Neurogenomic landscape of social status-dependent cooperative display behavior

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Abstract

The neurogenomic mechanisms modulating cooperation are largely unknown, and previous work has been largely constrained to considering few genes in limited neurological space. Here we leverage transcriptomic and behavioral data on birds that perform cooperative courtship displays to better understand the mechanisms underlying cooperative behaviors. Males of the neotropical wire-tailed manakin (*Pipra filicauda*) form cooperative display coalitions, and their cooperative behaviors are modulated by testosterone in a status-specific manner: Testosterone promotes cooperation in non-territorial birds, but suppresses it in territory holders. To understand the neurogenomic basis of individual and status-specific regulation of cooperative display behavior, we profiled gene expression in 10 brain nuclei and two key endocrine tissues known to regulate social behavior. We associated gene expression with each bird's detailed behavioral and endocrine profile derived from years of repeated measures taken from free-living birds in the Ecuadorian Amazon. We found distinct landscapes of constitutive gene expression were associated with social status, testosterone phenotype, and cooperation, reflecting the modular organization and engagement of neuroendocrine tissues. Sex-steroid and neuropeptide signaling appear important in mediating status-specific relationships between testosterone and cooperation, and suggests shared regulatory mechanisms with male aggressive and sexual behaviors. We also find that systemic gene expression across the brain that could form the basis of social status-specific neuro-physical phenotypes, which are potentially mediated by testosterone and growth hormone. Overall, our findings provide new, systems-level insights into the mechanisms of cooperative behavior, and suggest differences in neurogenomic state form the basis for individual differences in behavioral phenotypes.

Introduction

Cooperative interactions among individuals can determine social status and, ultimately, individual fitness. Although the evolutionary basis of cooperation has received much attention, the mechanistic basis of cooperation is understudied. Cooperation, like other social behaviors, is likely mediated by individual differences in circulating hormones, such as sex steroids, and aspects of hormone-signaling pathways, including the distribution and abundance of hormone receptors in neural tissues (Soares *et al.* 2010). Individual variation in cooperative behavior is likely achieved by some combination of hormonal pleiotropy and modularity (Ketterson *et al.* 2009; Cox 2020). Hormonal pleiotropy mediates phenotypic integration, whereby multiple traits covary with a hormonal signal (Ketterson *et al.* 2009; Cox 2020), sometimes in opposite directions. For example, elevated testosterone typically promotes aggression but inhibits parental care in males (Wingfield *et al.* 2001). In contrast, through variation in hormone receptor expression, tissues across the body are often modular in their response to hormone signals, enabling diverse and flexible responses to a hormonal signal and trait independence (Ketterson *et al.* 2009; Lipshutz *et al.* 2019). An example of the latter, and a key player involved in modulating cooperative behavior is the social behavior network (SBN) of the brain. These reciprocally interconnected brain nuclei are rich in steroid receptors, steroidogenic enzymes and steroid-modulated neuropeptide systems (Newman 1999; Goodson 2005; O'Connell and Hofmann 2011). The SBN is both modular and dynamic, whereby different combinations of brain regions are activated in response to different social contexts (Newman 1999; Goodson 2005).

Variation among individuals in the expression of neuroendocrine genes across the SBN likely underlies repeatable individual differences in social behavior, and sets conditions for behavioral phenotypes that respond differently to circulating hormones and social stimuli. For example, the up or down-regulation of genes involved in sex steroid-signaling pathways across nuclei of the SBN can alter the sensitivity of the male brain to testosterone and manifest as differential behavioral responses to a given hormone signal. Emerging research is showing that that repeatable, individual differences in other behaviors (e.g., alternative reproductive tactics) are correlated with differences in constitutive gene expression or neurogenomic state among individuals characterized by suites of genes coexpressed across behaviorally relevant brain regions (Kabelik *et al.* 2021; Lattin *et al.* 2021; Antunes *et al.* 2021). Here, we attempt to

characterize, for the first time, how gene expression patterns across multiple brain regions and neuroendocrine tissues relate to individual variation in testosterone phenotype and cooperative behavior among males.

The wire-tailed manakin (*Pipra filicauda*) is a neotropical lekking bird in which unrelated males perform cooperative displays to attract females (Figure 1A). Long-term display partnerships established between territorial and subordinate non-territorial (floater) males form the basis of complex social networks (Figure 1A, B) (Ryder *et al.* 2008; Ryder *et al.* 2011a). In this bird, territoriality is a pre-requisite to siring offspring, and floater males follow an age-graded queue for territorial status (Ryder *et al.* 2008; Ryder *et al.* 2009). Cooperation is directly linked to fitness, whereby territory holders with more display partners sire more offspring, and floaters with more partners are more likely to ascend to territorial status (Ryder *et al.* 2009). Individuals have a repeatable 'testosterone phenotype' in which territorial males tend to have higher testosterone than floaters (Figure 1D) (Ryder *et al.* 2011b; Ryder *et al.* 2020). Remarkably, circulating testosterone has a status-specific effect on the cooperative display behavior: experimental and observational evidence show that higher testosterone levels are antagonistic to cooperation in territorial males but promote cooperation in floater males (Ryder *et al.* 2020; Vernasco *et al.* 2020) (Figure 1C). To explore the neurogenomic basis of the status-specific androgenic modulation of cooperative behavior in these birds, we quantified transcriptome-level gene expression in the SBN and endocrine tissues of wild male wire-tailed manakins.

Results

Landscape of gene expression among neural and endocrine tissues

Using previously developed microdissection protocols (Horton *et al.* 2020), we conducted transcriptome-level sampling of the pituitary, testes, and 10 brain nuclei comprising the entire SBN and a portion of the mesolimbic reward system (MRS) (Lopes and König 2020; Bentz *et al.* 2021b; Kabelik *et al.* 2021; Bentz *et al.* 2021a; Antunes *et al.* 2021) (Figure 1F, Table S1.2) from nine territorial and seven floater males. A subset of these individuals were characterized for testosterone and cooperative phenotypes (Figure 1DE). In this population, territorial males have, on average, higher circulating testosterone than floaters (Ryder *et al.* 2011b; Ryder *et al.* 2020), and the males sampled for this study reflect that pattern (Figure 1D).

A male's testosterone phenotype is represented by mean testosterone level calculated from repeated sampling (Supplement 1). In the broader population, the relationship between testosterone and social network measures used as a proxy for cooperation was status-specific, with testosterone suppressing cooperation in territorial males but increasing cooperation in floaters (Ryder *et al.* 2020). The overall frequency of cooperative interactions, however, does not differ between territorial and floater males (Figure 1E). Here, we used an individual's social network strength (time spent displaying with other males) as a proxy for an individual's cooperative tendency.

Our analyses revealed substantial differences in gene expression among tissues and brain regions (Figure 1G; Figure S2.7). As expected, the pituitary was associated with the expression of genes key to hormonal regulation (e.g., PRL, GH1, TSHB, POMC; Figure S2.3), while the testes showed an upregulation of genes associated with germ cells and flagellar motility (e.g., PKDREJ, BOLL, DRC7; Figure S2.3). Within the brain, the majority of gene expression variation was explained by differences between brain regions (Figure 1G inset) and was reflected in brain region-specific gene co-expression networks (Figure S2.7). These expression patterns are more similar between adjacent nuclei, forming major brain-region groupings of the amygdala, hypothalamus, and midbrain (Figure 1G). We predicted that 18 candidate neuroendocrine genes (Table S1.3) known to be associated with social behavior, would be associated with social status and cooperation in the wire-tailed manakin. Our results confirmed that these genes relating to sex-steroid signaling and metabolism, as well as steroid sensitive neuropeptides, had tissue and brain region-specific expression (Table 2.2, Figures S4.1-4.2). Further, we predicted that gene expression patterns more broadly would be brain region-specific (Figure 1G) and would reveal the key nuclei and neurogenomic pathways involved in mediating social status, mean testosterone level, and cooperative behavior.

Social status and testosterone phenotype are associated with tissue and brain region-specific patterns of gene expression

We observed similar landscapes of gene expression associated with mean testosterone and social status (Figure 2A, 2D, Figure S3.7), patterns that were expected because testosterone and status themselves are correlated (Figure 1D) (Ryder *et al.* 2011b; Ryder *et al.* 2020). The testes (Supplement 3) and the nucleus taenia (TnA), for example, had over 1,000 genes

differentially expressed (uncorrected $p < 0.05$) in association with both testosterone and status. Among the differentially expressed genes, estrogen receptor 2 (ESR2; aka ERβ) was upregulated in the TnA of males with higher testosterone (Figure 2B). Further, our analysis of the medial preoptic area (POM), an SBN nucleus known to be involved in regulating male sexual behavior and parental care (Panzica *et al.* 1996), revealed differential expression for compelling gene ontology categories and candidate neuroendocrine genes associated with mean testosterone (Figure S3.25, Figure S4.10, Figure S5.7). Notably, males with higher testosterone exhibited upregulation of androgen receptor (AR) in POM (Figure 2C).

Multiple candidate neuroendocrine genes, including AR and ESR2, showed status-specific expression patterns. In the paraventricular nucleus (PVN), ESR2 was higher in floater males than in territorial males (Figure 2E), and AR expression was positively correlated with testosterone in floater males, but not in territorial males (Figure 2I). In the bed nucleus of the stria terminalis (BSTm), a region that bridges the SBN and MRS, AR expression was higher in territorial males than in floater males (Figure 2F). In TnA, floater males, but not territorial males, with higher testosterone had higher expression of vasoactive intestinal peptide receptor 1 (VIPR1; Figure 2G), indicating a positive link between circulating testosterone and sensitivity to VIP in this critical region. We also found status-specific differential expression of genes for enzymes involved in steroid metabolism, including 5-alpha reductase (SRD5A2) in the lateral septum (LS), and aromatase (CYP19A1) in the POM and midbrain central gray (GCt; Supplement 4). Indeed, GCt was a hotbed for status-specific gene expression (Figure S3.6: 419 genes, $q < 0.1$), including candidate neuroendocrine genes for VIP (Figure 2H), progesterone receptor (PGR), and estrogen receptor 1 (ESR1; aka ERα; Figure S4.14-4.15).

Cooperative behavior is associated with a distinct gene expression landscape

Network strength (the number of cooperative interactions between individuals) varies widely among territorial and floater males (Figure 1E), representing a key axis of variation in social behavior in this species. We predicted that the associations between gene expression and cooperative behavior would be different from those observed for social status and testosterone. Indeed, we found a distinctive landscape of constitutive gene expression across tissues relating to cooperative behavior (Figure 3A, Figure S3.5). Expression patterns in the pituitary were highly associated with network strength (109 genes, $q <0.1$), among these genes, prolactin (PRL) was

downregulated in highly cooperative males (Figure 3B). Gene expression in the LS was tightly associated with strength (98 genes q<0.1), and included genes involved in development and neural organization (Figure 3C). Although relatively few genes were differentially expressed in the medial bed nucleus of the stria terminalis (BSTm), the LS and BSTm shared many genes with similar patterns of expression in association with strength as shown by RRHO analysis (Cahill *et al.* 2018) (Figure 3D, E). In contrast, the LS and BSTm shared few genes with similar expression in association with testosterone (Figure 4F, G) or social status (Figure S6.1A), suggesting that these regions are uniquely engaged in modulating cooperative behavior. Within these nuclei we found differential expression in genes involved in neural development and organization (Figure 4C).

Social status is associated with systemic gene expression

Testosterone is pleiotropic in that it can effect multiple traits by influencing gene expression in in similar ways in many different target tissues (Ketterson *et al.* 2009; Cox 2020), and it is crucial to social status-specific regulation of behavior in the wire-tailed manakin (Ryder *et al.* 2020). We reasoned that these effects would be reflected in shared signatures of differential expression across brain regions. Social status was linked to the highest number of consistently differentially expressed genes (60 genes, median p<0.05). For example, there was global regulation of genes relating to the immune system, including a possible homolog of a C-type Lectin Domain gene (CLEC2B)upregulated in territorial individuals (Figure 4A, C). There was also a consistent signature of differential regulation of developmental genes between territorial and floater males, such as roundabout guidance receptor 4 (ROBO4) (Figure 4B, C). We found evidence for global differential regulation of telomere maintenance genes according to social status (e.g., POLD-1-like (LOC114000574) and RAD51 upregulation). These findings of global expression differences among status classes could be partly caused by the upregulation of growth hormone (GH1) in the pituitary of territorial males (Figure 4D).

Discussion

Using the most comprehensive transcriptome-level sampling of the SBN to-date (Lopes and König 2020; Bentz *et al.* 2021b; Kabelik *et al.* 2021; Bentz *et al.* 2021a; Antunes *et al.* 2021), our findings highlight both modular and integrative neurogenomic organization underlying individual variation in behavior. The relative roles of each are a central to the

development and evolution of any complex trait (Ketterson *et al.* 2009; Lipshutz *et al.* 2019; Cox 2020). We discuss our findings in relation to each theme in turn.

Different landscapes of gene expression with each trait reflect modular organization of neuroendocrine system

Modularity is reflected in how different brain nuclei, endocrine tissues, and genetic pathways within individuals are associated with different traits. Such neurogenomic landscapes, characterized by the upregulation of neuroendocrine genes in some brain regions and downregulation in others, would allow differential sensitivity to testosterone and other signaling molecules among SBN nuclei. These neurogenomic states can be likened to the classic 'landscape' model of immediate early gene activation across the SBN after a social stimulus (3, 4). In that landscape model, it is the pattern of gene expression across SBN nuclei that correlates with behavioral responses, not the up or downregulation of particular genes within a single nucleus. This original model was envisioned in the context of proximate responses to social stimuli; our findings extend this model to longer-term neurogenomic states that underlie individual variation in complex social behaviors. In the landscape of constitutive gene expression in *Pipra filicauda*, some brain regions appear to be more tightly associated with social status, testosterone phenotype, and their interaction (e.g., TnA, POM and GCt), whereas other regions are related to an individual's strength of cooperative behavior (e.g., LS). In our results large suites of genes show unique expression patterns in relation to each aspect (status, testosterone, and strength) in individual tissues and brain regions.

The importance of these particular brain regions is inferred from the number of differentially expressed genes, as well as the role of key neuropeptides and sex-steroid related genes. For example, the number of differentially expressed genes observed in TnA implicates this region of the SBN as a hub where multiple neuroendocrine pathways are involved in modulating individual variation in testosterone phenotype. As a key example, the TnA of males with high testosterone phenotype would be more sensitive to estrogens due to the upregulation of ESR2. The conversion of testosterone to estradiol by the enzyme aromatase and subsequent binding to estrogen receptors is a key pathway in the regulation of testosterone-dependent male behaviors more broadly (Schlinger and Balthazart 2013), which is reflected in our observed patterns of estrogen receptor expression as well as brain region specific differences in aromatase

expression. For example, within TnA itself, ESR1 has been shown to regulate status-specific aggression in birds (Horton *et al.* 2014; Merritt *et al.* 2020) and prosocial behavior in prairie voles (Cushing *et al.* 2008). ESR2 is not well characterized in birds, but it appears to be important in emotional state, social behavior, and sexual regulation broadly across the mouse central nervous system (Dombret *et al.* 2020), and specifically in the medial amygdala (the putative homolog of the avian TnA) (Nakata *et al.* 2016; Lymer *et al.* 2018). We also found evidence that status-specific regulation of behavior is modulated directly by testosterone through AR. Higher AR expression is linked to display performance in golden-collared manakins (Schlinger *et al.* 2013), and AR expression in one or more nuclei of the SBN (e.g., POM, PVN, BSTm) likely plays a key role in the testosterone-mediated social behavior of male wire-tailed manakins.

Our results also highlight the importance of neural VIP expression in modulating social behavior (Kingsbury and Wilson 2016), and VIP activity in GCt may play a pivotal role in the status-specific regulation of cooperation. Indeed, the GCt showed status-specific expression of VIP, ESR1, and PGR in relation to testosterone phenotype. Although tyrosine hydroxylase (TH) neurons in the GCt have been associated with courtship behaviors (Goodson *et al.* 2009; Ben-Tov *et al.* 2021), our data suggest a previously undescribed function of VIP for modulating courtship behavior (Kingsbury and Wilson 2016). The GCt coordinates social stimuli and downstream motor processes that control behavior. For example, in the songbird brain, GCt links POM to the song control system and thereby mediates androgen-dependent singing behavior (Haakenson *et al.* 2020). Given that cooperative display in manakins requires coordinated physical and vocal displays (Trainer *et al.* 2002), the status-specific differential expression of genes for steroid receptors and steroid-sensitive neuropeptides in the GCt suggest that this SBN region may play a unique role in mediating status-specific relationships between testosterone and cooperative behavior.

The amount of time an individual male spends displaying with other males (i.e., network strength), our proxy for cooperative behavior, is associated with a unique pattern of gene expression among tissues and brain regions. In particular, the LS showed a large number of differentially expressed genes associated with cooperation, and BSTm had a similar, but weaker, pattern of expression. BSTm has neural projections into the LS, and vasotocin (AVP) and VIP

neurons in the BSTm and receptors for these neuropeptides in the LS are associated with affiliative behavior and gregariousness in birds (Kelly *et al.* 2011; Kelly and Goodson 2013; Kingsbury and Wilson 2016). Those prior studies suggest a role for these brain nuclei in processing social information acutely, whereas our study suggests differences among individuals in cooperative tendency is related to constitutive differences in gene expression and neural structure, perhaps relating to excitability of these regions in response to social information. Thus, diverse affiliative behaviors across species may be regulated by conserved mechanisms and brain regions. In contrast with other studies on the neural mechanisms regulating vertebrate social behavior (Feldman 2012), we found no convincing evidence that key nonapeptides, vasotocin or mesotocin (OXT) or their receptors, are differentially expressed in any tissue or brain region in association with status, testosterone, or individual differences in behavior. This finding does exclude a role for these nonapeptides in regulating status-specific differences or sociality in our manakins, as perhaps they are relevant on timescales different from those we measured, and/or confer differences in activity of OXT or AVP neurons.

The candidate steroid and neuropeptide gene expression patterns across the SBN that we identify here may underlie variation in the testosterone-mediated cooperative behaviors of male wire-tailed manakins by differentially altering the metabolism of and sensitivity to testosterone among SBN nuclei. Expression patterns for these neuroendocrine genes present a useful basis for conceptualizing the role of other genetic pathways. For example, we found evidence for the differential expression of genes involved in broader neural development and metabolism (e.g., Figure 3C). Indeed, testosterone manipulations are known to induce changes in neuroanatomy in behaviorally relevant brain nuclei (Kabelik *et al.* 2008), suggesting a direct role for testosterone-mediated neural growth and flexibility in modulating male-male cooperation (Soares *et al.* 2010). Our results provide promising targets for further study on the mechanisms underlying cooperative behavior, including examination of neuroanatomical features and the use of hormonal manipulations and RNAi technologies to examine causal relationships between neural gene expression and behavior (e.g., (Merritt *et al.* 2020)).

Similarities in gene expression reflect the role of testosterone as a phenotypic integrator

Similarities in gene expression among tissues are predicted as a result of the pleiotropic effects of testosterone, and support the role of the hormone as a mediator of phenotypic integration. The extent of gene expression similarities and differences among tissues may reflect the degree of integration and independence (or modularity), respectively, that can either constrain or facilitate adaptive evolution (Ketterson *et al.* 2009; Lipshutz *et al.* 2019). We identified similarities in gene expression between brain regions that broadly recapitulated tissue ontology, including the striking similarity in gene expression between LS and BSTm in relation to an individual's cooperative tendency (network strength). We also identified consistent, brain-wide (systemic) gene expression differences associated predominantly with social status and testosterone phenotype. However, systemic results did not explain as much variation in overall neural gene expression as with some axes of personality (Kabelik *et al.* 2021; Lattin *et al.* 2021), suggesting that the brain region-specific patterns may be more important in modulating male wire-tailed manakin behavior. However, the broader associations of social status with metabolic, immune, and telomere gene ontology categories suggests differences in allostatic load among status classes in male-male cooperative systems (Goymann and Wingfield 2004; Vernasco *et al.* 2021; Jones and DuVal 2021). Further, differential global regulation of telomeres may explain the relative stability of telomere length in territorial wire-tailed manakin males (Vernasco *et al.* 2021), and collectively these results may represent metabolic adaptations for territoriality.

Our results highlight two additional hormones that may have a system-wide role in the status-specific modulation of cooperative display behavior. We found differences in the expression of genes for important behavioral and metabolic hormones (prolactin and growth hormone) in the pituitary. The anterior pituitary is the primary production site for both hormones, and thus gene expression patterns here are likely good predictors for circulating levels of those hormones and thus signal strength. Transcripts encoding prolactin, best known for its role in promoting parental care behaviors, were negatively correlated with cooperative behavior. While prolactin is emerging as important in other areas of social behavior, such as nest defense (Mohamed *et al.* 2016), the negative relationship observed appears contradictory to a general model of increased prolactin promoting broadly affiliative social interactions. We speculate that this relationship reflects that cooperative display behavior may have evolved from initially

aggressive or competitive interactions (Díaz-Muñoz *et al.* 2014). The other compelling phenotypic integrator for status-dependent behaviors in the wire-tailed manakin comes from the gene encoding growth hormone (GH1). Ascension in social rank in the cooperatively displaying manakins is associated with age (McDonald 1989; DuVal 2007; Ryder *et al.* 2008). It was therefore surprising that territorial males in our study showed increased expression of GH1 in the pituitary as compared to floater males. However, stimulation of the growth hormone axis is associated with increased testosterone that enables phenotypic divergence between male and female anoles (Cox *et al.* 2017). Without gross morphological differences in wire-tailed manakin status-classes, growth-hormone could have subtle effects on multiple systems enabling the status-specific regulation of behaviors. In particular, status differences in GH1 expression may explain repeated signatures of neural development and protein synthesis in our results (Sonksen 2006), and may reflect the increased metabolic demands on displaying territorial males through muscle performance and regulation of oxidative stress (Hu *et al.* 2019). Although the neuroethological effects of growth hormone in birds are understudied, it has been linked to feeding behavior in poultry (Tachibana *et al.* 2015). Growth hormone has been associated with behavioral and cognitive effects in mammals (Rollo *et al.* 1999). Perhaps most interesting is that peripheral growth hormone administration promotes increased aggression and brain dopaminergic activity in fish (Johansson *et al.* 2004). Thus, our results suggest that the growth hormone axis may play a role in the status-specific regulation of wire-tailed manakin cooperative displays, and warrant further consideration of the potential for growth hormone to play a role in modulating avian social behaviors more broadly.

Conclusion

The landscapes of gene expression and plurality of gene function, including sex-steroid and neuropeptide signaling, observed across the SBN of the wire-tailed manakin brain speaks to the modular nature of behavioral mechanisms (Newman 1999; Goodson 2005; Lipshutz *et al.* 2019) and highlights the continuum between complete phenotypic integration and modularity (Ketterson *et al.* 2009). Much of the previous work on the mechanisms of social and cooperative behaviors focus on relatively few candidate genes in broad-scale brain divisions (e.g., hypothalamus) or in one or a few particular nuclei. Our work presented here is one of the most comprehensive examinations to date of region-specific gene expression in all nuclei of the SBN.

Our findings help us to better understand cooperative behavior (Soares *et al.* 2010) by linking neurogenomics with endocrine mechanisms. We further demonstrate and emerging idea that constitutive landscapes in gene expression across the brain are associated with repeatable differences in behavioral phenotype (Kabelik *et al.* 2021; Lattin *et al.* 2021; Antunes *et al.* 2021). We also provide tantalizing glimpses into nuclei and genomic pathways involved in the status-specific regulation of this cooperative display behavior. Although questions remain on the acute mediators of cooperative display behavior in the wire-tailed manakin, we demonstrate the power and flexibility of a system-wide approach. We propose that landscapes of constitutive gene expression for multiple genes and across many tissues and brain regions, like those observed here, form the foundation of consistent inter-individual differences in any behavior, upon which flexible and acute responses to social stimuli are built.

Materials and Methods

All methods described here were approved by Smithsonian Animal Care and Use Committee (protocols 12-12,14-25,17-11), as well as the Ecuadorian Ministry of the Environment (MAE-DNB-CM-2015-0008), and samples were transported with export permit (006-016-EXP-1C-FAU-DNB/MA) and import permit (USDA APHIS 126133). Detailed methods can be found in the supplementary material, and full code from analyses in R v 4.0.2 are available on the GitHub site (https://periperipatus.github.io/PIFI_brain_transcriptome/).

Field Methods

For RNA sequencing we sampled 16 wild territorial and floater wire-tailed manakin males from Tiputini Biodiversity Station in the lowland Amazon rainforest of Ecuador (Table S1.1). To provide high-resolution data on cooperative social interactions, we used proximity data logging for 180 free-living male manakins. Social network structure and individual variation in behavior were characterized using tag proximity detections within territories (Ryder *et al.* 2012; Dakin and Ryder 2018; Ryder *et al.* 2020). On 12 of these 16 males, we used repeated testosterone sampling (Figure 1D, Supplement 1) to characterize each male's hormonal phenotype (mean testosterone) and tagging data to calculate the time spent interacting with other males (social network strength) (Figure 1E).

Microdissection and RNAseq

Methods for brain extraction, tissue preservation, cryosectioning, microdissection, and RNA extraction followed those published previously (Horton *et al.* 2020). Briefly, birds were euthanized by decapitation near the site of capture immediately after blood sampling for testosterone. Whole brains were extracted within 4-6 minutes of sacrifice and immediately frozen in powdered dry ice. To capture the full Hypothalamic-Pituitary-Gonadal axis critical to sex-steroid regulation, we additionally sampled the pituitary gland and testes, which were sampled immediately after the brain. Brains and testes were kept frozen on dry ice in the field (< 4 hours) until transferred into a 21-day liquid nitrogen-charged Arctic Express™ dry shipper (Thermo Scientific) where they were stored at cryogenic temperatures until imported into the United States. Pituitaries were preserved in RNAlater™ (Invitrogen) at ambient temperatures until imported into the United States.

The brains were cryosectioned, and frozen sections were microdissected to excise tissue from 10 different nuclei. Eight of these nuclei are nodes of the highly interconnected SBN of the vertebrate brain (Newman 1999; Goodson *et al.* 2005). The SBN nuclei included the VMH, AH, POM, TnA, and two midbrain regions (GCt and ICo) (Figure 1F). Two of the SBN nuclei sampled, BSTm and LS, are also nodes of the Mesolimbic Reward System (MRS), reflecting the interconnectivity of the two networks, both of which play key roles in the regulation of vertebrate social behavior (O'Connell and Hofmann 2011). We also sampled the arcopallium intermedium (AI), a region that may be, in part, homologous the basolateral amygdala of mammals, a node of the MRS (O'Connell and Hofmann 2011), which is hypothesized to play a role in androgen-dependent manakin display behavior (Fusani *et al.* 2014). Finally, we sampled the paraventricular nucleus (PVN), a region that is not yet formally recognized as part of the SBN, despite its neuropeptide projections to nodes of the SBN and established role as a major regulator of vertebrate social behavior (Goodson and Thompson 2010; Goodson and Kingsbury 2013). For a graphical representation of the tissues sampled see Figure 1G, with a full list of individuals collected in Table S1.1 and tissues in Table S1.2.

RNA was extracted and sequenced according to previously described methods (Horton *et al.* 2020). RNA libraries for the various tissues and field seasons were randomized amongst sequencing batches (Supplement 1, Table S1.1 & S1.2). All samples analyzed are associated

with Bioproject PRJNA437157, and SRR12660169-198, SRR19521260-271, SRR19521432-575. The file that matches SRA numbers with our code and metadata available on the GitHub.

Gene expression analyses

Reads were mapped to the annotated *Pipra filicauda* genome (GCA_003945595.1) using splice-aware mapper STAR v.2.7.5 (Dobin *et al.* 2013), and transcripts were counted against exons using featureCounts v.2.0.1 (Liao *et al.* 2014). For subsequent analyses we used a total of 170 libraries, and quality filtering of individuals and gene-counts was performed before the analysis of each tissue (Supplement 1 & 3).

We characterized differences in gene expression among tissues and nuclei using principal components analysis in PCAtool*s* and Weighted Gene Co-Expression Analysis (WGCNA) (Langfelder and Horvath 2008; Blighe and Lun 2020) (Supplement 2). Then, to explore associations with our traits of interest (social status, mean testosterone, and social network strength) we identified differentially expressed genes using separate models in DESeq2 (Love *et al.* 2014) (Supplement 3), and co-expression networks using WGCNA (Supplement 5).

We conducted further analysis on eighteen candidate genes with known involvement in social behavior (Table S1.3) (Supplement 4). These broadly divided into sex hormone metabolism and sex hormone receptors (Figure S4.1), and neuropeptides and their receptors (Figure S4.2). Any candidate gene identified by differential gene expression (DGE) analysis with an uncorrected $p<0.05$ was considered "significant". In addition to the three separate relationships above, we also explored whether candidate genes were involved in mediating status specific relationships between mean testosterone and social behavior (Ryder *et al.* 2020) through a final DGE interaction analysis (status*mean testosterone). The list of putative relationships between interest variables and candidate genes was further filtered by excluding any relationship with highly influential observations, or with $R^2<0.3$ (Supplement 4).

We characterized overlap in gene expression patterns by comparison of ranked gene-lists (Supplement 6). The gene-lists were ranked by $-\log_{10}$ transforming the p-values and multiplying by the direction of expression. These gene lists were then compared for similarity between each pair of tissues by calculating a) Pearson correlation b) rank-rank hypergeometric overlap (RRHO) test (Cahill *et al.* 2018). Finally, to explore systemic gene expression patterns (i.e., consistent patterns across the entire brain), we calculated the median of the transformed p-value.

Genes that had a median p-value <0.05 (transformed value >|1.3|) were considered consistently differentially expressed. All Gene Ontology enrichment for biological process was determined using custom annotations (Supplement 3) using *clusterProfiler* v3.16.1 (Yu *et al.* 2012).

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https://github.com/periperipatus/PIFI_brain_transcriptome

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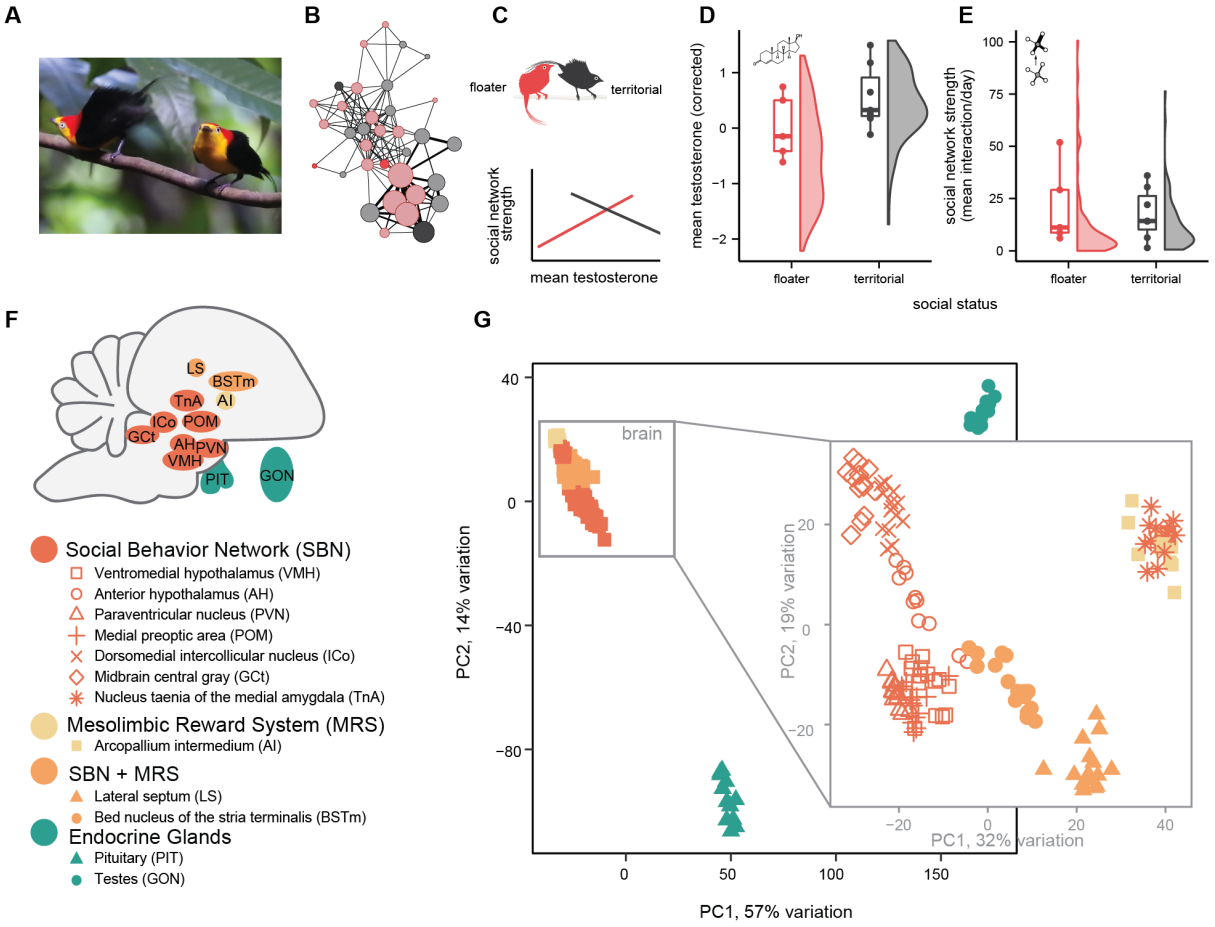

Fig. 1. Circulating testosterone and neuroendocrine tissues involved in the cooperative behavior of wire-tailed manakins. A) Males engage in cooperative displays (photo: Alice Boyle) that form the basis of B) complex social networks. The example shown is a lek over one week, where node size indicates a male's average social network strength, and edge width represents the frequency of interactions between a pair of individuals. Node color denotes floater (red) and territorial (gray) male status, where darker colors were included in the study. C) A male's testosterone phenotype influences his cooperative tendency differently in territorial and floater classes. D) Twelve individuals (points) were from the larger population with repeated measures of testosterone (density), and (E) proximity-data logging measures of social network strength. Note the total sample size for social status is N=16. F) RNAseq characterized gene expression from each individual in 10 testosterone-sensitive brain regions and two endocrine tissues. Structures shown are not to scale. Colors represent functional groups (O'Connell and Hofmann 2011). G & inset) principal component analysis of transcriptomic data reveals distinct gene expression profiles among brain regions and endocrine tissues (colors and shapes as F).

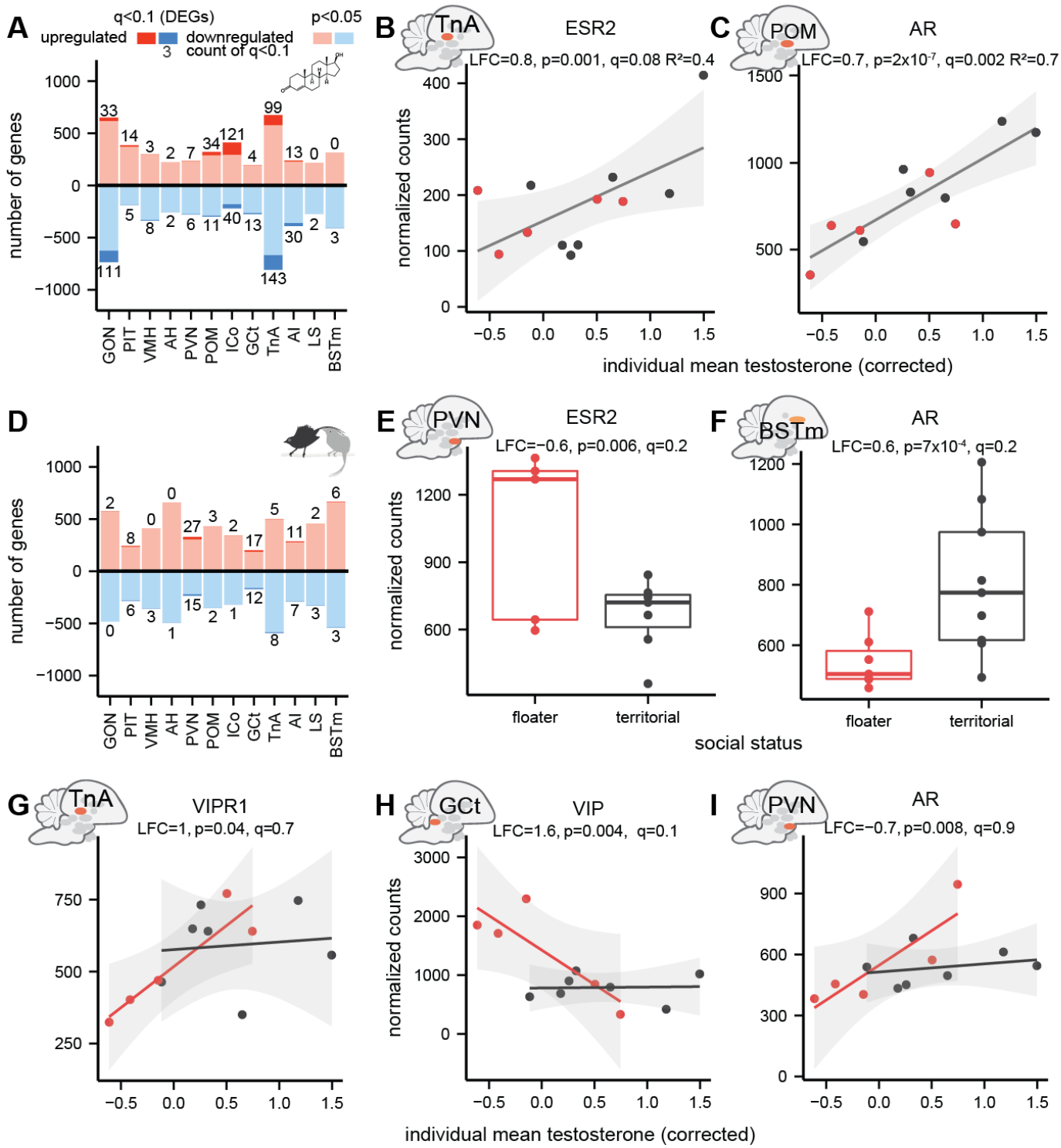

Fig. 2. Neurogenomic landscape of social status and hormone phenotype. A) There is a landscape of gene expression in male wire-tailed manakins with high mean testosterone relative to lower mean testosterone. Red bars indicate genes that are upregulated in males with higher testosterone, whereas blue indicates genes that are downregulated in males with higher testosterone. Darker colors and text indicate number of differentially expressed genes after FDR

correction. B) ESR2 is upregulated in TnA of males with higher testosterone, where point colors indicate social status (red = floater; black = territorial). C) AR was upregulated in POM of males with higher testosterone. D) A broadly similar landscape occurs in relation to social status, where red indicates genes upregulated in territorial males. E) ESR2 was downregulated in the PVN of territorial males. F) AR was upregulated in the BSTm of territorial males. G) VIPR1 expression shows a status-specific relationship with mean testosterone in the TnA. H) VIP (amongst others) showed status-specific gene expression in relation to mean testosterone in the GCt. I) AR shows status-specific expression in relation to mean testosterone in the PVN.

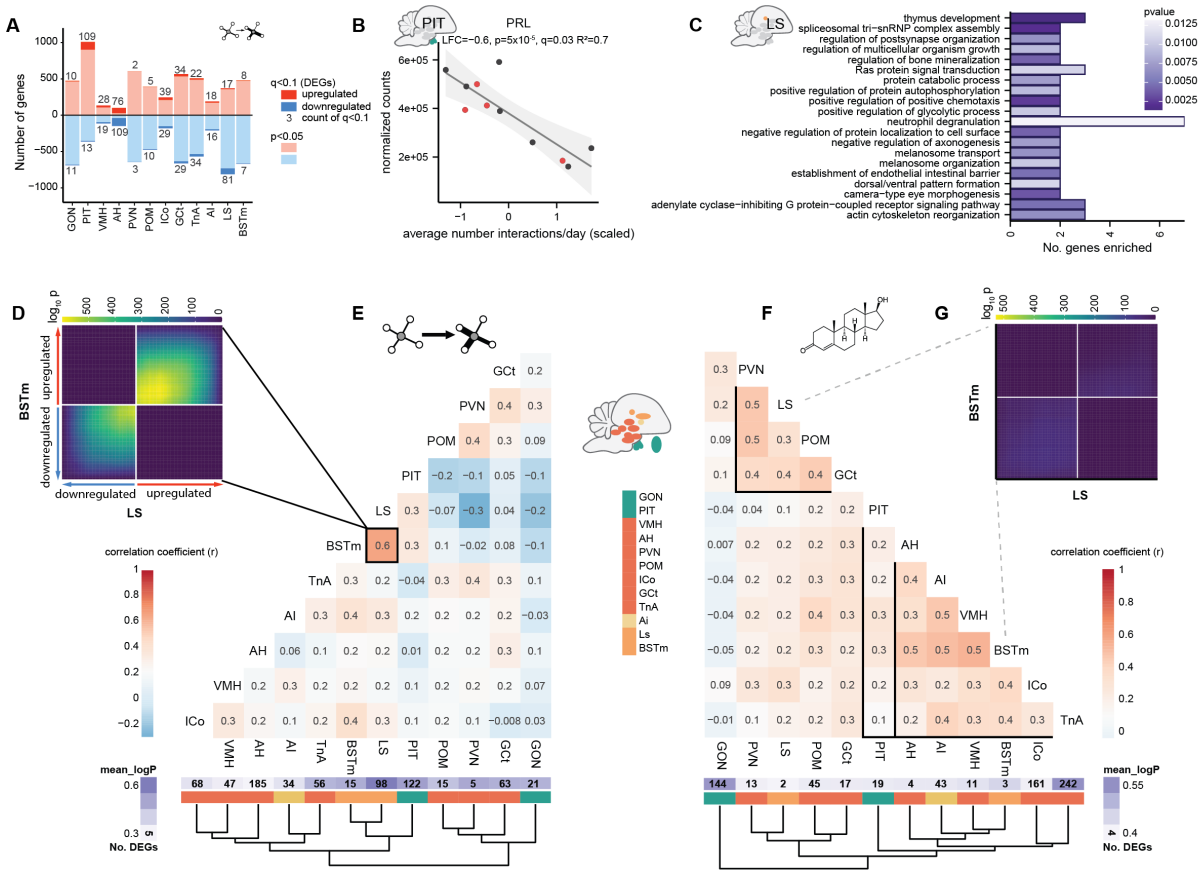

Fig. 3. A distinct landscape of gene expression in relation to cooperative behavior. A) Landscape of gene expression in male more cooperative wire-tailed manakins (social network strength). Darker colors and text indicate number of genes after FDR correction. Red indicates genes that are upregulated in more cooperative males, whereas blue indicates genes that are downregulated in these males. B) PRL in the pituitary shows decreased expression in males with higher cooperative tendencies. C) GO terms relating to neural development were among enriched categories in the differentially expressed genes of the Ls, where bar color indicates uncorrected p-value. D) RRHO2 analysis reveals similar profiles of gene expression associated with cooperation in the LS and BSTm, E) but otherwise generally low similarity of gene expression among tissues in association with cooperation. F) Similarity of gene expression among tissues in relation to testosterone phenotype roughly recapitulates ontogeny of brain regions, G) and there is no correlation between testosterone related gene expression profiles in the LS and BSTm. E,F) Cell color and number are the Pearson correlation coefficients of gene expression between tissues, correlating log10 transformed differential expression p-values (times the direction of differential expression). Black boxes outline significant tissue correlation clusters based on 1,000 bootstrap resamples of the correlation matrix.

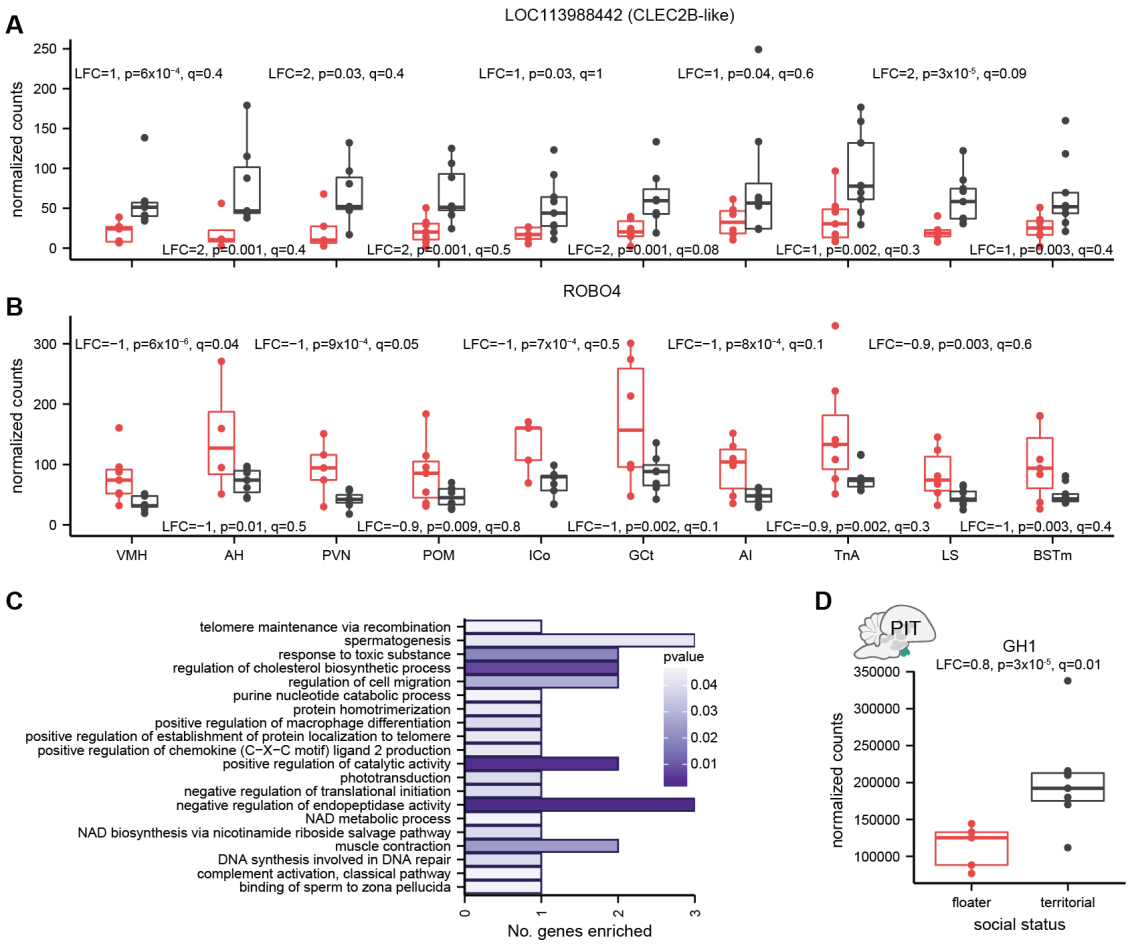

Fig. 4: Global neural gene expression differences between social classes

A) Immune gene CLEC2B-like was consistently upregulated in the brains of territorial individuals, and B) developmental gene ROBO4 was consistently downregulated in the brains of territorial males. C) Enriched GO categories among genes that were consistently up- or downregulated between territorial and floater males, where bar color indicates uncorrected p-value. D) These broader signatures of developmental differences could be explained by upregulation of growth hormone transcript in the pituitary of territorial males.