly lower expression in alpha males compared to both subdominant and subordinate males. This module included genes involved in regulation of the cell cycle.

3.3. Transcription profiles associated with despotism in alpha males

Despotism observed across eleven alpha males ranged from 0.33 to 0.63, with a mean of 0.49 and a median of 0.45 (Fig. 4A). Here we conducted differential gene expression (DGE) analysis against despotism only with transcriptomic data of alpha males (N = 11). The DEGs with the highest log 2-fold changes are listed in Fig. 4B and expression profiles of selected DEGs are visualized in Fig. 4C. In mPOA, 246 genes were significantly positively associated with despotism (i.e. expressed at higher levels in more despotic alpha males) and 331 genes significantly negatively associated with despotism. No GO terms for higher or lower expressed DEGs were identified. In vHYP, 242 genes were significantly positively associated with despotism and 284 genes were significantly negatively correlated. While there were no significant GO terms identified for negatively correlated genes, several GO terms for positively correlated genes were identified: cellular response to topologically incorrect protein, regulation of transcription from RNA polymerase II promoter in response to stress, regulation of DNA-templated transcription in response to stress, integrated stress response signaling. A total of 28 genes were identified as DEGs in both regions while 60% of those genes (17 genes) were differentially expressed in the same direction. Interestingly, one of the ribosomal protein genes, *Rpl21*, exhibited greater expression in highly despotic alpha males in both regions (Fig. 4B). We also investigated transcription profiles of the genes previously associated with aggression. We used a curated list of 40 genes identified as associated with aggression in mice and humans from Zhang-James et al., 2019. Among 40 genes, 36 genes were expressed significantly in mPOA and vHYP. In mPOA, none of these 36 genes were significantly associated with despotism. In vHYP, one gene, *Ache* (acetylcholinesterase) was positively associated with despotism (Fig. 4C). As *Cartpt* (Prepropeptide) exhibited lower expression in the vHYP of alpha males compared to subdominants and subordinate males, we further explored its expression profile according to variation in despotism. Interestingly, *Cartpt* expression was actually lower in more despotic alpha males (Fig. 4C).

4. Discussion

We identified over 3000 genes that were differentially expressed between mice of different social status in the mPOA and vHYP. Given the broad role of the mPOA in social behavior and rank-related behavior (i.e. scent-marking and aggression), it is not surprising that we identified differential expression of several genes important for social behavior in this region. Notably, many of these genes were differentially expressed in subdominant compared to dominant and subordinate mice (Fig. 1B). *Avp* was more highly expressed in the mPOA of subdominant mice



D)

	# of DEG	Top genes	GO terms
↑ in alpha vs. subdom	741	*Rp1* *Avp* *Pvalb,* *Pmfbp1* *Sp8Col3a1*	regulation of membrane potential neurotransmitter transport regulation of neurotransmitter levels import into cell regulation of neurotransmitter transport
↓ in alpha vs. subdom	578	*Padi4* *Foxd2* *Ghrh* *Npvf* *Wif1*	synapse organization locomotory behavior regulation of nervous system process regulation of neurotransmitter receptor activity
↑ in alpha vs. sub	542	*Stmnd1* *BC051019* *Ccdc33* *Krt8* *Scnn1a*	cilium organization cilium assembly microtubule-based movement
↓ in alpha vs. sub	459	*Agrp* *Crabp1* *Ghrh* *Foxd2* *Pomc*	regulation of membrane potential muscle system process muscle contraction synaptic transmission, glutamatergic positive regulation of behavior
↑ in subdom vs. sub	326	*Padi4* *Slc5a1* *Reg3g* *Krt8* *Rbp2*	cilium organization cilium assembly non-motile cilium assembly axoneme assembly intraciliary transport
↓ in subdom vs. sub	351	*Gnrhr* *Lhx8* *Scn4b* *Drd5* *Pvalb*	negative regulation of neuron projection development diol biosynthetic & metabolic process cAMP biosynthetic process cAMP metabolic process

Fig. 2. Results of differential gene expression (DGE) analysis in the vHYP. (A) Principal component analysis (PCA) of RNA-seq data in the vHYP. (B) Box plots show normalized expression levels of genes involved in feeding regulation across social status. *eFDR<0.05, **eFDR<0.01, ***eFDR<0.005. C) Volcano plots show significance (eFDR) for differentially expressed genes (DEGs) for each pairwise comparison of social status in neuroendocrine gene sets. DEGs met criteria if the absolute values of log2 fold change were greater than 15% at the empirical false discovery rate (eFDR) of 5%. Arrows indicate the direction of the change in gene expression for a given comparison. (D) Summary of DGE analysis by socials status and Gene Ontology Biological Process (GO-BP) analysis for upregulated or downregulated DEGs for each status comparison. Top genes include five genes with the highest absolute value of log2 fold change in gene expression. Alpha = alpha male, Subdom = subdominant male, Sub = subordinate male.

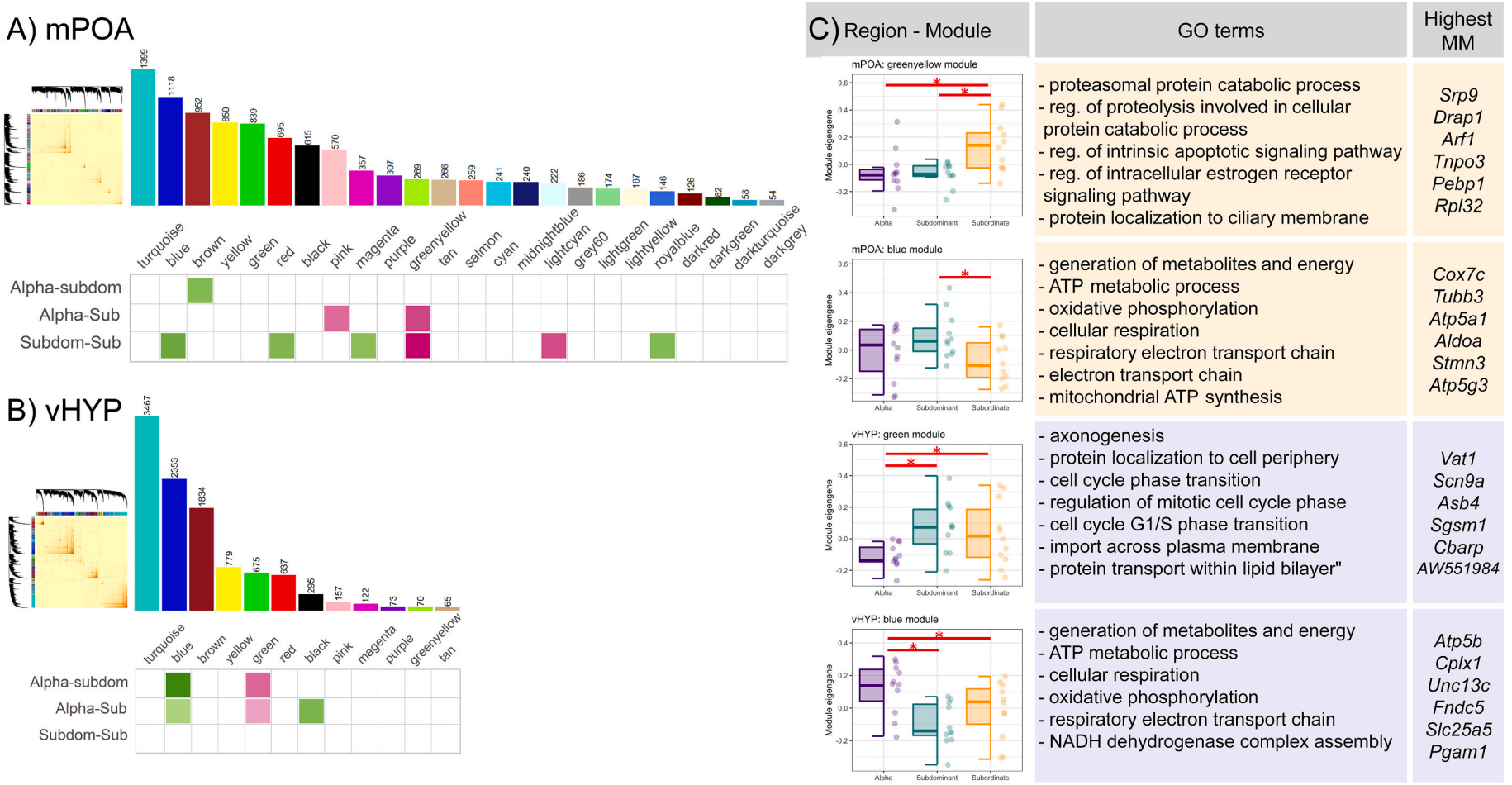


Fig. 3. Results of Weighted Gene Correlation Network Analysis (WGCNA). (A, B) Heatmaps representing linear regression estimates of eigengene expression of WGCNA modules against social status in the mPOA (A) and vHYP (B). Only significant differences in median eigengene expression are represented by colored boxes. Darker shades of green represent relatively higher expression in the social status listed first for each comparison. Darker shades of pink represent relatively higher expression in the social status listed second for each comparison. Numbers refer to the number of genes identified as belonging to each module. (C) Boxplots showing module eigengene expression by social status, summary of GO-BP analysis and list of genes with the highest module membership (MM). A red asterisk represents significant differences as defined by the 95% highest density interval (HDI) of the effect estimate not overlapping with zero. Alpha = alpha male, Subdom = subdominant male, Sub = subordinate male. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

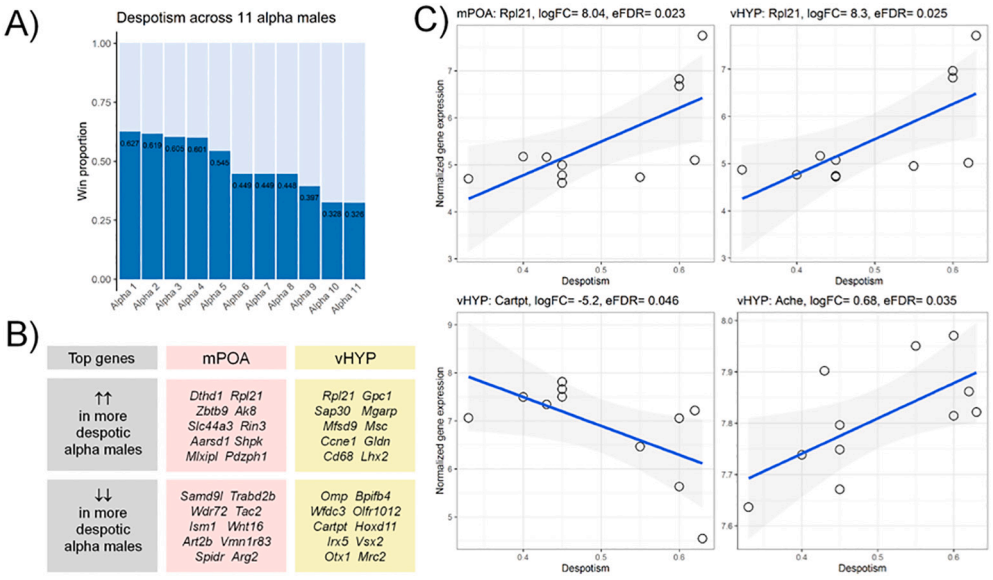


Fig. 4. Results for DGE × Despotism analysis. (A) Stacked bar plots showing despotism, measured as the proportion of wins that belonged to the highest-ranking mouse (Alpha) in each cohort out of the total number of agonistic interactions that resulted in a win for any individual. (B) Top ten genes with the highest positive and negative log2 fold change in gene expression associated with despotism. (C) Scatterplots showing significant relationship between normalized gene expression levels and despotism for selected genes.

compared to alpha mice and trended towards higher expression in subdominant mice compared to subordinates. Avp acting on V1a receptors in the hypothalamus promotes the expression of dominance behaviors (Albers, 2012; Terranova et al., 2017). For example, infusion of Avp in the mPOA controls contextually appropriate flank-marking behavior in dominant and subordinate hamsters (Ferris et al., 1986). Cichlid fish also show elevated activity of vasotocin neurons in the preoptic area following aggressive interactions (Loveland and Fernald, 2017). Thus, the promotion of dominant behavior by Avp may underlie higher *Avp* expression levels in subdominant compared to subordinate mice. However, the relevance of higher expression levels in subdominant compared to alpha mice is unclear. Previous work has shown that alpha male mice have lower V1aR binding in the lateral preoptic area (lPOA) compared to subordinate males (Lee et al., 2019), which also suggests that vasopressin in the preoptic area does not necessarily promote aggression in alpha males. Interestingly, we found that *Avp* was more highly expressed in alpha compared to subdominant mice in vHYP. This finding is consistent with studies in hamsters that have shown that injection of AVP into the ventromedial hypothalamus induces aggression (Delville et al., 1996). These findings leads us to propose that *Avp* may act independently in different hypothalamic subnuclei to support aggressive behavior in subdominants compared to alpha males, though this conjecture needs to be experimentally tested.

Similar to *Avp*, *Oxt* was significantly more highly expressed in the mPOA of subdominant compared to both alpha and subordinate mice. Oxytocin signaling has been positively associated with status in species as diverse as primates and fish (Almeida et al., 2012; Michopoulos et al., 2011), although work in mice and rats has shown that oxytocin signaling can be positively associated with both subordinate behavior and dominant behavior in a brain-region specific manner (Lee et al., 2019; Timmer et al., 2011). The role of oxytocin within the mPOA specifically is also inconsistent across rodent species and sex. Infusion of oxytocin into the mPOA of female hamsters reduces aggression (Harmon et al., 2002), but elevated oxytocin receptor binding in this region has been reported in aggressive compared to non-aggressive Wistar rats (Lukas et al., 2010). It should be noted that the majority of these studies only examined dyadic dominance relationships (i.e. dominant vs. subordinate status). The current study is the first to our knowledge to examine a range of social ranks and reveal a novel non-linear relationship between dominance and oxytocin.

Npy was also more highly expressed in the mPOA of subdominants compared to subordinates. *Npy* positively regulates the expression of gonadotropin-releasing hormone (GnRH) in preoptic neurons (Li et al., 1994) and thus mediates activity of the hypothalamic–pituitary–gonadal (HPG) axis. Previous work has shown that subdominant mice who ascend to alpha status following removal of the established alpha from a group have increased GnRH mRNA in the mPOA compared to those who did not ascend, which is proposed to indicate a shift towards greater reproductive activity associated with dominant status (Williamson et al., 2017b). An upregulation of HPG axis activity could explain elevated *Npy* expression levels in subdominant compared to subordinate mice. In contrast to the above genes, *Nos1*, was less expressed in subdominant mice compared to subordinate mice. *Nos1* is involved in the manufacture of the signaling molecule, nitric oxide (NO). While NO has a multitude of functions in the brain, there is evidence that it is particularly important for social behavior. In rodents, neonatal inhibition of *Nos1* has been linked to social interaction deficits in rats (Black et al., 2002) and impaired NO signaling within the mPOA has been linked to inappropriate aggressive and sexual behavior (Ma et al., 2005; Nelson et al., 1995). Our results suggest that *Nos1* may act as a regulator of social behavior that contributes to differences between subdominant and subordinate social status.

In the vHYP, genes that mediate food intake exhibited lower expression in alpha males compared to subdominant and subordinate mice. We found no correlation between body weight and status or gene expression. Our vHYP dissections included ARC, which is composed of populations of orexigenic neurons, whose activation promotes feeding, and anorexigenic neurons, whose activation inhibits feeding. Genes encoding orexigenic (*Npy*, *AgrP*, and *Gal*) peptides showed reduced expression in alphas compared to lower-ranking mice. Previously we have shown that alpha mice eat much more frequently than all other individuals in the hierarchy (Lee et al., 2018). We propose that higher orexogenic gene expression in subdominant and subordinate males compared to alphas is indicative of their readiness or motivation to feed. Subdominant and subordinate mice tend to forage when alpha males are inactive (e.g. sleeping), suggesting that they must vigilantly monitor their environment for opportunities to feed (Lee et al., 2018). Importantly, our vHYP dissections also included the VMH, which controls food-anticipatory activity in mice conditioned to expect restricted food at a specific time of day (Ribeiro et al., 2009), supporting the role of this region in food motivation in subordinate mice with restrained time windows for feeding.

Conversely, we were surprised to observe elevated vHYP anorexigenic gene expression (*Pomc* and *Cartpt*) in subdominant and subordinate mice compared to alpha males, as anorexigenic and orexigenic ARC neurons exert inhibitory effects onto each other (Luquet et al., 2005).

However, the relationship between these neuronal subpopulations is not necessarily dichotomous. Feeding peptides appear dysregulated in hyperphagic rats following early-life or neonatal stress, such that both *Npy* and *Pomc* mRNA are both upregulated (Maniam and Morris, 2012; Yam et al., 2017). Also, mice exposed to chronic social defeat stress exhibit elevated *Npy*, *Agrp*, and *Gal* (Patterson et al., 2013), though how social stress modulates anorexigenic genes is less clear. Subordinate mice in despotic hierarchies typically exhibit slightly elevated corticosterone levels compared to dominant mice (Williamson et al., 2017a) and in the present study we found that subordinate mice did indeed have higher concentrations of circulating corticosterone compared to dominant mice, and this was exacerbated in highly despotic hierarchies (Lee et al., 2022). We used Bayesian regression analyses to test whether social stress experienced by lower-ranking mice could potentially underlie their elevated levels of feeding pathway genes in the vHYP. This analysis revealed however that corticosterone levels did not predict feeding gene expression after controlling for status. This suggests that the differential expression of orexigenic and anorexigenic genes are likely induced by other features of social status related physiology other than stress.

We also identified genes associated with degree of despotism in alpha males. *Rpl21* expression was greater in more despotic males in both the mPOA and vHYP. *Rpl21* encodes a fundamental ribosomal protein and thus could have a myriad of downstream effects on translation of other genes and cellular processes. The behavioral implications of this particular gene are understudied, however a recent study found that *Rpl21* was differentially expressed throughout the brains of domestic versus wild rabbits, and more specifically, was less expressed in hypothalamus of wild rabbits (Sato et al., 2020). Reduced aggression is a hallmark of domesticated animals; therefore, these results suggest that hypothalamic *Rpl21* may mediate levels of aggression. Additionally, we found that the expression of only one gene (*Ache*), that was also included in a curated list of aggression-related genes (Supplemental Table S2, Zhang-James et al., 2019), was significantly higher in more despotic males. This result suggests that, at least in alpha males, upregulation of acetylcholinesterase, a cholinergic enzyme that hydrolyzes acetylcholine, may promote aggression as has been previously proposed (Bandler, 1969; Picciotto et al., 2015).

We also performed WGCNA to identify functionally related genes that are co-expressed, thus revealing potentially novel cell pathways that are differentially regulated across social status. In total, we identified 11 modules in the mPOA and vHYP that were associated with social status. In the mPOA, the greenyellow module was significantly more expressed in subordinates compared to subdominant and alpha mice. This module consists of genes involved in protein catabolism and regulation of the intracellular estrogen receptor (ER) signaling pathway. The role of protein catabolism in the mPOA is unknown but may represent changes in energy allocation. Interestingly, activation of estrogen sensitive neurons in the mPOA induces hypometabolism and hypothermia (Zhang et al., 2020), suggesting that shifts in this ER signaling pathway may also contribute to changes in energy regulation. ER signaling in the mPOA has been well-studied in the context of social behavior, particularly reproductive behaviors where estrogen promotes parental and sexual behavior in males (Trainor et al., 2003; Tsuneoka et al., 2017). Less is known as to how estrogen in the mPOA facilitates social dominance, though male mice lacking ERα and ERβ show reduced and increased aggression respectively (Nomura et al., 2002; Ogawa et al., 1999, 1997). We have also previously reported that female subordinate mice have elevated expression of estrogen receptors in the ventromedial hypothalamus, but not the mPOA, compared to dominant mice (Williamson et al., 2019). Although we did not find any effect of social status on the expression of estrogen receptor alpha or beta, we did observe significantly higher expression of estrogen-related receptors alpha and gamma in alpha males in the vHYP. These orphan nuclear receptors share transcriptional targets with ERs and appear to interact with ERs in the regulation of both metabolic processes and social behaviors including social dominance (Cui et al., 2015; Saito and Cui, 2018). These findings suggest that increased expression of genes involved in promoting intracellular estrogen signaling may reduce aggression and promote subordinate behavior, although this remains to be tested in future studies.

Other modules in the mPOA were more highly expressed in dominant mice. The brown module was more highly expressed in alpha mice compared to subdominant mice and included a wide variety of genes, including those involved in renal system processes. This is noteworthy as we have previously observed that alpha mice urinate more frequently than subdominant and subordinate mice as a function of marking territory (Lee et al., 2017) and the mPOA has also been shown to partially regulate territorial urine marking in male lab mice (Hou et al., 2016). Thus, we hypothesize that this module consists of candidate genes that control micturition.

We identified fewer modules in the vHYP. Expression of the green module, which consists of genes involved in cell division and turnover was significantly higher in subdominant and subordinate mice compared to dominant males. Like protein catabolism in the mPOA, upregulation of cell turnover in the vHYP could have many different physiological implications. Interestingly, the blue modules in both the mPOA and vHYP consisted of genes involved in energy generative processes such as ATP metabolism and the electron transport chain. In the mPOA, this module was more highly expressed in subdominant compared to subordinate mice, but in the vHYP it was more highly expressed in alpha mice compared to subdominant and subordinate mice. *Cox5a* has high module membership (MM) in both regions, indicating that it is highly co-expressed with the genes in each module. *Cox5a* encodes cytochrome *c* oxidase subunit 5A, a constituent of Complex IV, the last enzyme in the mitochondrial electron transport chain (Fukuda et al., 2007). One possible explanation for these results is that different brain regions have differential social status induced energetic demands. Considering the role of the vHYP in feeding behavior and thermoregulation (Nagashima et al., 2000), elevated blue module expression in alphas could reflect a positive shift in activity levels and energy metabolism as has been documented in dominant animals (Biro and Stamps, 2010; Emery Thompson et al., 2009; Lee et al., 2018). The benefit of WGCNA analysis is to identify biological pathways that are differentially expressed between different social statuses that would otherwise be difficult to parse out of our thousands of DEGs. However, these modules represent a diverse array of genes and their functional groups are also quite broad. Further investigation into these new candidate genes is needed to determine their more specific contribution to subordinate and dominant phenotypes.

In summary, we used Tag-seq to identify differentially expressed genes in the mPOA and vHYP of mice of distinct social rank: alpha, subdominant, and subordinate. We also identified possible functional gene networks in which expression was highly correlated and associated to social rank. A novel pattern between social rank and expression level was identified in several social behavior genes in the mPOA, such that subdominant mice generally exhibited relatively higher gene expression. In the vHYP, we identified a negative linear correlation between social rank and several genes involved in feeding regulation that could potentially underlie food motivation in these lower-ranking animals. While we did not use approaches that enable the assessment of non-coding genes or specific cell-types, such as single-cell RNA-sequencing, and therefore cannot draw conclusions about transcriptional differences with single cell resolution, we identified numerous novel candidate genes for further investigation into potential pathways within the hypothalamus that plastically coordinate physiological processes and behaviors in mice of varying social status. Further investigation should also include studies in female hierarchies to determine if similar gene expression differences underlie divergent phenotypes in these groups as well. This study is an important hypothesis-generating step that will inform future investigations into how variation in gene expression across different hypothalamic nuclei is functionally integrated to regulate critical adaptive shifts in behavior and physiology among animals of

varying social status.

Data accessibility

Data and code used in this paper are available at the following repository: https://github.com/jalapic/hypothal_rna

Data availability

I have shared the link to data and code in the main manuscript

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105176.

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