Neurogenomic landscape associated with status-dependent cooperative behavior

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Abstract

The neurogenomic mechanisms mediating cooperation in animals are understudied. Here we leverage extensive 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. That is, testosterone promotes cooperation in non-territorial birds, but suppresses it in territory holders. We sought to understand the neurogenomic underpinnings of these three related traits: social status, cooperative behavior, and testosterone phenotype. To do this, we profiled gene expression in 10 brain nuclei spanning the social decision-making network (SDMN), 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 3 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 to be important in mediating status-specific relationships between testosterone and cooperation, which suggests shared regulatory mechanisms with male aggressive and sexual behaviors. We also identify differentially regulated genes that are involved in cellular activity and synaptic potentiation, suggesting that multiple mechanisms underpin these genomic states. Finally, we identify SDMN-wide gene expression differences between territorial and floater males that could form the basis of "status-specific" neurophysiological phenotypes, potentially mediated by testosterone and growth hormone. Overall, our findings provide new, systems-level insights into the mechanisms of behavior, and suggest that differences in neurogenomic state are the basis for individual differences in social behavior.

Significance Statement

Cooperative behavior is an important determinant of fitness in many animals, yet its neurogenomic underpinnings remain poorly understood. We leveraged an unprecedented dataset describing gene expression in multiple brain nuclei and endocrine tissues from male wire-tailed manakins (*Pipra filicauda*) characterized for social status, behavior, and testosterone phenotype in the Ecuadorian Amazon. In this species, testosterone influences an unusual male-male cooperative display, but the nature of the relationship between cooperation and circulating testosterone depends on social status. We found evidence that individual behavioral phenotypes are determined by a complex neurogenomic landscape across interconnected brain nuclei. We show, on a transcriptomic scale, that brain modularity and hormone pleiotropy drive status-specific differences in gene expression resulting individual differences in cooperative behavior.

Introduction

Cooperation is the synchronized behavior(s) of two or more individuals to produce a net fitness benefit for one or more individuals (Taborsky and Taborsky 2015). An individual's decisions about cooperation are based on the immediate social stimulus and individual condition, which is based on recent and distant past experiences, developmental effects, and underlying genetics. These effects can ultimately produce repeatable, among-individual variation in behaviors – a behavioral phenotype – where some individuals tend to be more cooperative than others. To understand the mechanisms of cooperation, one needs to understand the neurophysiological signal processing of the stimulus through to the behavioral response, as well as the genetic and neurophysiological components that underpin behavioral phenotypes. Indeed, understanding these mechanisms enables us to link how genetic and neurophysiological architecture can constrain and facilitate the evolution of cooperation (Soares *et al.* 2010; Hofmann *et al.* 2014; Taborsky and Taborsky 2015). Differences in cooperative behavioral phenotype are in part achieved by the pleiotropic yet modular effects of hormone signaling systems (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 hormonal and social signals and trait independence (Ketterson *et al.* 2009; Lipshutz *et al.* 2019).

Cooperation, like other social behaviors, appears to be mediated by 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; Kasper *et al.* 2017). The neural substrates modulating social behaviors are conserved across vertebrates (O'Connell and Hofmann 2011). Known as the social decision making network (SDMN), 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 SDMN is both modular and dynamic, whereby different combinations of brain regions are activated in response to different social contexts (Newman 1999; Goodson 2005). This model means the common practice of measuring the

85 neurological response in a single nucleus is not reflective of all systems engaged in modulating a
86 given social behavior. Variation among individuals in the expression of neuroendocrine genes
87 across the SDMN likely underlies repeatable individual differences in social behavior and sets
88 conditions for behavioral phenotypes that respond differently to circulating hormones and social
89 stimuli. For example, the up or down-regulation of genes involved in sex-steroid signaling
90 pathways across nuclei of the SDMN can alter the sensitivity of the male brain to testosterone
91 and manifest as differential behavioral responses to a given hormone signal. Emerging research
92 is showing that behavioral phenotypes are correlated with differences in constitutive gene
93 expression or neurogenomic state among individuals characterized by suites of genes co-
94 expressed across behaviorally relevant brain regions (Kabelik *et al.* 2021; Lattin *et al.* 2021;
95 Antunes *et al.* 2021). However, given the diversity of behavioral components associated with
96 cooperation (Díaz-Muñoz *et al.* 2014; Kasper *et al.* 2017), questions remain on how
97 neuroendocrine mechanisms operate to produce the variety of social and cooperative behaviors
98 among individuals. Indeed, our understanding of the neuroendocrine mechanisms underlying
99 seemingly paradoxical male-male reproductive cooperative behaviors is only emerging (DuVal
100 and Goymann 2011; Díaz-Muñoz *et al.* 2014; Vernasco *et al.* 2020; Ryder *et al.* 2020; Jones and
101 DuVal 2021; Loveland *et al.* 2021). For the first time, we characterize gene expression across the
102 neuroendocrine system in relation status-specific androgenic modulation of a male-male
103 cooperative behavior.

104 The wire-tailed manakin (*Pipra filicauda*) is a neotropical lek breeding bird species in which
105 unrelated males perform cooperative displays to attract females (Figure 1A). Long-term display
106 partnerships established between territorial and subordinate non-territorial (floater) males form
107 the basis of complex social networks (Figure 1A, B) (Ryder *et al.* 2008; Ryder *et al.* 2011a). In
108 this bird, territoriality is a pre-requisite to siring offspring, and floater males follow an age-
109 graded queue for territorial status (Ryder *et al.* 2008; Ryder *et al.* 2009). Cooperation is directly
110 linked to fitness, whereby territory holders with more display partners sire more offspring, and
111 floaters with more partners are more likely to ascend to territorial status (Ryder *et al.* 2009).
112 Individuals have a repeatable 'testosterone phenotype' in which territorial males tend to have
113 higher testosterone than floaters (Figure 1D) (Ryder *et al.* 2011b; Ryder *et al.* 2020).
114 Remarkably, circulating testosterone has a status-specific effect on the cooperative display
115 behavior: experimental and observational evidence show that higher testosterone levels are

116 antagonistic to cooperation in territorial males but promote cooperation in floater males (Ryder
117 *et al.* 2020; Vernasco *et al.* 2020) (Figure 1C).

118 To explore the neurogenomic basis of the status-specific androgenic modulation of
119 cooperative behavior in these birds, we quantified transcriptome-level gene expression in the
120 SDMN and pituitary and testes of free-living male wire-tailed manakins sampled in the
121 Ecuadorian Amazon. Captive studies can obscure ecologically and evolutionary relevant
122 variation in environmental and behavioral responses (Kasper *et al.* 2017; Griffith *et al.* 2017), so
123 we demonstrate the exciting applicability of neuro-transcriptional methods in the challenging
124 conditions of the rainforest. In this study, we aimed to characterize the genes and neuroendocrine
125 pathways that modulate individual variation in cooperative behavior, as well as those related
126 social status and individual variation in testosterone phenotype as a modifier of both social status
127 and cooperative behavior. We characterized the constitutive gene expression for each of these
128 traits within and between nodes of the SDMN and the endocrine tissues. Given the androgenic
129 regulation of cooperative display (Ryder *et al.* 2020; Vernasco *et al.* 2020), we hypothesized that
130 differences in gene regulation would manifest in SDNM nuclei with high density of sex-steroid
131 receptors and pituitary and gonadal tissues (Newman 1999; Goodson 2005). We further reasoned
132 that androgenic regulation would relate to differential expression of genes relating to sex-steroid
133 signaling and metabolism, as well as three key neuropeptide systems involved in social behavior
134 (Goodson and Thompson 2010; Kingsbury and Wilson 2016). Given the modular nature of the
135 SDNM, we predicted that many gene expression patterns would be brain region-specific (Figure
136 1G). However, given the pleiotropic effects of testosterone (Ketterson *et al.* 2009; Cox 2020), we
137 also predicted that there would be signatures of testosterone's systemic influence on gene
138 expression patterns across the brain. We discuss our results in the context of modes of
139 phenotypic evolution and similarities with mechanisms of other social behaviors.

140 Materials and Methods

141 All methods described here were approved by Smithsonian Animal Care and Use Committee
142 (protocols 12-12,14-25,17-11), as well as the Ecuadorian Ministry of the Environment (MAE-
143 DNB-CM-2015-0008), and samples were transported with export permit (006-016-EXP-1C-
144 FAU-DNB/MA) and import permit (USDA APHIS 126133). Detailed methods can be found in
145 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/) and on FigShare (10.25573/data.22186516).

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). Males were categorized by social status as either territorial or floater. Territorial males (n=9) are males in definitive plumage that hold a consistent display territory over the study period, while floaters (n=7) have either definitive or pre-definitive plumage and do not occupy consistent territories within leks (Ryder *et al.* 2008). On 12 of these 16 males, we used repeated testosterone sampling (Figure 1D, Supplement 1) to characterize each male's testosterone phenotype (mean testosterone). In this population, territorial males have, on average, higher circulating testosterone than floaters (Ryder et al., 2020; Ryder, Horton, et al., 2011), and the males sampled for this study reflect that pattern (Figure 1D). A male's testosterone phenotype is represented by mean of the residuals of a linear regression from repeated sampling of testosterone concentration (Supplement 1 for more detail, see also (Ryder *et al.* 2020)). Mean testosterone as a measure of testosterone phenotype is repeatable within individuals (Ryder *et al.* 2020). In the broader population, the relationship between testosterone phenotype 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) (Figure 1C).

Social network structure and individual variation in behavior were characterized using autonomous radio-telemetry and tag proximity detections within territories on a total population of 180 male manakins over multiple years (Ryder *et al.* 2012; Dakin and Ryder 2018; Ryder *et al.* 2020). We used this tagging data to calculate social network strength for the same 12 males with repeated testosterone measures. Social network strength is a proxy for cooperative tendency and is the time spent interacting with other males averaged over the study period (Figure 1E), and was the most repeatable within individuals (Ryder *et al.* 2020). This social network proxy for cooperation was most strongly status specific relation to an individual's testosterone phenotype (Ryder *et al.* 2020) (Figure 1C), but the overall frequency of cooperative interactions does not differ between territorial and floater males (Figure 1E).

175 ***Microdissection and RNAseq***
176 Methods for brain extraction, tissue preservation, cryosectioning, microdissection, and RNA
177 extraction followed those published previously (Horton *et al.* 2020b). Briefly, birds were
178 euthanized by decapitation near the site of capture immediately after blood sampling for
179 testosterone. Whole brains were extracted within 4-6 minutes of euthanasia and immediately
180 frozen in powdered dry ice. To capture the full Hypothalamic-Pituitary-Gonadal axis critical to
181 sex-steroid regulation, we additionally sampled the pituitary gland and testes, which were
182 sampled immediately after the brain. Brains and testes were kept frozen on dry ice in the field (<
183 4 hours) until transferred into a 21-day liquid nitrogen-charged Arctic Express™ dry shipper
184 (Thermo Scientific) where they were stored at cryogenic temperatures until imported into the
185 United States. Pituitaries were preserved in RNAlater™ (Invitrogen) at ambient temperatures
186 until imported into the United States.

187 The brains were cryosectioned, and frozen sections were microdissected to excise tissue from 10
188 different nuclei involved in social behavior – nine of which are from the SDMN. The SDMN
189 reflects the interconnectivity of two networks involved in regulating social behavior (O'Connell
190 and Hofmann 2011), constituting nuclei from both the Social Behavior Network (SBN)
191 (Newman 1999; Goodson 2005) and the Mesolimbic Reward System (MRS) (Olds and Milner
192 1954; O'Connell and Hofmann 2011). Figure 1F indicates the nomenclature used and the
193 relationships of these nuclei to the SBN and MRS. To clarify bird specific nomenclature: the
194 arcopallium intermedium (AI) is a region that may be, in part, homologous the basolateral
195 amygdala of mammals, a node of the MRS (O'Connell and Hofmann 2011), which is
196 hypothesized to play a role in androgen-dependent manakin display behavior (Fusani *et al.*
197 2014); and the nucleus taenia (TnA) is homologous to the mammalian medial amygdala
198 (O'Connell and Hofmann 2011). Finally, we sampled the paraventricular nucleus (PVN) for a set
199 of 10 nuclei sampled. This is a region that is not yet formally recognized as part of the SDMN,
200 despite its neuropeptide projections to nodes of the SDMN and established role as a major
201 regulator of vertebrate social behavior (Goodson and Thompson 2010; Goodson and Kingsbury
202 2013). For a graphical representation of the tissues sampled see Figure 1F, with a full list of
203 individuals collected in Table S1.1 and tissues in Table S1.2.

204 RNA was extracted and sequenced according to previously described methods (Horton *et*
205 *al.* 2020b). RNA libraries for the various tissues and field seasons were randomized amongst

206 sequencing batches (Supplement 1, Table S1.1 & S1.2). All samples analyzed are associated
207 with Bioproject PRJNA437157, and SRR12660169-198, SRR19521260-271, SRR19521432-
208 575. The file that matches SRA numbers with our code and metadata available on the GitHub.

209 ***Gene expression analyses***

210 Reads were mapped to the annotated *Pipra filicauda* genome (GCA_003945595.1) using
211 splice-aware mapper STAR v.2.7.5 (Dobin *et al.* 2013), and transcripts were counted against
212 exons using featureCounts v.2.0.1 (Liao *et al.* 2014). For subsequent analyses we used a total of
213 170 libraries, and quality filtering of individuals and gene-counts was performed before the
214 analysis of each tissue (Supplement 1 & 3). To further explore tissue and brain nucleus function
215 in this species (Horton *et al.* 2020b), we characterized differences in gene expression among
216 tissues and brain nuclei using principal components analysis in PCAtool*s* and Weighted Gene
217 Co-Expression Analysis (WGCNA) (Langfelder and Horvath 2008; Blighe and Lun 2020)
218 (Supplement 2). The number of individuals used in each tissue varies owing to quality filtering
219 and variable sampling of individuals across all brain regions (Table S1.1 & S1.2).

220 To describe the role of tissues and brain nuclei in relation to these hormonal and
221 behavioral phenotypes we conducted filtering (supplement 1), differential expression
222 (Supplement 3) and co-expression (Supplement 5) analysis in each tissue and nucleus separately.
223 For a subset of tissues, we had an extra four individuals characterized for social status from a
224 previous study (Horton *et al.* 2020b), for which behavioral and hormone sampling data were
225 unavailable. Owing to this, and to not overparameterize the models that account for batch effects
226 we identified differentially expressed genes (DEGs) associated with each of our traits of interest
227 (social status, testosterone phenotype and social network strength) separately. These were run as
228 separate negative binomial generalized linear models for each trait that accounted for batch
229 effects where necessary using DESeq2 (Love *et al.* 2014) (For detailed description see
230 Supplement 3) in R v 4.0.2 (R Core Team 2020). Mean testosterone and social network strength
231 were input to DESeq2 as continuous variables. In these cases, significant DEGs are interpreted to
232 be correlated with the predictor variable, and Log_2 Fold Change (LFC) value represents the LFC
233 of gene expression per unit of the predictor variable. In all cases, significant DEGs were
234 identified using the Wald Test and the default FDR corrected p-value of 0.1 ($q<0.1$). Further, for
235 each tissue and nucleus we identified gene co-expression networks using WGCNA. The identity
236 of genes of interest identified by WGCNA were similar to those in DESeq2. For brevity and

237 simplicity we focus mostly on the differential expression results, but the full results are presented
238 in Supplement 5.

239 Due to the key role testosterone plays in modulating behavior in this species (Ryder *et al.*
240 2020), we conducted further detailed analysis on nine candidate genes involved in sex-hormone
241 metabolism and signaling (Figure S4.1), and a further nine steroid sensitive neuropeptides and
242 their receptors (Figure S4.2). Any candidate gene identified by differential gene expression
243 (DGE) analysis with an uncorrected $p < 0.05$ was considered "significant" and examined further.
244 In addition to analyzing models of status, mean testosterone and social network strength
245 separately, we also explored whether candidate genes were involved in mediating status specific
246 relationships between mean testosterone and social behavior (Ryder *et al.* 2020) by extracting
247 "significant" candidate genes from a final DGE interaction analysis (model form: expression ~
248 status x mean testosterone). Owing to the overfitting concerns discussed above, we primarily
249 considered this model as a method for identifying which of our candidate genes showed status-
250 specific expression with respect to mean testosterone, rather than identifying new DEGs. The list
251 of putative relationships between genes and traits were plotted (Supplement 4), and any
252 relationship with highly influential observations, or with $R^2 \leq 0.2$, was excluded from our final
253 set. For candidates derived from the interaction effect models, and for genes that were associated
254 with multiple traits, we decided the most convincing relationship based on a stepdown AIC
255 procedure (Supplement 4). Given the substantially smaller set of candidate genes we considered
256 uncorrected p-values to be significant ($p<0.05$), but report the FDR corrected value as well.

257 We characterized overlap in gene expression patterns among brain nuclei by comparison
258 of ranked gene-lists (Supplement 6). The gene-lists were ranked by $-\log_{10}$ transforming the p-
259 values and multiplying by the direction of expression. These gene lists were then compared for
260 similarity between each pair of tissues by calculating a) Pearson correlation and b) rank-rank
261 hypergeometric overlap (RRHO) test (Cahill *et al.* 2018).

262 Testosterone is pleiotropic in that it can affect multiple traits by influencing gene
263 expression in in similar ways in many different target tissues (Ketterson *et al.* 2009; Cox 2020),
264 and it is crucial to social status-specific regulation of behavior in the wire-tailed manakin (Ryder
265 *et al.* 2020). We reasoned that these effects would be reflected in shared signatures of differential
266 expression across brain nuclei. To explore systemic gene expression patterns (i.e., consistent

267 patterns across the entire brain), we used the transformed p-values from the aforementioned
268 analysis and calculated the median p-value across all brain nuclei. Genes that had a median p-
269 value < 0.05 (transformed value > |1.3|) were considered consistently differentially expressed
270 across the brain. Further, the eigenvalues for each principal component (PC) from the whole-
271 brain PCA analysis (Figure 1G inset) were Pearson correlated with our variables of interest using
272 pcatools::eigencorplot() (Blighe and Lun 2020) (Supplement 1). Correlations observed with
273 social status were further validated by a linear mixed model in package 'nlme' (Pinheiro *et al.*
274 2016). The model form included individual as a random effect and incorporated batch variables
275 that were also correlated with the same PC (Supplement 1).

276 We used *clusterProfiler* v3.16.1(Yu *et al.* 2012). to determine patterns of functionality in
277 DEGs and WGCNA modules with Gene Ontology enrichment analyses. If the total number of
278 FDR corrected DEGs was less than 40, we used gene set enrichment analysis instead which
279 leverages the rank order of p-values and direction of expression (Supplement 3).

280

281 **Results**

282 ***Landscape of gene expression among neural and endocrine tissues***

283 Our analyses revealed substantial differences in gene expression among tissues and brain
284 regions (Figure 1G; Figure S2.7). As expected, the pituitary was associated with high expression
285 levels of genes key to hormonal regulation (e.g., PRL, GH1, TSHB, POMC; Figure S2.3), while
286 the testes showed high expression levels of genes associated with germ cells and flagellar
287 motility (e.g., PKDREJ, BOLL, DRC7; Figure S2.3). Within the brain, the majority of gene
288 expression variation was explained by differences between brain regions (Figure 1G inset) and
289 was reflected in brain region-specific gene co-expression networks (Figure S2.7). These
290 expression patterns are more similar between adjacent nuclei, forming major brain-region
291 groupings of the amygdala, hypothalamus, and midbrain (Figure 1G). Our 18 candidate
292 neuroendocrine genes (Table S1.3) also showed tissue and brain region-specific expression
293 (Table 2.2, Figures S4.1-4.2).

294 ***Social status and testosterone phenotype are associated with tissue and brain region-specific***
295 ***patterns of gene expression***
296 We observed a landscape of gene expression in association with testosterone phenotype
297 (Figure 2A) and social status (Figure 2B), that is characterized by a high number of DEGs (or co-
298 expression modules) identified in some tissues, but not others. Similarly, across brain regions we
299 identified relationships between many of our 18 candidate genes and status, testosterone
300 phenotype or their interaction (Figure 2C). Mean testosterone was a continuous predictor in
301 DESeq2, as such DEGs show up-or downregulation with a higher testosterone phenotype (Figure
302 2A), while DEGs in social status indicate up or down regulation in territorial males (Figure 2B).
303 The number of trait-associated co-expression modules were similar to our results from
304 differential gene expression, so we primarily focus on the tissues with large numbers of DEGs
305 and significant candidate genes for the main manuscript, but the full suite of results can be
306 viewed in supplements 3 and 5. The number of DEGs in each tissue was more similar between
307 testosterone phenotype and status than any other comparison of traits examined ($R = 0.5$, $p =$
308 0.08, Figure S3.7), and this pattern appears to hold across co-expression modules (Supplement 5)
309 and candidate gene analysis (Figure 2C & Figure 3B). This similarity was expected because
310 testosterone phenotype and status themselves are correlated (Figure 1D) (Ryder *et al.* 2011b;
311 Ryder *et al.* 2020).

312 The testes and the nucleus taenia (TnA) each had over 1,000 genes differentially
313 expressed (at uncorrected p-value < 0.05) in association with both testosterone phenotype and
314 status (144 and 252 genes after FDR correction $q<0.01$ in association with mean T; 2 and 13
315 after FDR correction in association with social status). DEGs identified in association with
316 testosterone phenotype in the testes were enriched for genes involved with response to
317 corticosterone (GO:0051412) and synaptic transmission and organization (e.g., GO:0007268,
318 GO:0050808) among others (Figure S3.20). Notable DEGs included steroidogenic acute
319 regulatory protein (STAR, LFC = -1.1, $p = 2x10^{-7}$, $q = 0.0003$, $R^2 = 0.6$) and candidate gene
320 AVPR1A (LFC = 0.8, $p = 5x10^{-5}$, $q = 0.01$, $R^2 = 0.7$; Figure S4.4). Note that Log_2 Fold Change
321 (LFC) represents change in gene expression per unit of the predictor variable. However, these
322 transcriptional differences do not correspond to significant differences in testis size in relation to
323 testosterone phenotype (Table S3.2), nor social status (Table S3.1 & S3.2).

Among the 252 DEGs associated with testosterone phenotype in the TnA, there was non-significant enrichment for genes involved in neural crest migration (GO:0001755), learning and memory (GO:0007613, GO:0007612), as well as long-term synaptic potentiation (e.g., GO:0060291) (Figure S3.28). Among the DEGs associated with testosterone phenotype in the TnA, candidate gene estrogen receptor 2 (ESR2; aka Erβ) was upregulated in the TnA of males with higher testosterone (Figure 2C; LFC = 0.8, p = 0.001, q = 0.08, $R^2 = 0.4$). Other notable DEGs in the TnA included upregulation of immediate early gene JUN, also a 'coral1' hub gene (module membership (MM) = 0.93; LFC = 0.61, p = 0.0004, q = 0.05) and corticotropin releasing hormone receptor 2 (CRHR2, LFC = 1, q = 0.006, $R^2 = 0.5$).

Not all nuclei showed similar patterns between status and testosterone phenotype. For example, the intercollicular nucleus (ICo) of the midbrain showed 161 DEGs associated with testosterone phenotype after FDR correction, but did not stand out in association with social status (3 DEGs). Most of the significantly enriched GO terms were biological processes relating to motor proteins and cilia (e.g., GO:0003341, Figure S3.26). Intriguingly, although the POM did not reveal a high number of DEGs in association with testosterone phenotype (45), our analysis revealed differential expression for compelling gene ontology categories and candidate neuroendocrine genes (Figure S3.25, Figure S4.10, Figure S5.7). Notably, males with higher testosterone exhibited upregulation of candidate gene androgen receptor (AR) in POM (Figure 2D). Indeed, the 'floralwhite' module comprised many of the upregulated DEGs and was significantly associated with both testosterone phenotype and to a lesser extent social status (Figure S5.6). This module showed enrichment for biological processes relating to G protein-coupled receptor signaling pathways (GO:0007186) and neuropeptide signaling pathway (GO:0007218), with statistically non-significant enrichment for steroid receptor signaling (GO:0030518), learning and memory (GO:0007611), and nervous system development (GO:0007399) (Figure S3.7). Indeed, many of the module hub genes were also DEGs in association with testosterone phenotype, and interact with candidate sex hormone pathways, prolactin releasing hormone (PRLH, MM = 0.91, LFC = 2.4, q = $5x10^{-5}$, $R^2 = 0.6$, Figure S3.25A) and growth regulating estrogen receptor binding 1 (GREB1, MM = 0.86, LFC = 0.6, q = $3x10^{-5}$, $R^2 = 0.7$, Figure S3.25A), gonadotropin releasing hormone 1 (GNRH1, MM = 0.76, LFC = 0.4, p = 0.006, q = 0.2, $R^2 = 0.4$). Other notable 'floralwhite' hub genes include the candidate gene progesterone receptor (PGR, MM = 0.81) which was also associated with social status

355 (Figure 2C; LFC = 0.4 p = 0.006, q = 0.7) and serotonin receptor 5A (HTR5A, MM = 0.87, LFC
356 = 0.4, p = 0.04, q = 0.6). Candidate gene aromatase (LOC113993669/CYP19A1) was also
357 upregulated in males with higher testosterone phenotype (Figure 2C, LFC = 0.6, p = 0.02, q =
358 0.4, R^2 = 0.5), but was found in the 'lightcoral' co-expression module.

359 Despite overall similarity in gene expression associations with testosterone phenotype
360 and status, some brain regions were more strongly associated with status or status × testosterone
361 phenotype interaction (Figure 2B). Likewise, expression in many of the 18 candidate
362 neuroendocrine genes were associated with status or the interaction with testosterone in different
363 nuclei (Figure 2C). The paraventricular nucleus (PVN) of the hypothalamus showed the most
364 DEGs after FDR correction (42), which showed enrichment for cytoskeletal movement genes
365 (Figure S3.12B) involved in intracellular transport and cilia (e.g., GO:0003341, GO:0061640),
366 and non-significant enrichment for cholesterol biosynthesis (GO:0006695, GO:0045540).
367 Candidate gene expression also revealed status-specific associations with testosterone phenotype
368 in the PVN, that is a statistical interaction between status and testosterone phenotype (Figure
369 2C). In particular, AR expression was positively correlated with testosterone phenotype in floater
370 males, but not in territorial males (Figure 2F; LFC = -0.7, p = 0.008, q = 0.9). The PVN of
371 territorial males also showed higher VIPR1 (Figure S4.8; LFC = 0.3, p = 0.02, q = 0.4) and lower
372 ESR2 expression (LFC = -0.6, p = 0.006, q = 0.2). In the bed nucleus of the stria terminalis
373 (BSTm), a region that bridges the SDMN and MRS, there were more than 1000 putative DEGs,
374 but only nine after FDR correction. Among these, AR expression was higher in territorial males
375 than in floater males (Figure 2E; LFC = 0.6, p = 0.0007, q =0.2). Further, testosterone
376 metabolism gene 5-alpha reductase (SRD5A2) was upregulated in the lateral septum (LS) of
377 territorial males (Figure S4.20; LFC = 0.7, p = 0.0005, q = 0.4). The VIP system is also
378 implicated in status-specific responses to testosterone in the TnA, where VIPR1 was upregulated
379 in association with testosterone in territorial, but not floater males (Figure S4.17; LFC = 1, p =
380 0.04 q = 0.7).

381 GCt was a hotbed for gene expression associated with social status and status ×
382 testosterone phenotype interaction (Figure 1B, Figure S3.6). In association with social status,
383 gene set enrichment analysis revealed there was a significant tendency for downregulation of
384 genes relating to synapse assembly (GO:0051965), and response estradiol (GO:0032355), and

385 upregulation of genes relating to RNA processing (e.g., GO:0000184, GO:0006396) and
386 translation (e.g., GO:0006413, GO:0006412) (Figure S3.15). While, candidate neuroendocrine
387 genes showed status specific expression in association with testosterone phenotype in this region
388 including progesterone receptor (PGR, Figure S4.15; LFC = 1.9, p= 0.003, q = 0.1), estrogen
389 receptor 1 (ESR1; aka Erα; LFC = 1.1 p = 0.04, q = 0.4), prolactin receptor (PRLR; LFC = 0.7, p
390 = 0.01, q = 0.2), and vasoactive intestinal peptide (VIP; Figure S4.15; LFC = 1.6, q = 0.005, q =
391 0.1). Further, candidate gene aromatase was downregulated with higher testosterone phenotype
392 in the GCt (LFC = -1.3, p = 0.002, q = 0.3, R^2 = 0.4).

393 ***Cooperative behavior is associated with a distinct gene expression landscape***

394 Network strength (the number of cooperative interactions between individuals) varies
395 widely among territorial and floater males (Figure 1E), representing a key axis of variation in
396 social behavior in this species. We predicted that the associations between gene expression and
397 cooperative behavior would be different from those observed for social status and testosterone
398 phenotype. Indeed, we found a distinctive landscape of constitutive gene expression across
399 tissues relating to cooperative behavior (Figure 3A, Figure S3.5), as well as among candidate
400 genes (Figure 3B). These results are based on results from a continuous predictor and can be
401 interpreted as genes with significant up-or downregulation with increasing social network
402 strength. The Anterior Hypothalamus (AH) had the largest number of DEGs associated with a
403 male's social network strength. In this nucleus there were no significantly enriched GO terms,
404 but there was non-significant enrichment for genes involved in RNA metabolism (e.g.,
405 GO:0043928) and regulation of translation (e.g., GO:0017148), development (e.g., GO:0061061,
406 GO:0042472) and synaptic potentiation (e.g., GO:0007274) (Figure S3.35). The pituitary also
407 stood out in the abundance of differentially expressed genes associated with male's social
408 network strength (109 genes after FDR correction q < 0.1). There were no significantly enriched
409 GO categories after FDR correction (Figure S3.33B), but among the non-significant terms were
410 actin filament organization (GO:0007015), neural differentiation (e.g., GO:0031103,
411 GO:0045664) and the cell cycle (e.g., GO:0043065, GO:0071158.) Among the DEGs in the
412 pituitary, candidate genes prolactin (PRL) (Figure 3C; LFC = -0.6, p=$5x10^{-5}$ q = 0.03, R^2 = 0.7),
413 ESR2 (Figure S4.5; LFC = -0.7, p = 0.04, q = 0.5, R^2 = 0.5) and VIPR2 (Figure S4.5; LFC = -
414 0.4, p = 0.008, q = 0.3, R^2 = 0.4) were downregulated in highly cooperative males. Intriguingly,
415 the PVN showed a weak correlation of one of our candidate genes: oxytocin (OXT) expression

416 decreased with a male's social network strength (Figure 3D; LFC = -0.3, $p = 0.04$, $q = 0.6$, $R^2 =$
417 0.2). Further, although there was less representation of sex-steroid related pathways in the
418 candidate gene analysis (Figure 2B), PGR was downregulated in males with higher social
419 network strength in the BSTm (Figure 2E; LFC = -0.3, $p = 5x10^{-4}$, $q = 0.2$, $R^2 = 0.5$).

420 Gene expression in the LS was tightly associated with an individual's social network
421 strength (98 genes after FDR correction $q < 0.1$). There were no GO categories significantly
422 enriched after FDR correction (Figure S3.42), but among the top non-significant terms were
423 those broadly involved in development (e.g., GO:0048538, GO:0048593), and neural
424 organization (e.g., GO:0099175, GO:0050771). However, the expression of our candidate genes
425 in the LS was not linked to social network strength. Although relatively few genes were
426 differentially expressed in the medial bed nucleus of the stria terminalis (BSTm) with respect to
427 social network strength, the LS and BSTm shared many genes with similar patterns of expression
428 in association with strength as shown by RRHO analysis (Cahill *et al.* 2018) (Figure 3G, F). In
429 contrast, the LS and BSTm shared few genes with similar expression in association with
430 testosterone phenotype (Figure 4H, I) or social status (Figure S6.1A), suggesting that these
431 regions are uniquely engaged in modulating cooperative behavior. Indeed, pairwise correlations
432 of gene expression in association with testosterone phenotype broadly recapitulates similarity of
433 tissues sampled (Figure 3H) and perhaps reflects similarities in pathways related to sex-steroid
434 signaling (Figure 2C).

435 ***Testosterone phenotype and social status are associated with systemic gene expression***
436 We reasoned that the pleiotropic effects of testosterone and its role in social status in the
437 manakins (Ryder *et al.* 2020) would also produce systemic effects on gene expression, or similar
438 patterns of expression across multiple regions. Social status and testosterone phenotype were
439 linked to the highest number of consistently differentially expressed genes (60 and 66 genes with
440 median uncorrected $p < 0.05$ respectively), while only 10 genes were consistently differentially
441 expressed in association with social network strength (Supplement 6). Further, PC5 of the whole
442 brain analysis was significantly associated with social status in a linear mixed model that
443 corrected for batch confounds and repeated sampling of individuals (Figure S2.5). There was
444 overlap between the genes and GO categories between the consistently differentially regulated
445 genes with social status and testosterone phenotype and those identified in PC5 (Supplement 6),
446 but we focus on these common themes in relation to social status in the main manuscript for

447 simplicity. The top two most consistently differentially regulated genes are the upregulation of
448 an immune system gene C-type Lectin Domain gene (CLEC2B) (Figure 4A) and down
449 regulation of development gene roundabout guidance receptor 4 (ROBO4) (Figure 4B) in
450 territorial males. These signatures are reflected in the patterns of GO biological process
451 enrichment, though none of these terms are significant after FDR correction (Figure 4C). There
452 was also evidence for global differential regulation of telomere maintenance genes (Figure 4C),
453 with downregulation of POLD-1-like (LOC114000574) and upregulation of RAD51 in territorial
454 males. Further, there is evidence for global regulation of cholesterol biosynthesis (Figure 4C),
455 and glutathione transferases (Figure S2.6, Table S6.2), both of which are relevant in steroid
456 hormone biosynthesis. These findings of global expression differences among status classes
457 could be partly caused by the upregulation of growth hormone (GH1) in the pituitary of
458 territorial males (Figure 4D; LFC = 0.8, $p = 3x10^{-5}$, q = 0.01). This result holds in the model that
459 accounts for the interaction between social status and mean T (LFC = 0.9, $p = 6x10^{-5}$, q = 0.05).

460 **Discussion**

461 Using the most comprehensive transcriptome-level sampling of the SDMN to-date (Lopes and
462 König 2020; Bentz *et al.* 2021b; Kabelik *et al.* 2021; Bentz *et al.* 2021a; Antunes *et al.* 2021),
463 our findings highlight both modular and integrative neurogenomic organization underlying
464 individual variation in cooperative behavior. The relative roles of each are central to the
465 development and expression of any complex trait (Ketterson *et al.* 2009; Lipshutz *et al.* 2019;
466 Cox 2020). We discuss our findings in relation to each theme in turn.

467 **Different landscapes of gene expression with each trait reflect modular organization of the**
468 **neuroendocrine system**

469 Modularity is reflected in how different brain nuclei, endocrine tissues, and genetic
470 pathways within individuals are associated with the expression of different traits. Such
471 neurogenomic landscapes, characterized by the upregulation of neuroendocrine genes in some
472 brain regions and downregulation in others, would allow differential sensitivity to testosterone
473 and other signaling molecules among SDMN nuclei. These neurogenomic states can be likened
474 to the 'landscape' model of immediate early gene (IEG) activation across the SDMN after a
475 social stimulus (Newman 1999; Goodson 2005). In that landscape model, it is the pattern of IEG
476 expression across brain nuclei that correlates with behavioral responses, not the up or
477 downregulation of particular genes within a single nucleus. This original model considered this

478 landscape of IEG expression (and later neuropeptides) in the context of proximate responses to
479 social stimuli. Our findings extend this model in two ways: by considering transcriptomic
480 variation and how long-term neurogenomic states underlie individual variation in social
481 behavior.

482 In the landscape of constitutive gene expression in male wire-tailed manakins, total
483 number of DEGs and candidate gene expression implicate constitutive differences in most brain
484 regions, but the brain regions with higher numbers of DEGs or candidate gene expression may be
485 more important for mediating certain traits. Some brain regions showed associations between
486 gene expression and both social status and testosterone phenotype (e.g., POM) or only
487 testosterone phenotype (e.g., TnA, testes). This may be because these centers are involved in
488 testosterone mediated traits irrespective of social status (O'Connell and Hofmann 2012).
489 Meanwhile, others appeared to be more related to social status and status specific relationships
490 with testosterone phenotype (e.g., PVN, GCt), or an individual's strength of cooperative
491 behavior (e.g., PIT, LS). Our results also suggest that the identity and function of these genes
492 vary across tissues and brain regions (Figure 3GH). However, most analyses revealed DEGs
493 relating to neural development, synaptic potentiation or organization and genes relating to
494 cellular metabolism, or transcription/translation regulation. Thus, individual variation modulated
495 by neurogenomic states that encode synaptic modifications and connectivity (mechanism of
496 memory formation), larger scale shifts in cellular activity, and neural structure (Clayton *et al.*
497 2019; George *et al.* 2020)). These mechanisms may be supported or feedback on the variation in
498 elements of sex-steroid signalling we investigated in detail (*sensu* "neuroendocrine action
499 potential" (Clayton *et al.* 2019)).

500 We found ample evidence for modularity in steroid related gene expression, particularly
501 in association with social status, testosterone phenotype or their interaction. Unsurprisingly, we
502 found evidence that status-specific regulation of behavior could be modulated directly by
503 testosterone through AR in multiple nuclei (e.g., POM, PVN, BSTm). Higher AR expression is
504 linked to display performance in golden-collared manakins (Schlinger *et al.* 2013). Further, the
505 conversion of testosterone to estradiol by the enzyme aromatase and subsequent binding to
506 estrogen receptors is a key pathway in the regulation of testosterone-dependent male behaviors
507 more broadly (Schlinger and Balthazart 2013), and found evidence for their role in multiple

508 nuclei. Our results also highlight the importance of neural VIP expression in modulating multiple
509 aspects of social behavior (Kingsbury and Wilson 2016; Horton *et al.* 2020a), as it was the only
510 neuropeptide system with widespread patterns of differential expression with respect to both
511 social status, testosterone phenotype and social network strength.

512 Notably, VIP and other steroid receptors may play a role in status-specific responses to
513 testosterone in GCt. Although tyrosine hydroxylase (TH) neurons in the GCt have been
514 associated with courtship behaviors (Goodson *et al.* 2009; Ben-Tov *et al.* 2021), our finding of
515 association with VIP and courtship behaviors broadly speaking in GCt is novel (Kingsbury and
516 Wilson 2016). GCt coordinates social stimuli and downstream motor processes that control
517 behavior. For example, in the songbird brain, GCt links POM to the song control system and
518 thereby mediates androgen-dependent singing behavior (Haakenson *et al.* 2020). Given that
519 cooperative display in wire-tailed manakins requires coordinated physical (and vocal displays in
520 cooperative *Chiroxiphia* manakins (Trainer *et al.* 2002)), the status-specific differential
521 expression of genes for steroid receptors and steroid-sensitive neuropeptides in GCt suggest that
522 this SDMN region may play a unique role in mediating status-specific relationships between
523 testosterone and cooperative behavior.

524 The pattern of co-upregulation of steroid related genes in the POM of males with high
525 testosterone phenotype (and territorial males) point to this region's role as a potent steroid
526 regulator. The POM is known to be involved in regulating male aggression, sexual behavior and
527 parental care (O'Connell and Hofmann 2011), and stimulates gonadal testosterone production
528 through gonadotropin releasing hormone (*GnRH*). Indeed, the observed increase in GNRH1
529 transcription may relate to transcriptional changes indicating enhanced steroid production in the
530 testes. Co-upregulation of AR and GNRH1 would seem contradictory due to the negative
531 regulatory effects of androgens and AR on *GnRH* (Brayman *et al.* 2012), though these may not
532 interact at the cell population level or may show different temporal patterns of translation.
533 Regardless, activation and steroid receptor concentration in POM is associated with social status
534 differences and social status ascension cichlid fish (Maruska *et al.* 2013), and mice (Lee *et al.*
535 2022). In the same cichlid species, different preoptic cell types, including homologs for PVN,
536 showed higher activation in one of the two partners in cooperative territorial defense
537 (Weitekamp and Hofmann 2017). Indeed, testosterone can link the POM to the Hypothalamic

538 Pituitary Adrenal (HPA) axis via the PVN (Williamson *et al.* 2010), which in our study also
539 showed evidence of status specific gene expression, including AR. This previous work, and the
540 close linkages between the PVN and POM (Williamson *et al.* 2010), suggest these nuclei in
541 wire-tailed manakins could be involved in multiple elements of behavior depending on cell
542 types, social contexts and timescales investigated.

543 The number of differentially expressed genes observed in TnA implicates this region as a
544 hub for neurogenomic pathways involved in regulation of testosterone mediated traits. The avian
545 homolog of the medial amygdala (O'Connell and Hofmann 2011), the TnA integrates sensory
546 information and has been associated with the regulation of diverse social behaviors (Raam and
547 Hong 2021). Indeed, our results may reflect persistent activation of this area, based on the
548 upregulation of immediate early genes such as JUN and SYT7 in males with high testosterone
549 phenotype, and differential regulation of other genes involved in long-term synaptic potentiation
550 (Clayton 2000; Marrone *et al.* 2008; O'Connell *et al.* 2012; Clayton *et al.* 2019). Further, our
551 results implicate potential estrogenic regulation in this region via increased ESR2 in males with
552 higher testosterone phenotype. Indeed, estrogenic regulation through ESR1 has been shown to
553 regulate status-specific aggression in birds (Horton *et al.* 2014; Merritt *et al.* 2020) and prosocial
554 behavior in prairie voles (Cushing *et al.* 2008). Although ESR2 is not well characterized in birds,
555 it may function in the medial amygdala of mammals to regulate social recognition (Lymer *et al.*
556 2018), and sexual regulation (Nakata *et al.* 2016), and is associated with higher social rank in the
557 forebrains of cichlid fish (Burmeister *et al.* 2007). Further, the differential expression of stress
558 steroid receptor (CRHR2) hints again at the previously unexplored role of the HPA axis in
559 modulating display behavior in this species. Indeed, social ranks often differ in their
560 glucocorticoid profiles (Goymann and Wingfield 2004) or regulatory responses to
561 glucocorticoids (Snyder-Mackler *et al.* 2019). The related cooperative displaying lance-tailed
562 manakins (*Chiroxiphia lanceolata*) vary in testosterone as well as corticosterone profiles
563 according to social status (DuVal and Goymann 2011; Jones and DuVal 2021). Finally, the fish
564 homolog of TnA appears mediate aggression and cooperation in cooperative territory defense in
565 an actor-specific manner (Weitekamp and Hofmann 2017; Weitekamp *et al.* 2017), supporting a
566 role for modulating cooperation in wire-tailed manakins as well.

567 The frequency an individual male spends displaying with other males (i.e., network
568 strength), our proxy for cooperative behavior, is associated with a unique pattern of gene
569 expression among tissues and brain regions. Some of these signatures are difficult to parse, for
570 example many genes are differentially expressed in the AH, yet lack clear GO enrichment. Prior
571 to our study AH has associated with regulation of aggression rather than cooperation in males,
572 and acting via AVP receptors and VIP (O'Connell and Hofmann 2011; Horton *et al.* 2020a).
573 Although we do not find any evidence for the role of these genes in the AH, our findings may
574 suggest that cooperative displays are derived from displays of territorial aggression, and/or gene
575 expression is inhibiting aggression in these displays. Indeed, cooperative displays may act as a
576 way of maintaining dominance hierarchies in wire-tailed manakins.

577 The variety of pathways differentially expressed in the pituitary suggests there are whole-
578 organismal effects of social bond strength mediated by the pituitary. Indeed, previous work on
579 this species showed that more social males had shorter telomeres (Vernasco *et al.* 2021), and
580 social bond strength has been associated with signatures of energy metabolism and stress
581 (Anderson *et al.* 2022; Simons *et al.* 2022). Interestingly, PRL transcript was downregulated in
582 more cooperative males. Prolactin is best known for its role in promoting parental care
583 behaviors, including in cooperative breeders (Schoech *et al.* 1996), but emerging studies suggest
584 it could play a role in inhibiting aggression in cooperative breeders (Medger *et al.* 2019; Gilbert
585 *et al.* 2022) and promotion of nest defense behavior (Mohamed *et al.* 2016).

586 The LS showed many differentially expressed genes associated with cooperation, and
587 highly cooperative individuals may show differences in neural structure based on the enriched
588 GO terms. Patterns of expression in the BSTm were similar, but weaker, as it showed very few
589 statistically significant DEGs. BSTm has neural projections into the LS, and vasopressin (AVP)
590 and VIP neurons in the BSTm and receptors for these neuropeptides in the LS are associated
591 with affiliative behavior and gregariousness in birds (Kelly *et al.* 2011; Kelly and Goodson 2013;
592 Kingsbury and Wilson 2016). Those prior studies suggest a role for these brain nuclei in
593 processing social information acutely, whereas our study suggests differences among individuals
594 in cooperative tendency is related to constitutive differences in gene expression and neural
595 structure, perhaps relating to excitability of these regions in response to social information. Thus,
596 cooperative display is regulated by some of the same conserved brain regions as other types of

597 affiliative behavior. That we only observed a large number of DEGs in the LS and not in other
598 nuclei may suggest that other unsampled nuclei in the mesolimbic reward system are important
599 for modulation of cooperative tendency (e.g., (Paula *et al.* 2015; Weitekamp *et al.* 2017; de
600 Abreu *et al.* 2020; Lopes and König 2020).

601 We did not find convincing evidence for any variation in sensitivity to nonapeptides
602 (vasopressin and oxytocin) in the brain (AVPR2 in hypothalamic nuclei showed transcript counts
603 <30). However, the density of these receptors in particular brain regions may be just as important
604 as their abundance in modulating behavior (Goodson and Thompson 2010). We did find
605 evidence for differential sensitivity to AVP in the pituitary, again implicating stress response
606 pathways in mediating social status and cooperation in this species. Further, we did find a weak
607 negative relationship between oxytocin expression and social network strength in the PVN.
608 Although in apparent contrast to the widely held belief that oxytocin promotes sociality (as in
609 humans (Feldman 2012)), this among our other observations may indicate that cooperative
610 displays are a derivative of aggressive displays. However, nonapeptides have complex and
611 sometimes species-specific relationships with affiliative behavior (Goodson and Thompson
612 2010; Maruska *et al.* 2022), and can also be associated with social status (Lee *et al.* 2022).
613 Further, the ratio of peptide to mRNA can be an important factor indicating higher turnover
614 (usage) in certain contexts (Maruska *et al.* 2022). Therefore, we do not have sufficient evidence
615 that oxytocin inhibits cooperation in this species, and further experimental work is needed.

616 Our results establish a variety of mechanisms that could underpin individual variation in
617 cooperative behavior, and its status specific modulation by testosterone. We find evidence for
618 multiple forms of neuroplasticity, through steroid receptor variation, synaptic potentiation and
619 neural development, as well as cellular activity (Clayton *et al.* 2019). Indeed, testosterone
620 manipulations are known to induce changes in neuroanatomy in behaviorally relevant brain
621 nuclei (Kabelik *et al.* 2008), suggesting a direct role for testosterone-mediated neural growth and
622 flexibility in modulating male-male cooperation (Soares *et al.* 2010). Our results provide
623 promising targets for further study on the mechanisms underlying cooperative behavior,
624 including examination of neuroanatomical features and the use of hormonal or other
625 pharmaceutical manipulations and RNAi technologies to examine causal relationships between
626 neural gene expression and behavior (e.g., (Merritt *et al.* 2020)).

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

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

646 Our results highlight two additional hormones that may have a system-wide role in the
647 status-specific modulation of cooperative display behavior. We found differences in the
648 expression of genes for important behavioral and metabolic hormones (prolactin and growth
649 hormone) in the pituitary. The anterior pituitary is the primary production site for both hormones,
650 and thus gene expression patterns here are likely good predictors for circulating levels of those
651 hormones and thus signal strength. Transcripts encoding prolactin, best known for its role in
652 promoting parental care behaviors, were negatively correlated with cooperative behavior. While
653 prolactin is emerging as important in other areas of social behavior, such as nest defense
654 (Mohamed *et al.* 2016), the negative relationship observed appears contradictory to a general
655 model of increased prolactin promoting broadly affiliative social interactions. We speculate that
656 this relationship reflects that cooperative display behavior may have evolved from initially
657 aggressive or competitive interactions (Díaz-Muñoz *et al.* 2014). The other compelling

658 phenotypic integrator for status-dependent behaviors in the wire-tailed manakin comes from the
659 gene encoding growth hormone (GH1). Ascension in social rank in the cooperatively displaying
660 manakins is associated with age (McDonald 1989; DuVal 2007; Ryder *et al.* 2008). It was
661 therefore surprising that territorial males in our study showed increased expression of GH1 in the
662 pituitary as compared to floater males. However, in anolis lizards, stimulation of the growth
663 hormone axis is associated with increased testosterone that enables phenotypic divergence
664 between males and females (Cox *et al.* 2017). Without gross morphological differences in wire-
665 tailed manakin status-classes, growth-hormone could have subtle effects on multiple systems
666 enabling the status-specific regulation of behaviors. In particular, status differences in GH1
667 expression may explain repeated signatures of neural development and protein synthesis in our
668 results (Sonksen 2006), and may reflect the increased metabolic demands on displaying
669 territorial males through muscle performance and regulation of oxidative stress (Hu *et al.* 2019).
670 The neuroethological role of growth hormone are not well known, but its release may be linked
671 to stress (Greenwood and Landon 1966; McCormick *et al.* 1998). Growth hormone
672 administration promotes increased aggression in mice (Matte 1981), and fish (Jönsson *et al.*
673 1998; Johansson *et al.* 2004). Indeed, growth hormone inhibitor somatostatin and its receptors
674 have also been implicated in regulating aggression and social status in cichlid fish independent of
675 androgens (Hofmann and Fernald 2000; Trainor and Hofmann 2006) whilst natal androgen
676 treatment increased aggression and somatostatin receptor expression in zebra finches (Bentz *et*
677 *al.* 2021b). We did some evidence for negative relationship between SSTR1 and SSTR2 with
678 testosterone phenotype in the testes, and positive association between SSTR1 and testosterone
679 phenotype in the POM but these were not significant after FDR correction. Thus, our results
680 suggest that the growth hormone axis may play a role in the status-specific regulation of wire-
681 tailed manakin cooperative displays, and increased GH in territorial males may be an important
682 inhibitor of cooperation by promoting aggression. Future work on social behavior should
683 consider the role of growth hormone and somatostatin in conjunction with sex steroids and
684 glucocorticoids to gain novel insights into its function in behavioral regulation.

685 ***Conclusion***

686 Across the SDMN and HPG of the male wire-tailed manakin we observed correlations with
687 social status, cooperation and testosterone phenotype in patterns of gene regulation and a

plurality of gene functions, including sex-steroid and neuropeptide signaling. This landscape of gene expression 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 SDMN, albeit correlative only. Our correlative findings help us to better understand cooperative behavior (Soares *et al.* 2010) by linking neurogenomics with endocrine mechanisms, and generate further hypotheses to test in further experiments to implicate causality. 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.

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719 **Data and materials availability:** Raw read data are available associated with BioProject
720 PRJNA437157, and all code used to conduct analyses is available here:
721 https://github.com/periperipatus/PIFI_brain_transcriptome

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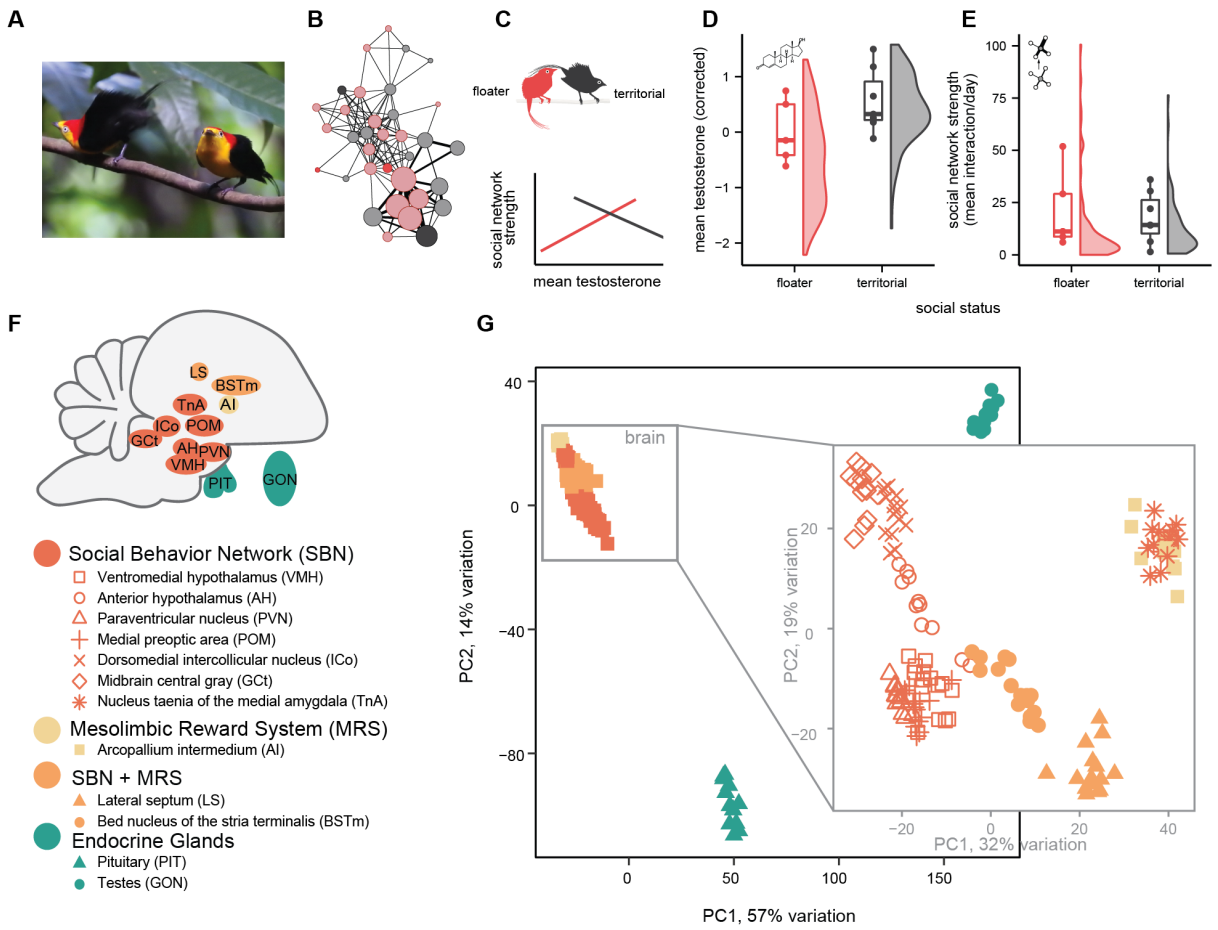

Fig. 1. Circulating testosterone and neuroendocrine tissues are involved in the cooperative behavior of wire-tailed manakins.
A) Males engage in cooperative displays that form the basis of B) complex social networks. The example shown is a single lek over one week, where node size indicates a male's average social network strength (measure of overall time spent interacting), 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 (mean testosterone (corrected)) 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 individuals characterized for social status is N=16 is larger than for testosterone or sociality (N=12). 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). (photo by Alice Boyle).

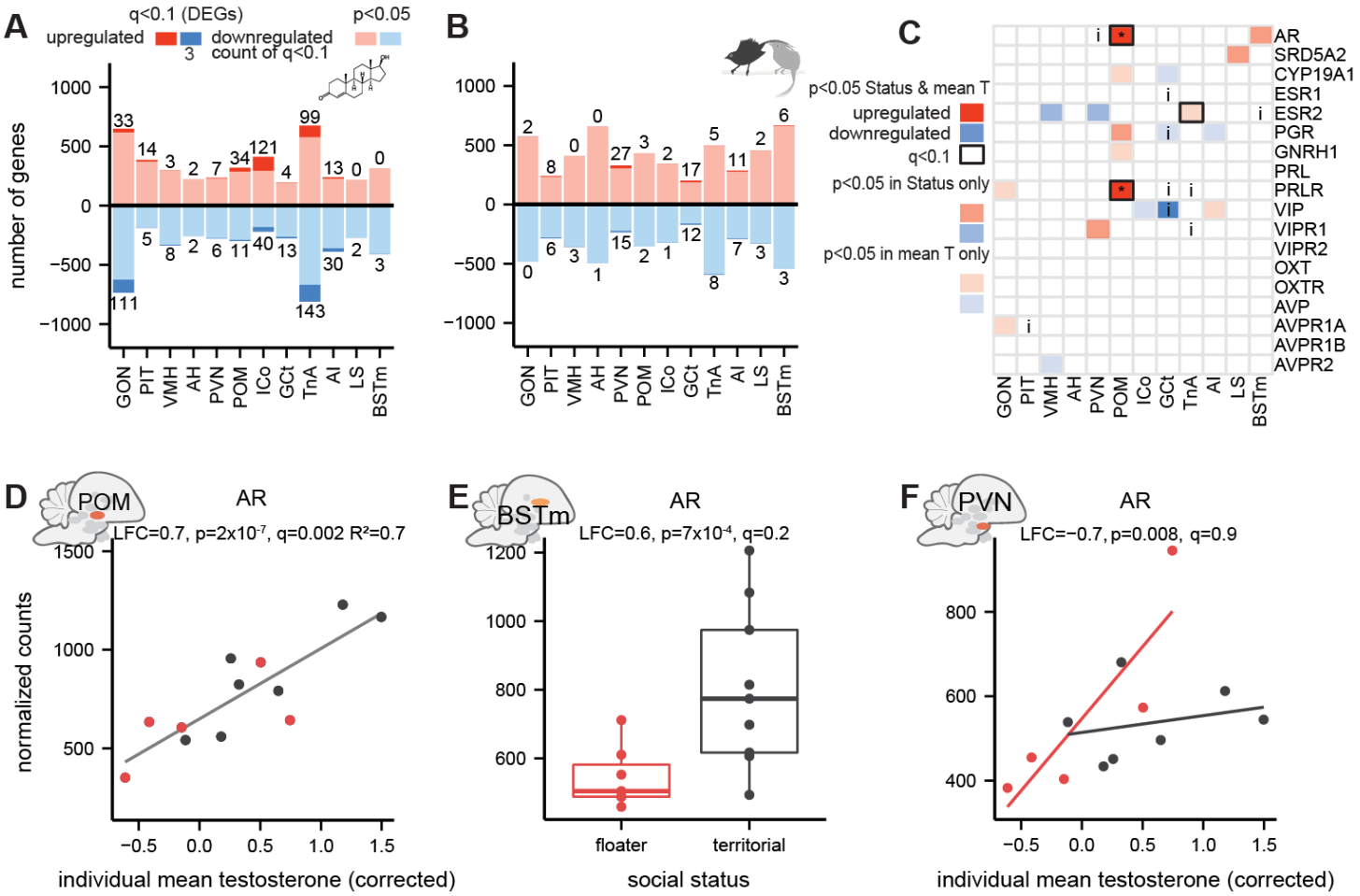

Fig. 2. Neurogenomic landscape of social status and hormone phenotype.
A) There is a landscape of gene expression in male wire-tailed manakins with high testosterone phenotype (mean testosterone (corrected)) relative to lower testosterone phenotype. Red bars indicate number of genes that are upregulated 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) The landscape of gene expression in relation to social status, where red indicates genes upregulated in territorial males. C) Summary of candidate gene analysis and filtering, where colors indicate direction of expression (as previous panels), but intensity reflects which of the models were significant, and "i" indicates there was an interaction effect, and a black box indicates result was significant after FDR correction for all genes in that region's transcriptome. D) AR was upregulated in POM of males with higher testosterone. where point colors indicate social status (red = floater; black = territorial. Fitted line from linear regression. Subheader indicates key results from DESeq2: Log_2 Fold Change (LFC), uncorrected p-value (p), and correction for multiple testing (q) and R^2 correlation coefficient calculated separately. E) AR was upregulated in the BSTm of territorial males. F) AR shows status-specific expression in relation to testosterone phenotype in the PVN. Grey area represents the 95% confidence interval as determined by linear regression of gene ~ Status×mean_T. Note that maximum sample size for analysis of social status was N=16, while

only 12 individuals were sampled for testosterone and behavior, but the actual sample size varies slightly in each tissue owing to quality filtering.

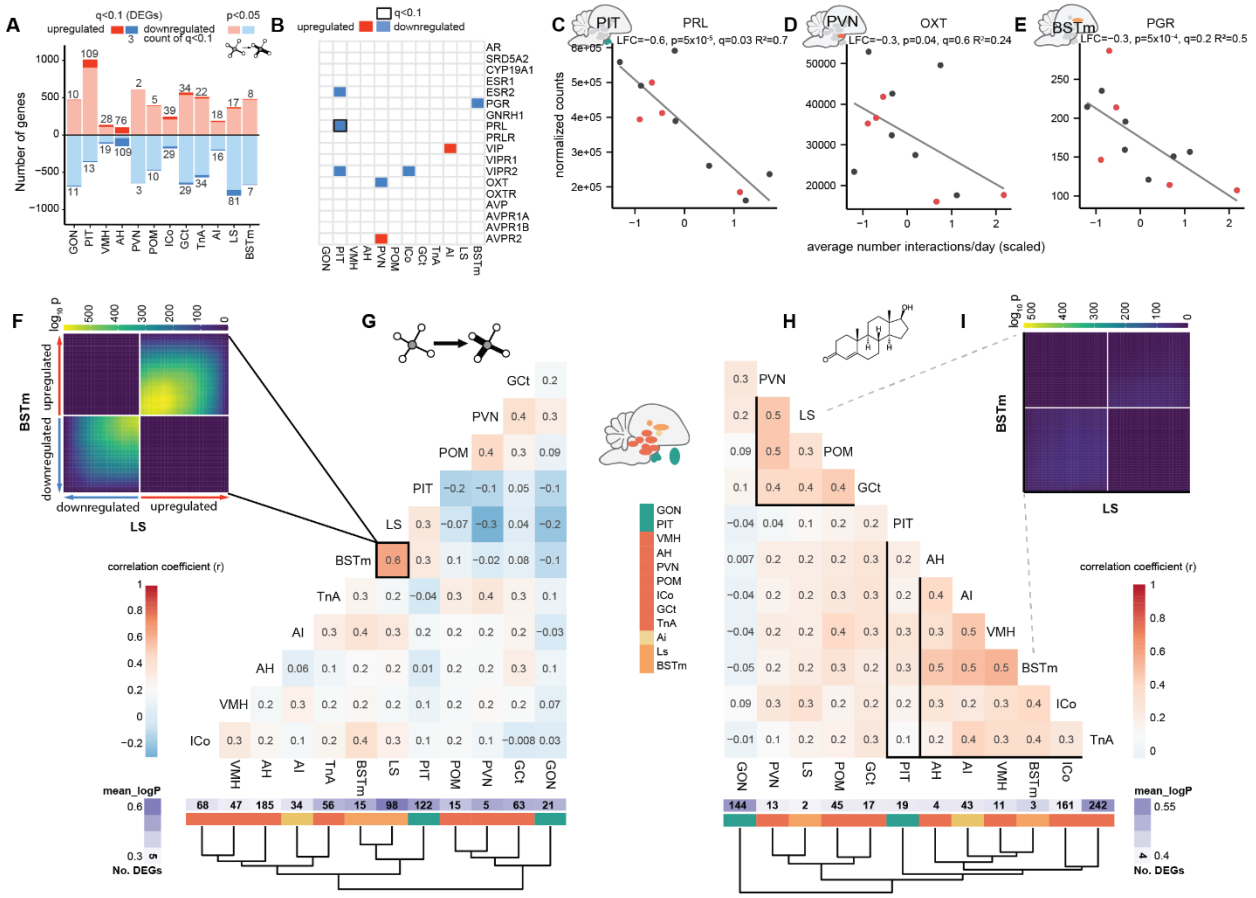

Fig. 3. A distinct landscape of gene expression in relation to cooperative behavior.
A) Landscape of gene expression in male more cooperative wire-tailed manakins (social network strength). Darker colors and text indicate number of genes after FDR correction. Red indicates genes that are upregulated in males with higher social network strength, whereas blue indicates genes that are downregulated in these males. B) Summary of candidate gene analysis and filtering, where colors indicate direction of expression (as previous panels) in relation to individuals with higher social network strength. A black box indicates the result remained significant after FDR correction for all genes in that region's transcriptome. C) Prolactin (PRL) in the pituitary shows decreased expression in males with higher social network strengths. D) Oxytocin (OXT) transcript expression decreases in males with higher social network strength. E) Progesterone Receptor (PGR) has lower transcript expression in males with higher social network strength. F) RRHO2 analysis reveals similar profiles of gene expression associated with cooperation in the LS and BSTm, G) but otherwise generally low similarity of gene expression among tissues in association with cooperation. H) Similarity of gene expression among tissues in relation to testosterone phenotype roughly recapitulates ontogeny of brain regions, I) 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 $\log_{10}$ 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.

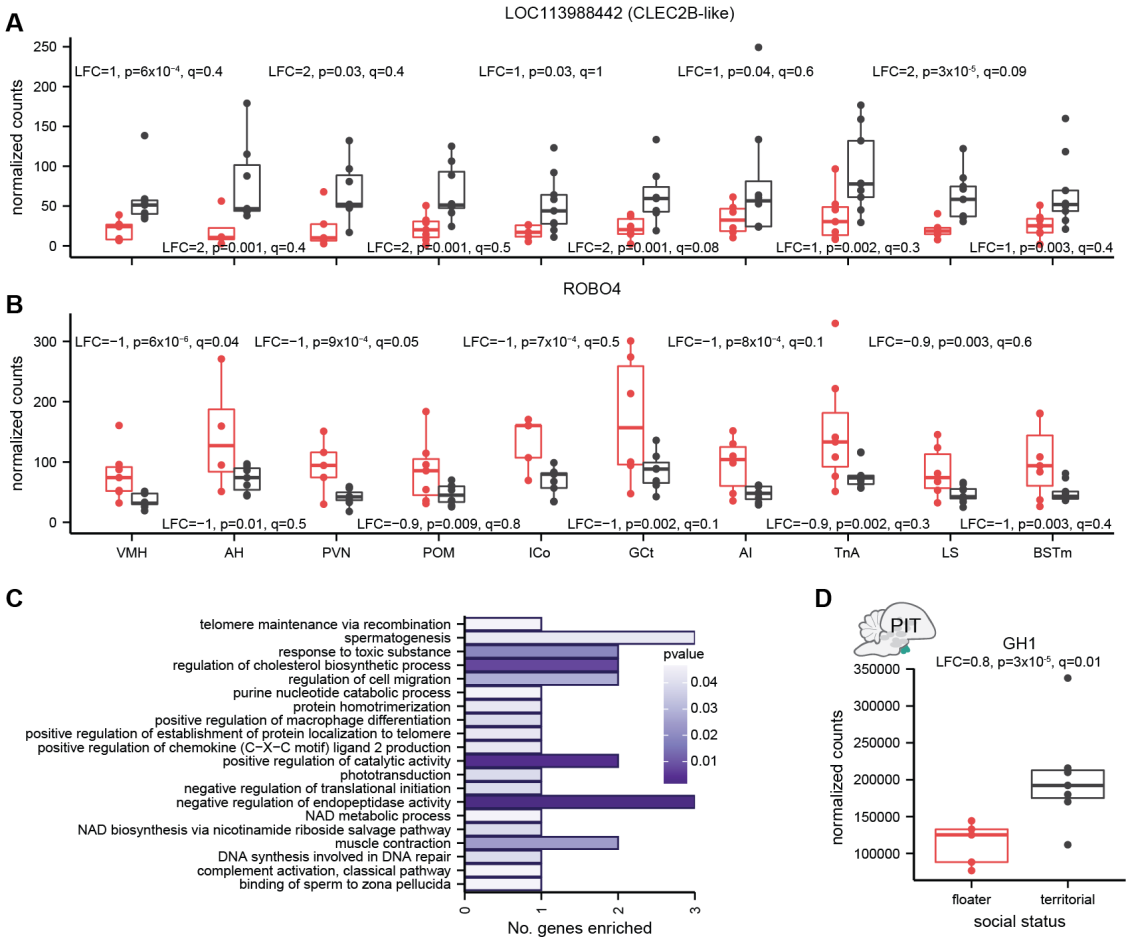

Fig. 4: Global neural gene expression differences between social classes
A) Immune gene CLEC2B-like was consistently upregulated in the brains of territorial individuals, and B) developmental gene ROBO4 was consistently downregulated in the brains of territorial males. C) Enriched GO categories among 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.