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Transcriptomic Analysis Reveals Sex-Dependent Expression Patterns in the Basolateral Amygdala of Dominant and Subordinate Animals After Acute Social Conflict

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Abstract

The basolateral amygdala (BLA) is a critical nucleus mediating behavioral responses after exposure to acute social conflict. Male and female Syrian hamsters both readily establish a stable dominant-subordinate relationship among same-sex conspecifics, and the goal of the current study was to determine potential underlying genetic mechanisms in the BLA facilitating the establishment of social hierarchy. We sequenced the BLA transcriptomes of dominant, subordinate, and socially neutral males and females, and using de novo assembly techniques and gene network analyses, we compared these transcriptomes across social status within each sex. Our results revealed 499 transcripts that were differentially expressed in the BLA across both males and females and 138 distinct gene networks. Surprisingly, we found that there was virtually no overlap in the transcript changes or in gene network patterns in males and females of the same social status. These results suggest that, although males and females reliably engage in similar social behaviors to establish social dominance, the molecular mechanisms in the BLA by which these statuses are obtained and maintained are distinct.

Keywords RNA-Seq · Sex differences · Social defeat · Social stress · Agonistic behavior · Transcriptional signature

Introduction

Social stress is argued to be the most prevalent stressor experienced by humans [1] and is thought to increase the risk or to stimulate the onset of many neuropsychiatric disorders including anxiety disorders, posttraumatic stress disorder, and depression [2–6]. The most commonly studied form of social stress in preclinical studies is social defeat. Acute and repeated exposure to social defeat stress causes many of the same outcomes in rodents (e.g., increased social avoidance, anxiety, and anhedonia) as are observed in humans after stressful experiences [5, 7–12]. Sex differences in response to social stress are also prevalent across taxa, including in humans wherein many stress-related neuropsychiatric disorders present with sexually dimorphic prevalence and symptoms [13–16]. It can be challenging to study female

responses to social stress in rodent species, however, because female rats and mice do not display spontaneous aggressive behavior, and males do not normally attack conspecific females [17]. Therefore, it is difficult to study sex differences in mechanisms and responses to social stress in ethologically meaningful ways using these animal models. In Syrian hamsters (*Mesocricetus auratus*), however, both sexes readily engage in agonistic behavior with a same-sex conspecific in the wild and in the laboratory [18–20]. Both males and females use the same agonistic behaviors to rapidly establish stable dominant-subordinate relationships allowing for the study of "winners" (dominants) and "losers" (subordinates) in both sexes. Fortuitously, agonistic encounters in hamsters rarely result in physical injury, as contrasted with commonly used mouse models of defeat, permitting the direct study of the effects of social stress without the confounding trauma of physical injury.

Our laboratory has extensively studied the behavioral and neurochemical correlates of responses to social conflict in hamsters and have found sex differences in some of the behavioral responses to social stress. While both sexes display behavioral responses to social stress, defeated males paired with a new opponent often exhibit more overt submissive behaviors after defeat (e.g., flees and risk assessments), while defeated females exhibit less submission and more social and communicative behaviors (e.g., sniffing

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and flank marking) [21–24]. In both males and females, social defeat stress elicits similar levels of social avoidance of a confined opponent, however, suggesting that both sexes exhibit at least some of the same behavioral responses to defeat [21, 25]. We have defined a neural circuit that facilitates the behavioral responses to acute social conflict in hamsters and have identified the basolateral amygdala (BLA) as a critical nucleus mediating these changes. The BLA is necessary for the acquisition and expression of conditioned defeat, a striking change in behavior in which a defeated, subordinate animal abandons all territorial aggression and produces only submissive and defensive behavior [26, 27]. We have also demonstrated that de novo protein synthesis in this nucleus is required [28] and that overexpression of cyclic AMP binding protein in the BLA during social defeat enhances subsequent conditioned defeat [29]; thus, it appears clear that gene regulation in the BLA mediates, at least in part, behavioral responses to defeat. Less is known, however, about the specific genes or gene networks involved in mediating behavioral responses to acute social conflict and the subsequent formation of a dominant-subordinate relationship in males *and* females.

While hamsters are an ideal model for the study of acute social conflict, as outlined above, there is a dearth of genetic tools available for the investigation into genetic mechanisms underlying stress-induced behavioral changes in this species. Our lab recently published the transcriptome of male and female Syrian hamster whole brain [30], providing a starting point with which to begin genetic exploration of behavior using Syrian hamsters as a model. To further investigate the genetic mechanisms underlying the establishment of social status, we sequenced the transcripts in the BLA of dominant and subordinate males and females and compared transcript expression and gene network patterns to that of same-sex socially neutral controls. The primary purpose of this study was to identify genes that may mediate the formation of social status and to determine whether these patterns of gene regulation are similar in males and females.

Results

Females and Males Exhibit Similar Behavioral Patterns During Acute Social Conflict

As is often true with weight-matched pairs, residence did not confer dominance. In fact, in this study, intruders were dominant 65% of the time. There was no difference in the latency to first attack between males and females (Student's t test with Welch's correction: $t(5.942) = 1.915$, $p = 0.1045$), nor was there a significant difference in the duration of agonistic behavior in males and females before a hierarchy was established (Student's t test: $t(10) = 0.8742$, $p = 0.4025$) in Trial 1 (Fig. 1a). Overall, across all three trials, there was no main effect of sex in dominants (two-way ANOVA: $F(1, 40) = 9.18e-013$, $p > 0.9999$; Fig. 1b) or subordinates (two-way ANOVA: $F(1, 40) = 1.652e-011$, $p > 0.9999$; Fig. 1c), suggesting no significant sex differences in overall duration of each behavior within social status. There was a main effect of behavior in dominants ($F(3, 40) = 34.91$, $p < 0.0001$) and subordinates ($F(3, 40) = 454.1$, $p < 0.0001$), with dominant animals primarily displaying aggressive behavior and subordinate animals displaying almost exclusively submission (Fig. 1b, c). There were subtle behavioral differences observed during Trial 1, but overall, there were no substantial sex differences observed within each of the three behavioral trials (Supplemental Fig. 1).

De Novo Transcriptome Assembly and Optimization

The initial de novo assembly was created using high-quality samples (Supplemental Table 1 and Supplemental Fig. 2) and generated 1.2 M possible genes. The assembly was then optimized, as described in the Supplemental Materials and Supplemental Fig. 3, resulting in 120,003 transcripts mapping to 14,493 unique BLAST identifiers.
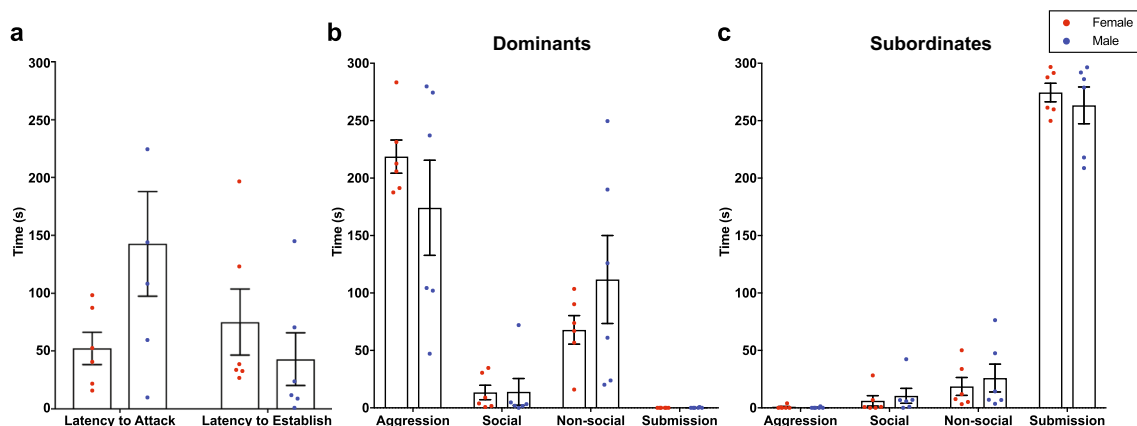

Fig. 1 Females (red) and males (blue) show similar behavior during acute social conflict. **a** There were no differences in latency to attack in the first trial or in latency to establish a hierarchy after attack between males and females. **b**, **c** There were no sex differences in behavior in dominant animals (**b**) or subordinate animals (**c**)
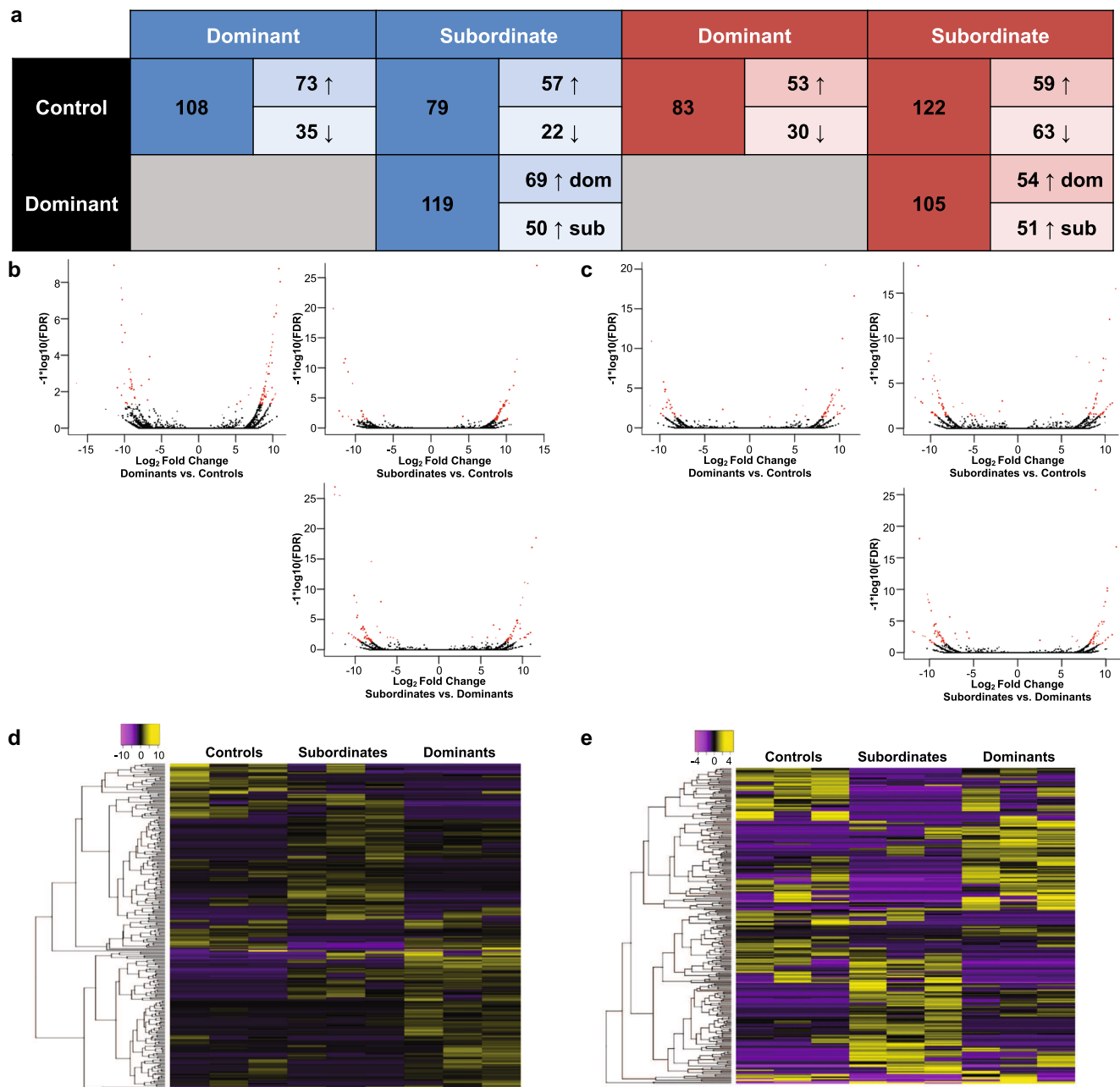
Fig. 2 The distribution of differentially expressed transcripts (DETs) in males and females follow similar patterns despite little overlap in the identity of the actual transcripts that were changed. **a** Table depicting the number of transcripts that were differentially expressed in males (left, blue) and females (right, red). Arrows indicate the number of transcripts that were higher or lower in dominants and subordinates compared with controls. **b**–**e** The distribution of transcripts in each comparison in males (**b**, **d**) and females (**c**, **e**). Red dots in the volcano plots (**b**, **c**) indicate the transcripts that were significantly different (FDR < 0.05). The heatmaps (**d**, **e**) show the transcript clustering dendrograms to the left. Purple indicates transcripts that had a negative log_2 fold change, and yellow indicates transcripts that had a positive log_2 fold change. Note the difference in scale in males (− 10 to 10) and females (− 4 to 4)

Differential Expression, Gene Ontology, and Network Analyses Reveal Little Transcriptional Overlap Between Males and Females

Expected read counts from each sample were calculated to determine which transcripts were most highly expressed. The most highly expressed transcripts in hamster amygdala (both male and female) are listed in Supplemental Table 2 and largely overlapped with those found in the whole brain of males and females [30]. Differential expression analyses were then completed. Samples from dominant and subordinate hamsters were compared to samples from same-sex controls, and Supplemental Table 3 lists all the differentially expressed transcripts (DETs). There were similar numbers of DETs in males

and females of different social status (Fig. 2a). In total, 258 unique transcripts were differentially expressed in males (with 48 transcripts present in more than one comparison) and 263 unique DETs in females (with 47 transcripts present in more than one comparison). The majority of DETs present in more than one comparison were in both dominants and subordinates compared with controls, suggesting that these transcripts are altered by social interaction and not necessarily by social status (see Supplemental Table 3). Figure 2 shows the distribution of all DETs in males (Fig. 2b, d) and females (Fig. 2c, e).

After analyzing the DETs in males and females, we determined that very few overlapped between the sexes. Table 1 lists the transcripts that were differentially expressed in both males and females; however, it is the case that some of these changed in opposite directions. In fact, of the 521 differentially expressed transcripts, only 22 overlapped between the sexes, leaving 499 unique DETs across sex and social status (Supplemental Table 3). To determine if this lack of overlap was due to divergent expression patterns in males and females, or, alternatively, if these transcripts were regulated similarly

but just failed to reach significance in both sexes, we determined the degree of overlap between expression profiles of males and females of different social status using rank rank hypergeometric overlap (RRHO). This analysis revealed no correlation between the DETs in male and female dominants (Spearman's rho = −0.0576; Fig. 3a, b) or subordinates (Spearman's rho = 0.0256; Fig. 3c, d), suggesting that there was no significant overlap in the expression patterns of transcripts after the establishment of social status. The RRHO heat maps indicate that any overlap that exists in the DETs at all is in a set of transcripts that are regulated in opposite directions in males and females (reds in Fig. 3b, d). Supplemental Figure 4 shows the degree of overlap in the expression of all transcripts in the BLA between male and female dominants and subordinates.

The optimized assembly and the subsets of DETs were then analyzed using PANTHER gene ontology (GO) analysis to determine which molecular functions, biological processes, protein classes, and pathways were most represented. Catalytic activity and binding were the highest represented

Table 1 Differentially expressed transcripts that reached significance in both males and females $^+$ 2 isoforms were differentially expressed in both males and females

| Gene (Uniprot ID)	Expression Change in Females			Expression Change in Males		
	Dom v. Sub	Dom v. Con	Sub v. Con	Dom v. Sub	Dom v. Con	Sub v. Con
AT-hook-containing transcription factor (AKNA_MOUSE)	↑ Dom	-	-	↑ Sub	-	-
ATP-dependent 6-phosphofructokinase, muscle type (PFKAM_MOUSE)	-	-	↓	-	-	↑
Cullin-3 (CUL3_RAT)	-	↓	-	↓ Dom	↓	-
DNA repair protein RAD51 homolog 4 (RA51D_MOUSE)	↑ Sub	-	-	-	↑	-
Exportin-4 (XPO4_MOUSE)	↑ Dom	-	-	↑ Dom	↑	-
Glycylpeptide N-tetradecanoyltransferase 2 (NMT2_MOUSE)	↑ Sub	-	-	-	-	↓
Histidine triad nucleotide-binding protein 3 (HINT3)	↑ Sub	-	-	↑ Sub	-	-
Inactive ubiquitin carboxyl-terminal hydrolase 53 (UBP53_MOUSE)	-	↓	-	-	-	↑
Integral membrane protein 2C (ITM2C_RAT)$^+$	-	↑	↓	-	↑	↑
Junctophilin-1 (JPH1_MOUSE)	-	-	↓	↑ Sub	-	-
Kelch repeat and BTB domain-containing protein 4 (KBTB4_MOUSE)	-	↑	↑	-	↓	-
Magnesium transporter NIPA1 (NIPA1_MOUSE)	-	-	↓	↑ Dom	-	-
MAM domain-containing glycosylphosphatidylinositol anchor protein 2 (MDGA2_RAT)	↑ Dom	↑	-	↑ Sub	-	↓
Meprin A subunit alpha (MEP1A_RAT)	-	↓	-	-	-	↑
NEDD4-binding protein 2-like 1 (N42L1_MOUSE)	↑ Dom	-	-	-	↑	-
Protein transport protein Sec24A (SC24A_MOUSE)	-	-	↓	↑ Dom	-	-
Serine/threonine-protein kinase PAK 2 (PAK2_RAT)	-	-	↑	↑ Dom	-	-
TM2 domain-containing protein 1 (TM2D1_MOUSE)	↑ Sub	-	↑	-	↓	-
Tubby-related protein 4 (TULP4_MOUSE)	↑ Sub	-	-	-	-	↓
Voltage-dependent R-type calcium channel subunit alpha-1E (CAC1E_RAT)	-	↓	-	-	↓	↓
Voltage-dependent T-type calcium channel subunit alpha-1H (CAC1H_RAT)	↑ Sub	-	-	-	-	↑
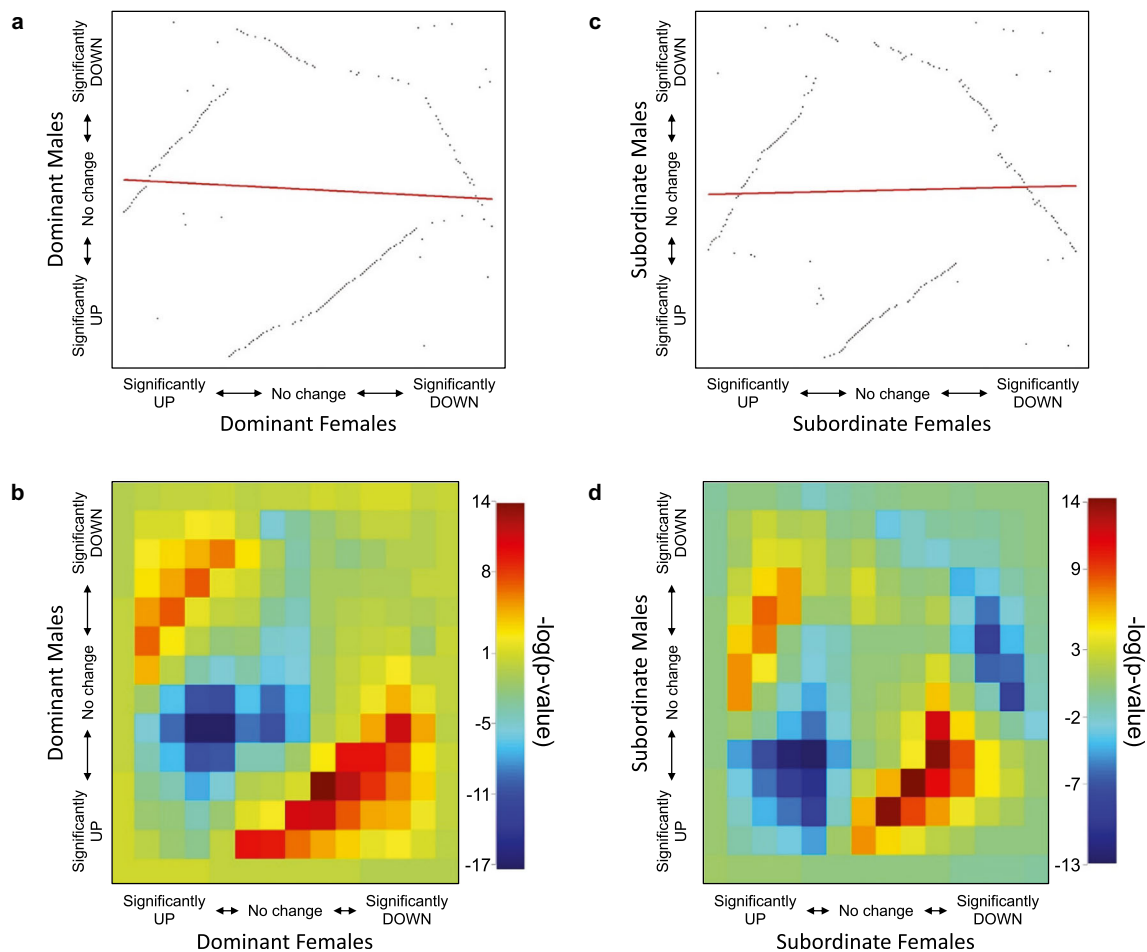
Fig. 3 There was no correlation in dominant males and females (**a**, **b**) or in subordinate males and females (**c**, **d**) when the differentially expressed transcripts (DETs) were compared using rank rank hypergeometric overlap (RRHO). RRHO ranks the DETs with the significantly increased transcripts first (bottom left) and the significantly decreased transcripts last (top right) on each panel (**a**, **c**). The heatmaps (**b**, **d**) show the $\log_{10}$-transformed p values of the overlapping transcripts in males and females

according to the hypergeometric distribution. Higher (reds) values indicate transcripts that overlap more than expected by chance while lower (blues) values indicate lower than expected overlap. The reds in the lower right and upper left corners (**b**, **d**) indicate that the overlapping transcripts are, in fact, regulated in opposite directions in males and females

molecular functions, whereas metabolic and cellular processes ranked highest in biological processes (Supplemental Fig. 5). The top protein classes (Supplemental Fig. 5) and pathways (Supplemental Fig. 6) were more evenly distributed. Analysis of the DETs within each sex revealed these GO terms were also present in the highest number of transcripts, with some sex-specific differences (Figs. 4 and 5). GOSeq enrichment analysis further revealed that the regulation of chromatin disassembly (under Biological Processes) was significantly enriched in transcripts that were lower in subordinate males (FDR = 0.02). Furthermore, 4 GO terms, all under Molecular Function, were enriched in transcripts that were significantly higher in dominant and subordinate males (cystine: glutamate antiporter activity (FDR = 0.02), cystine secondary active transmembrane transporter activity (FDR = 0.02), sulfur-containing amino acid secondary active transmembrane transporter activity (FDR = 0.02), and neutral L-amino acid

secondary active transmembrane transporter activity (FDR = 0.04)). In addition, 33 terms (24 biological processes, 8 molecular functions, and 1 cellular component) were enriched in transcripts that were higher in dominant and subordinate females, including dopaminergic synaptic transmission (FDR = 0.01), negative regulation of synaptic plasticity (FDR = 0.007), and receptor activator activity (FDR = 0.04) (Supplemental Table 4).

Finally, we used a weighted gene correlation network analysis (WGCNA) to determine the similarity in gene network expression patterns of the dominant, subordinate, and control samples in males and females. Analyzing transcript expression in the optimized assembly allowed us to graph the connectivity of our samples based on overall expression patterns. WGCNA grouped the 120,003 transcripts into 138 distinct modules (Supplemental Fig. 7). Of these, 10 significantly correlated with subordinate status (Fig. 6a), while 9 correlated
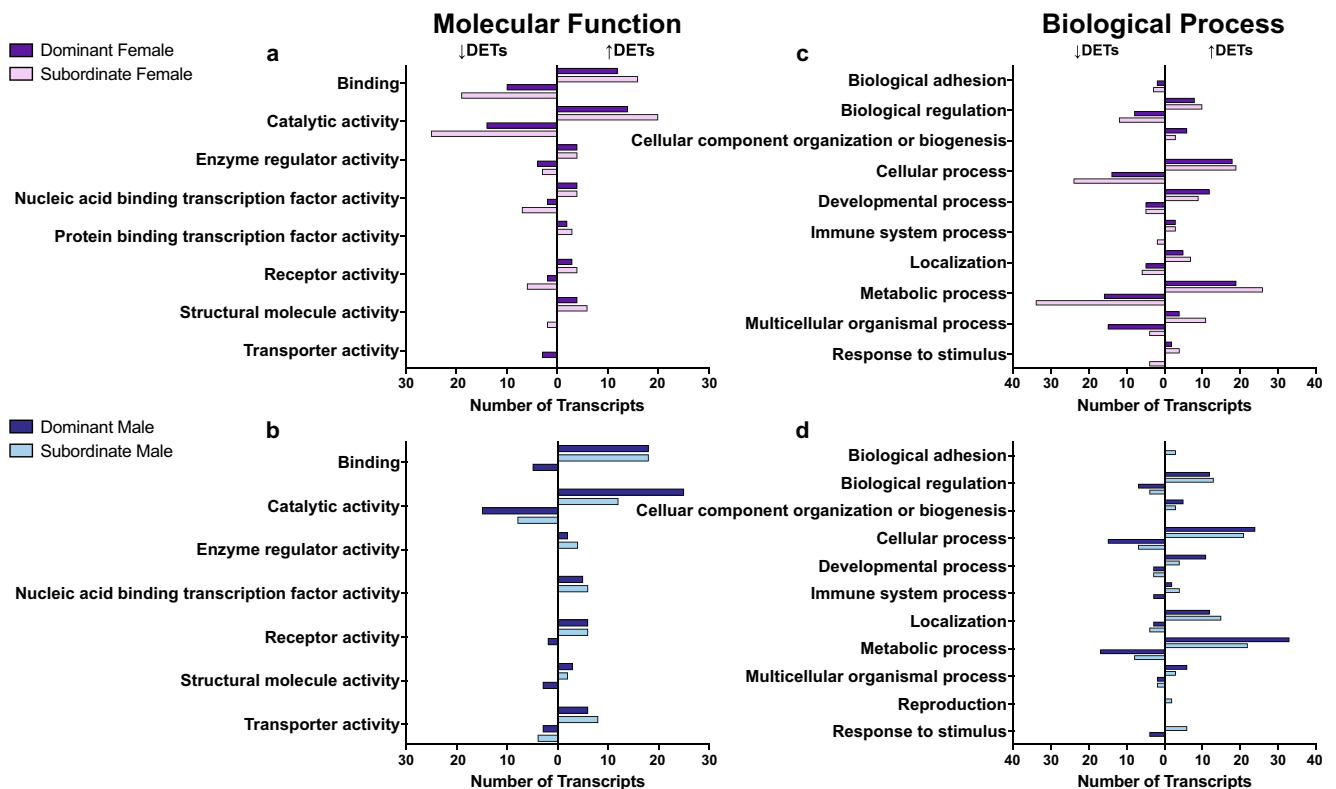
Fig. 4 GO analysis revealed Molecular Functions (**a**, **b**) and Biological Processes (**c**, **d**) that were represented by the differentially expressed transcripts (DETs) that were higher (bars to the right) or lower (bars to the left) in dominant (dark bars) and subordinate (light bars) animals

with dominant status (Fig. 6b). These correlations, however, were largely driven by only one sex, as can be seen in Fig. 6, further suggesting minimal overlap in transcript or gene network expression patterns in females and males after social conflict and the establishment of social status.

Discussion

Transcriptomic analysis of hamster amygdala revealed striking sex differences in the transcripts and pathways that change in response to the establishment of social status in males and females. In fact, there was virtually no overlap in overall expression patterns between the sexes. In this study, we investigated the transcriptomic profiles of dominant and subordinate males and females 24 h after acute social conflict because this is the time frame when we observe marked behavioral changes in defeated hamsters. Both males and females reliably and rapidly engaged in similar agonistic behaviors upon encountering a conspecific; however, the transcripts that were regulated between the sexes after this establishment were markedly different, suggesting that while the establishment of a social hierarchy is readily observed in both sexes, the underlying genetic mechanisms are distinct. In the rare instances when the same transcripts were differentially regulated by status in males and females, it was often in opposite directions. The transcripts, pathways, and gene networks highlighted here provide a more concrete understanding of how these behaviors might be mediated in male and female hamsters. Future experiments will further test these mechanisms, focusing on the specific genes and networks that are differentially expressed between males and females of different social status.

The sex-specificity of our data supports the hypothesis that male and female brains may act via different mechanisms to produce similar behavior. These results are consistent with previous data and theories that similar behavioral outcomes may be attained via activation of very different, sex-specific pathways, or by the same pathways but in opposite directions, to reach the same ultimate goal [31, 32], which in the present instance is the establishment of a stable dominance relationship within pairs of hamsters. Consistent with the data presented here, it was recently found that male and female mice have distinct gene expression patterns in hippocampal area CA3 in response to an acute stressor [33], suggesting that distinct brain regions have unique patterns of gene expression that are sexually dimorphic in response to stress. Furthermore, it was recently reported that males and females suffering from major depressive disorder have markedly different transcriptional profiles in several brain regions, including the prefrontal cortex and nucleus accumbens [34]. This study did not analyze the amygdala, but the results, in conjunction with the data presented here, further reinforce the need to include both
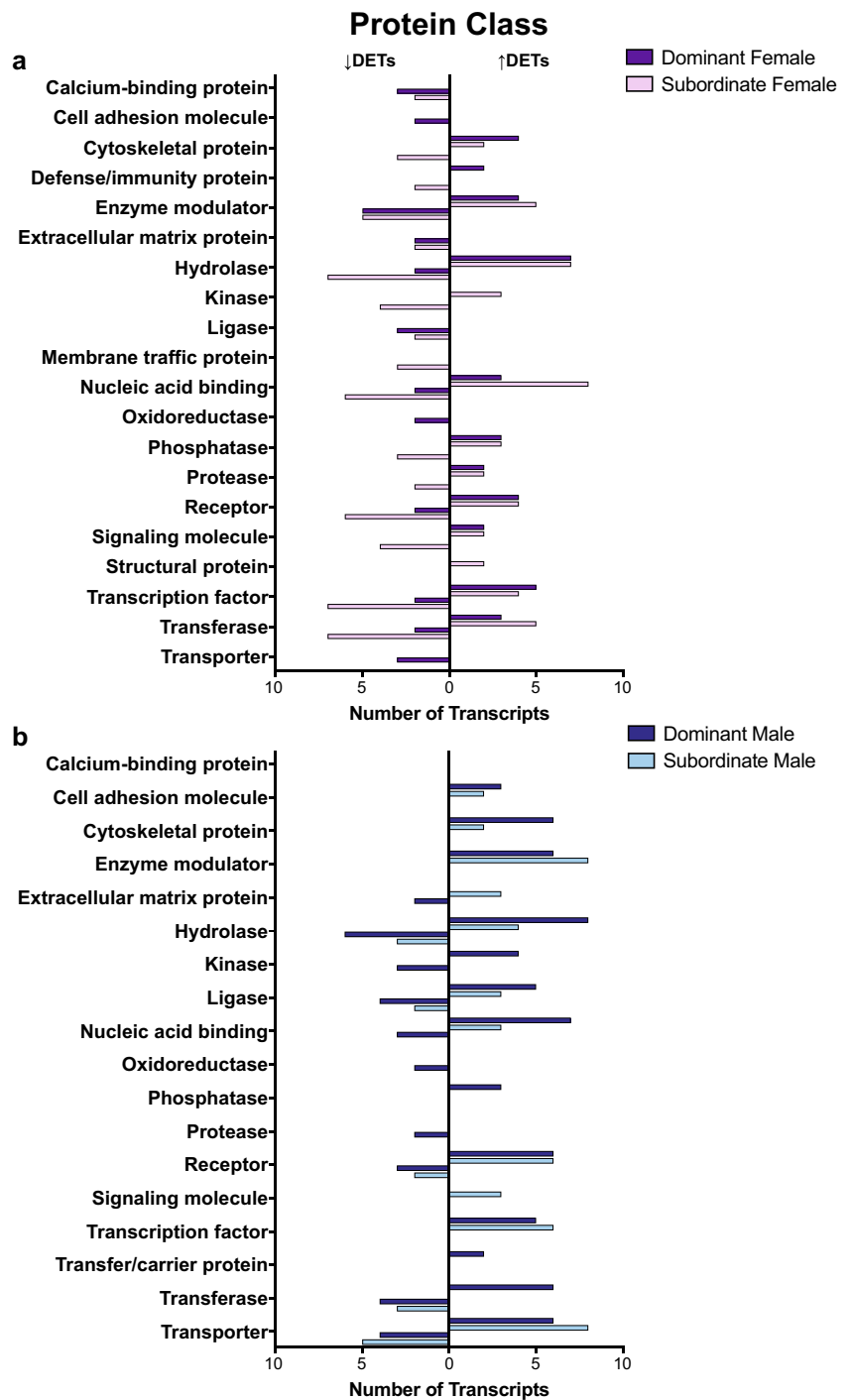
males and females and underscore the importance of using animal models that allow the direct study of sex differences in the regulation of behavior in species wherein both sexes produce similar, ethologically relevant behaviors.

Of note, several transcripts that were differentially expressed between animals of different social status were GABA- or glutamate-related genes that have been reported to have differential expression patterns in males and females in various animal models of neuropsychiatric disorders as well

as in clinical populations. These differences are often confined to specific brain regions, and our data further support sexually dimorphic patterns of expression in the amygdala following acute social conflict. For example, *Gria2*, which codes for an ionotropic glutamate receptor, was significantly higher in dominant and subordinate males compared with controls, suggesting that this gene may play a role in the processing of cues during social interactions in males. *Gria2* is associated with stimulus-reward learning [35] and is linked to sex differences
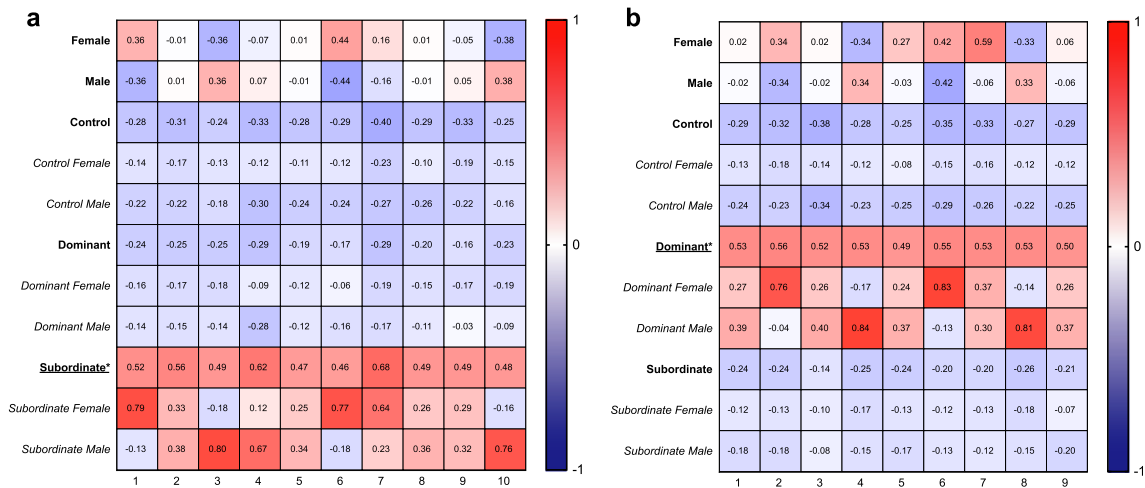
a

	1	2	3	4	5	6	7	8	9	10
Female	0.36	-0.01	-0.36	-0.07	0.01	0.44	0.16	0.01	-0.05	-0.38
Male	-0.36	0.01	0.36	0.07	-0.01	-0.44	-0.16	-0.01	0.05	0.38
Control	-0.28	-0.31	-0.24	-0.33	-0.28	-0.29	-0.40	-0.29	-0.33	-0.25
Control Female	-0.14	-0.17	-0.13	-0.12	-0.11	-0.12	-0.23	-0.10	-0.19	-0.15
Control Male	-0.22	-0.22	-0.18	-0.30	-0.24	-0.24	-0.27	-0.26	-0.22	-0.16
Dominant	-0.24	-0.25	-0.25	-0.29	-0.19	-0.17	-0.29	-0.20	-0.16	-0.23
Dominant Female	-0.16	-0.17	-0.18	-0.09	-0.12	-0.06	-0.19	-0.15	-0.17	-0.19
Dominant Male	-0.14	-0.15	-0.14	-0.28	-0.12	-0.16	-0.17	-0.11	-0.03	-0.09
Subordinate*	0.52	0.56	0.49	0.62	0.47	0.46	0.68	0.49	0.49	0.48
Subordinate Female	0.79	0.33	-0.18	0.12	0.25	0.77	0.64	0.26	0.29	-0.16
Subordinate Male	-0.13	0.38	0.80	0.67	0.34	-0.18	0.23	0.36	0.32	0.76

b

	1	2	3	4	5	6	7	8	9
Female	0.02	0.34	0.02	-0.34	0.27	0.42	0.59	-0.33	0.06
Male	-0.02	-0.34	-0.02	0.34	-0.03	-0.42	-0.06	0.33	-0.06
Control	-0.29	-0.32	-0.38	-0.28	-0.25	-0.35	-0.33	-0.27	-0.29
Control Female	-0.13	-0.18	-0.14	-0.12	-0.08	-0.15	-0.16	-0.12	-0.12
Control Male	-0.24	-0.23	-0.34	-0.23	-0.25	-0.29	-0.26	-0.22	-0.25
Dominant*	0.53	0.56	0.52	0.53	0.49	0.55	0.53	0.53	0.50
Dominant Female	0.27	0.76	0.26	-0.17	0.24	0.83	0.37	-0.14	0.26
Dominant Male	0.39	-0.04	0.40	0.84	0.37	-0.13	0.30	0.81	0.37
Subordinate	-0.24	-0.24	-0.14	-0.25	-0.24	-0.20	-0.20	-0.26	-0.21
Subordinate Female	-0.12	-0.13	-0.10	-0.17	-0.13	-0.12	-0.13	-0.18	-0.07
Subordinate Male	-0.18	-0.18	-0.08	-0.15	-0.17	-0.13	-0.12	-0.15	-0.20

Fig. 6 WGCNA analysis revealed several gene networks that significantly correlated with social status. Ten modules correlated with subordinate status (**a**) and 9 modules correlated with dominant status (**b**). Correlations are reported within each cell, with positive correlations shown in red and negative correlations shown in blue. All 138 modules from WGCNA, along with p values for each module, are shown in Supplemental Fig. 7. Each of the modules shown here had a p value < 0.05 for either subordinate (**a**) or dominant (**b**) status (indicated by asterisk). These correlations were primarily driven by one sex, as can be seen in the subcategories below when separated by sex within status

in major depressive disorder (MDD), in that *Gria2* expression is higher in the prefrontal cortex of female patients with MDD [36]. Our data suggest that *Gria2* in the amygdala is not significantly altered in females and may in fact play a more significant role in males during social interactions. We also found that the alpha-4 subunit of the GABA-A receptor was more highly expressed in dominant males when compared with subordinate males. Expression of this subunit increases in the amygdala of females but also in the hippocampus of both males and females after progesterone withdrawal and is linked to increases in anxiety-like behavior [37, 38]. Our data, in conjunction with these previous findings, reinforce the importance of sex- and site-specificity when interpreting differences in gene expression patterns after stress exposure.

In addition, *Gad2*, the gene that encodes the protein GAD65, was higher in dominant males after an acute agonistic interaction. This gene is reduced in patients with MDD [39], and low GAD65 expression is correlated with spatial learning deficits after stress in the medial amygdala of males. Conversely, high GAD65 expression in the CA1 region of the hippocampus is correlated with spatial learning deficits in females [40]. Glutamic acid decarboxylase (GAD) catalyzes the formation of GABA from glutamate, and GAD65, in particular, is involved in GABA synthesis specifically for phasic inhibition of neurotransmission [41]. GAD65 increases in several nuclei after acute and chronic stressors, including specific nuclei within the bed nucleus of the stria terminalis and hypothalamus [42], respectively, and we demonstrated here that *Gad2* was higher in the amygdala of dominant males. This suggests a potential increase in GABA stores available for neurotransmission in the abundant GABAergic neurons in the amygdala. An increase in GABA neurotransmission in the amygdala during social conflict would suppress the social stress-induced behavioral phenotype, thus potentially providing a protective effect in dominant animals against the stress of the encounter. This finding is also consistent with data showing that previous dominance confers resilience to subsequent stress [43] and suggests that increased GABAergic tone in the amygdala may be one mechanism by which this resilience is obtained and maintained.

We also examined genes associated with learning and memory, mood and anxiety disorders, and social behavior. Several transcripts that had lower expression in animals that experienced an agonistic encounter have been linked to bipolar disorder (*Akap5*) [44], general mood disorders (*Aldh1a1*) [45], anxiety (*Kif13a*) [46], and depression (*Mgat5*) [47]. Other genes linked to major depressive disorder (*Gad2*, *Gria2*) [36, 39], PTSD (*Dicer1*) [48], and anxiety (*Spock3*) [49] had higher expression in dominant and/or subordinate animals when compared with controls. Specifically, *Dicer1*, a gene directly involved in the expression of other genes by regulating the production of microRNAs, was higher in dominant males, and, consistent with this effect, increases in this gene have been linked to stress resilience [50]. Relatedly, decreases in *Dicer1* are observed in patients suffering from PTSD and depression compared with healthy controls [48]. These results suggest that *Dicer1* could be another gene of interest, along with *Gad2*, regulating the resilient behavioral phenotype obtained from achieving dominance. On the other hand, in regulating subordinate status, we found that *Uba6* was lower in subordinate males. This is consistent with previously observed increases in anxiety-like behavior and social avoidance in animals with a depletion of this gene [51] and is worth future investigation into how this gene, and others up- and down-stream in its network, may correlate with social avoidance after acute social stress.

We have previously shown that dopamine in the nucleus accumbens modulates the acquisition and expression of conditioned defeat [52]. Three transcripts representing dopamine-mediated signaling pathways (*E4l1l*, *E4l2*, *Cdk5*) emerged from the gene ontology analysis as being higher in subordinate females, suggesting that these genes may be directly involved in mediating defeat-induced behavioral responses in females. In addition, the gonadotropin-releasing hormone pathway was represented by four transcripts that were lower in subordinate females (*Nab1*, *Nfyb*, *Bmr1a*, *Plcb1*) and three transcripts that were higher in dominant males (*Bmr1b*, *Pp2ba*, *Tba1b*). It is not surprising that social stress might change these types of genes in a sexually dimorphic manner; indeed, we have previously reported that gonadal hormones modulate social stress-induced behavioral responses in both males and females [22, 24]. Several additional pathways were represented in the DETs, including multiple glutamate receptor pathways, beta 1 and 2 adrenergic receptor signaling pathways, 5HT2-type receptor-mediated signaling pathway, oxytocin receptor-mediated signaling pathway, and GABA synthesis. Further defining and investigating these gene pathways will provide a solid framework for determining the precise molecular mechanisms underlying dominant and subordinate status in males and females and will help us to understand the sex-specific mechanisms that ultimately lead to similar behavioral outcomes.

Overall, the data presented here are the first to show that there are sex-dependent mechanisms in the amygdala underlying dominant and subordinate status after acute social conflict in hamsters. Both sexes readily establish a social dominance hierarchy, but these data indicate that the genes and gene networks by which social statuses are established and maintained are genetically distinct in the two sexes.

Methods

Animals and Behavior

Adult male and female Syrian hamsters, approximately 10 weeks old and weighing between 120 and 140 g, were obtained from Charles River Laboratories (Danvers, MA). Animals were singly housed upon arrival under a 14:10 h light/dark cycle, as is standard in this species to maintain gonadal patency. Single housing in hamsters is standard and is not stressful for this species [53]. All animals were handled daily for at least 8 days before the beginning of the study. During handling, estrous cycles of females were monitored for at least 2 cycles (8 days) via vaginal swab to confirm estrous cycle stage and stability. Before social defeat training, animals were weight-matched and randomly assigned as a resident, intruder, or home-cage control. All females were paired on diestrus 1, and brains were collected on diestrus 2 because females on diestrus 2 show the most pronounced social avoidance after

defeat [21]. An equal number of males were paired each day that the females were run. Intruders were placed in the resident's home cage three times for 5 min to ensure a stable hierarchy; each pairing was separated by an inter-trial interval of 3 min during which the intruder was returned to its home cage (Supplemental Fig. 1). The timing for the 5-min trial during the first pairing began immediately after the first agonistic behavior was displayed, as described previously [54]. All behavior emitted during each trial was scored separately for each animal. Behavior was separated into the following categories: aggression (side or upright attack, chase, bite), submission (flee, side or upright submissive posture, tail lift), and social (greet, sniff, allogroom) and non-social behavior (self-groom, cage exploration, general locomotion), as described in detail in Albers, Huhman, and Meisel [55]. Latency to the initial agonistic interaction and time to establish a stable dominance relationship were also recorded. Home cage controls were left alone in their home cage during training and were not exposed to the behavioral suite during defeat training. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee and are in accordance with the standards outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Latencies to attack and to establish a hierarchy were analyzed using non-paired *t* tests, with Welch's correction for unequal variances. Behaviors scored during the agonistic encounters were analyzed using two-way ANOVAs with Sidak's post hoc analysis. All behavioral statistics were completed using Prism 7 for Mac OS X, version 7.0c.

Tissue Collection, RNA Isolation, and Sequencing

Paired animals, as well as home cage controls, were anesthetized via isoflurane exposure and decapitated 24 h after the final agonistic encounter. This is the time when we would normally test for the presence of defeat-induced social avoidance and for conditioned defeat [19, 56, 57]. Brains were quickly extracted, frozen immediately in isopentane on dry ice, and stored at − 80 °C until processing. Bilateral tissue punches (1 mm) aimed at the basolateral amygdala (BLA) were extracted from frozen brains and pooled for RNA isolation processing. RNA extractions followed a modified protocol using Trizol (Life Technologies, Grand Island, NY). Bilateral amygdalae from two animals of the same sex and social status (four total amygdala punches per sample) were pooled together for each RNA extraction in order to minimize the effect of individual variability, as described previously [30, 58–61]. This resulted in three biological replicates for each of six groups (dominant males, dominant females, subordinate males, subordinate females, control males, control females). Tissue was homogenized on ice with 1 mL Trizol. After full homogenization, homogenate was allowed to settle at room temperature for 5 min. Homogenate was then mixed with

200 μl of chloroform, allowed to stand at room temperature for 2–3 min, and then centrifuged at 12,000×g for 15 min at 4 °C to separate the phases. The aqueous RNA phase was removed and dispensed into a new 2-mL microcentrifuge tube. The aqueous phase was washed with 200 μL of chloroform, mixed well, allowed to stand 2–3 min, and then centrifuged at 12,000×g for 10 min at 4 °C. For enhanced visualization of the pellet, 3 μL of GlycoBlue (Life Technologies, Grand Island, NY) was added and mixed gently. For RNA precipitation, 500 μL of 100% isopropanol was added, mixed gently, and allowed to stand at room temperature for 10 min. To obtain an RNA pellet, the solution was centrifuged at 12,000×g for 20 min at 4 °C. The remaining liquid was carefully removed and the pellet was washed twice in 1 mL 75% ethanol in RNase-free water and centrifuged at 7500×g for 5 min at 4 °C. The pellet was allowed to air dry for approximately 5 min and was then re-suspended in 20 μL of ultrapure water. Samples were stored at − 80 °C until downstream processing. RNA quality and concentration was determined using the Agilent Bioanalyzer, and samples were then sent for sequencing to Beckman Coulter Genomics (Danvers, MA). Amygdala sequencing was completed in paired-end 100 bp reads, averaging 37 M reads per sample.

Transcriptome Assembly and Optimization

The amygdala de novo transcriptome was assembled as described previously [30]. All data was acquired using the computing resources at Georgia State University [62]. Briefly, we used Trinity [63, 64] (https://github.com/trinityrnaseq/trinityrnaseq/wiki) for the initial de novo assembly using all 18 samples from both males and females. The assembly was then optimized using TransDecoder [64] (https://transdecoder.github.io) with a minimum cut-off of 50 amino acids [65] and BLASTx [66] (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the Uniprot-rodent database from January 21, 2016 [67] (http://uniprot.org). The optimized assembly was annotated using the Trinity-recommended platform, Trinotate (https://trinotate.github.io). PANTHER (Protein Analysis Through Evolutionary Relationships, http://www.pantherdb.org) was used for functional annotation of the optimized assembly, using *Mus musculus* as the reference organism. All sequences are available under NCBI SRA Accession Number SRP074844.

Differential Expression Analysis and Statistics

Differential expression analysis was completed using expected read counts from RNA-Seq by Expectation-Maximization (RSEM) [68] (http://deweylab.github.io/RSEM) in an exact test using the Bioconductor package edgeR [69] (https://bioconductor.org/packages/release/bioc/html/edgeR.html), as described previously [30]. Transcripts were considered to significantly differ if the false discovery rate (FDR) was < 0.05.

FDR is adjusted for multiple comparisons in RNA-Seq data and is thus a more stringent threshold to use when determining significance when compared with unadjusted *p* values.

For further analysis of our differential expression data, we entered the data into RRHO (Rank Rank Hypergeometric Overlap; https://bioconductor.org/packages/release/bioc/html/RRHO.html) [70], using the recommended settings, to determine the degree of overlap in the expression patterns between males and females. We also used PANTHER (pantherdb.org) [71–73] and GOSeq (https://bioconductor.org/packages/release/bioc/html/goseq.html) [74] for gene ontology analyses. Lastly, we used a signed, weighted gene co-expression network analysis (WGCNA, https://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/) to cluster our individual samples by gene expression patterns in the amygdala following the recommended settings [75, 76].

Compliance with Ethical Standards

All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee and are in accordance with the standards outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Conflict of Interest The authors declare they have no conflict of interest.

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