1 **Brain transcriptomic changes in Japanese quail reveal a role for neurotensin in**
2 **avian parental care**

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Abstract

For many species, parental care critically affects offspring survival. But what drives animals to display parental behaviours towards young? In mammals, pregnancy-induced physiological transformations seem key in preparing the neural circuits that lead towards attraction (and reduced-aggression) to young. Beyond mammalian maternal behaviour, knowledge of the neural mechanisms that underlie parental care is severely lacking. We took advantage of a domesticated bird species, the Japanese quail, for which parental behaviour towards chicks can be induced through a sensitization procedure, a process that is not effective in all animals. We used the variation in parental responses to study neural transcriptomic changes associated with the sensitization procedure itself, with the outcome of the procedure (i.e., presence of parental behaviours) and with spontaneous parental care (i.e., in the absence of sensitization). Out of the brain regions studied, we found that most differences in gene expression were located in the hypothalamus. Our results highlight several molecular pathways that may contribute to the modulation of avian parental care. We identified one gene, neurotensin, that was previously only demonstrated to be causally associated with parental care in mammals. Our work opens new avenues of research into understanding the neural basis of parental care in non-mammalian species.

Keywords: avian parental care, bed nucleus of the stria terminalis, hypothalamus, Neurotensin, nucleus taeniae, prolactin, RNA-seq, Urocortin

1. Introduction

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35 While parental care is rare in many vertebrate taxa, it is essential for survival in the

36 majority of avian species, with biparental care being the most frequent modality

37 observed [1]. As a group, birds show an incredible diversity in forms of parental care,

38 from brood parasitism, where care is lost, to cooperative breeding, where non-

39 reproductive individuals provide care to offspring that are not their own [1]. The neural

40 underpinnings of young-directed parental care in birds are, however, severely

41 understudied. In terms of neuroendocrine mechanisms associated with incubation and

42 caring for young, the majority of attention has been paid to a single pituitary hormone:

43 prolactin [2,3]. In contrast to birds, uniparental maternal care is the most frequently

44 observed mode of care in mammals and, therefore, the neuroendocrine basis of this

45 behaviour is more well studied and understood than any other form of parental care

46 across vertebrate taxa.

47

48 Using a sensitization procedure developed by [4], a recent experiment by our group

49 confirmed that it is possible to induce parental behaviours in Japanese quail through a

50 single overnight exposure to chicks [5]. After the sensitization procedure, both sexes

51 display chick brooding behaviour and reduced aggression towards chicks (pecking

52 behaviour). While the change in behaviour after sensitization is drastic, it is not

53 observed in all animals, i.e., some animals fail to respond to the sensitization.

54 Conversely, a few animals not exposed to the sensitization spontaneously show some

55 parental care when paired with chicks.

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57 In the current study, we take advantage of this variation in responses to the

58 sensitization treatment to understand, from a neural molecular perspective, a) what

59 modifications occur due to the sensitization treatment, b) how animals that are unable to

60 show care after this treatment differ from parental ones, and c) how some animals are

61 capable of spontaneously showing care during their first encounter with chicks. We

62 focused our analysis on the hypothalamus, the bed nucleus of the stria terminalis and

63 the nucleus taeniae. We targeted these brain regions because they contain nuclei

64   important for parental care, affiliative interactions, and agonistic interactions in birds and

65   other vertebrates [6–11]. Our experiments highlight the importance of using intra-

66   species variation in parental responses to elucidate neural mechanisms of parental

67   behaviour and to discover new molecular pathways involved in avian parental care,

68   several of which we believe are conserved across other vertebrate taxa.

69

70   **2. Materials and methods**

71

72   **(a) Parental care induction procedure and behaviour quantification**

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74   Japanese quail (*Cortunix japonica*) were raised in the lab from eggs obtained from AA

75   Lab Eggs, Inc. (Westminster, CA) following the procedures described in [5]. Adult birds

76   were kept in an 8L:16D light cycle and maintained in groups of 4-5 same sex animals in

77   cages (100 × 40 × 50 cm). Two days prior to carrying out the experiments, a replica of

78   the wooden box (18 × 18 × 18 cm) used in the parental care sensitization procedure

79   was added to those cages. The day prior to the procedure, animals were separated

80   individually into cages identical to the group cages, which also contained a wooden box.

81   The wooden box was always open during these days, allowing the birds to inspect it

82   and go in and out. Food and water were provided *ad libitum* during the entire

83   experiment.

84

85   The day of the parental care sensitization procedure, each adult bird was locked inside

86   the wooden box in their individual cage starting at one hour before lights were off. Under

87   the parental induction treatment, two chicks (1-3 days old) were added to this box just

88   before lights off. Under the control treatment, no chicks were added to the box. Animals

89   were left undisturbed overnight. The morning after, the wooden box was opened, the

90   overnight chicks were removed and two new chicks (1-3 days old) were added to the

91   cage. This swap of chicks ensured that birds in both treatments (controls and

92   sensitized) were being tested for their behaviours towards novel chicks (in case

93   familiarity influenced the outcome). The novel chicks were allowed to stay in the cage

94   for 20 min, during which videos were continuously recorded (Axis M1065L network

95 camera by Axis Communications). At the end of the 20 min, adults were euthanized by

96 isoflurane inhalation and decapitation. The brain was removed from the skull, flash

97 frozen and then stored at -80 °C until further processing. Chicks were returned to their

98 cages. In total, 50 adults (ages 90-110 days old) were subjected to the experimental

99 procedures, of which 11 females and 13 males were randomly assigned to the

100 sensitization treatment and 19 females and 7 males to the control treatment. Two

101 control females were removed from the study due to methodological failures during the

102 experiments, bringing the final number of control females to 17. In total, 77 chicks were

103 used. Behaviours towards chicks within the 20 min period of observation were scored

104 by observers blind to the treatment, as described in De Bruijn et al. (2020). We used

105 these behavioural data to place birds in different categories for the subsequent

106 transcriptomics analysis. In particular, we used the duration of parental behaviour (chick

107 brooding, measured as time spent sitting on top of chicks) to define whether sensitized

108 birds were responders (>= 180 sec of parental behaviour) or non-responders (< 60 sec

109 of parental behaviour) to this treatment. Similarly, we classified controls birds as

110 showing spontaneous parental behaviour (>= 180 sec of parental behaviour) or no

111 parental behaviour (< 60 sec of parental behaviour). Animals with chick brooding

112 duration between 60 and 180 sec were removed from the data for the responsiveness

113 comparisons, but not for the treatment comparisons, during the transcriptomic analysis.

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115 **(b) Brain dissection**

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117 Brains were coronally sectioned on a Leica CM1860UV cryostat, at -18 °C. We used

118 separate surgical micropunches (EMS Rapid Core Instruments) to punch out the three

119 brain regions of interest from 100 µm slices, spaced apart by three 30 µm slices

120 collected onto microscope slides (Fisherbrand, item 12–550-15) for future use. The

121 brain regions collected were the entire hypothalamus, the nucleus taeniae (Tn) and the

122 bed nucleus of the stria terminalis (BnST). These regions were identified based on use

123 of both the quail and the chicken brain atlases [12,13]. Punches from each of these

124 brain regions were placed in individual tubes containing 2 mm size beads (ZR

125 BashingBeads Lysis Tubes, Zymo Research, item S6003-50) and 1 mL of QIAzol lysis

126   reagent (Qiagen, item #79306). Tissue homogenization was done by agitating the tubes

127   for 20 s at a 7 m s^{-1} speed (Beadbug 6 homogenizer, Benchmark Scientific),

128   followed by a 5 min rest period. The homogenate was then transferred into a new tube

129   and preserved at − 80 °C until RNA isolation.

130

131   **(c) RNA isolation, library preparation and sequencing**

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133   Total RNA was extracted from the aqueous layer formed after chloroform precipitation,

134   using the RNA Clean & Concentrator Kit-5 (Zymo Research, item # R1013) following

135   manufacturer's instructions and including the DNase I in-column treatment step. These

136   samples were then shipped on dry-ice to Novogene Corporation Inc. (Chula Vista, CA,

137   USA), where RNA quantity, purity and integrity were assessed on an Agilent 2100

138   Bioanalyzer (Agilent Technologies, Santa Clara, CA), cDNA libraries were generated

139   using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) and cDNA

140   fragments (150 ~ 200 bp in length) were then purified using the AMPure XP System

141   (Beckman Coulter, Beverly, USA). Paired-end sequencing of libraries (PE150; Illumina

142   Novaseq 6000) was performed according to standard protocols. An average of 52

143   million paired-end raw reads were obtained for each sample.

144

145   **(d) Mapping and differential gene expression analysis**

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147   An average of 85.3% of clean (post adapter removal and quality filtering) reads were

148   mapped to the Japanese quail reference genome (Coturnix_japonica_2.0, INSDC

149   Assembly Mar 2016, downloaded from Ensembl), representing an average of 43.7

150   million mapped reads per sample (Table S1 contains information on mapping statistics

151   per sample). Mapping was done using HISAT2 [14], and HTSeq was used to count the

152   number of mapped reads to each gene [15]. Differential gene expression analysis was

153   performed using the DESeq2 R package [16,17].

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155   For each sex and for each brain region, the following pairwise comparisons were

156   performed: birds subjected to the sensitization treatment relative to control treated birds;

157   birds responsive to the sensitization treatment (responders) versus non-responsive

158   birds (non-responders); and birds subjected to the control treatment that showed

159   spontaneous parental care (spontaneous) relative to birds in the same treatment that

160   showed no care (no-care). As only one control male showed spontaneous parental

161   behaviour, comparisons of this male relative to the non-parental males in the control

162   group were not performed. To control for the false discovery rate due to multiple

163   testing, *p*-values were adjusted using the Benjamini-Hochberg procedure. Genes were

164   considered as statistically differentially expressed when adjusted *p*-values

165   were < 0.05. Boxplots for a subset of the differentially expressed genes were

166   prepared using the ggplot2 R package [18], using DESeq2 normalized counts. A

167   heatmap was produced for the differentially expressed genes in the hypothalamus of

168   control females using the pheatmap package (version 1.0.12; [19]) in R, using variance

169   stabilized DESeq2 counts.

170

171   **(e) Gene ontology (GO) enrichment analysis and KEGG pathway enrichment**

172   **analysis of differentially expressed genes**

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174   GO enrichment analysis was done only for the differentially expressed genes (DEGs)

175   found in the hypothalamus of control females with spontaneous versus control females

176   with no parental care. The analysis was done separately for up- and down-regulated

177   DEGs, using ShinyGO (v0.61; [20]) with the Japanese quail as the species, and FDR

178   cut-off of 0.05. ShinyGO can also group genes by functional categories defined by high-

179   level GO terms, which are often more easily interpretable and therefore, also reported.

180   KEGG pathway enrichment analysis was also performed using ShinyGO with an FDR

181   cut-off of 0.05, but this time with mouse as the reference species, as this analysis tool is

182   not well developed for Japanese quail.

183

184   **3. Results**

185

186   **(a) Effect of sensitization treatment**

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188   ***Behavioural response.*** In response to spending an overnight enclosed in a box with

189   two chicks (sensitization treatment), 72.7 % of females responded with parental

190   behaviours (responders) and all others were non-responders. Concerning the males,

191   69.2 % males were responders, 23.1% non-responders and one male undefined (> 60

192   sec of parental behaviour but < 180 sec). Out of control treated animals (no chicks

193   overnight), 25 % of the females showed spontaneous parental behaviour, 62.5% of

194   females showed no parental behaviour and two females were undefined. The duration

195   of parental behaviour in control animals is, however, shorter (average ± s.e.m = 396 ±

196   119 sec) than that of parental females in the sensitization treatment (744 ± 54 sec). Of

197   the control males, only 12.5 % showed spontaneous parental behaviour and all others

198   were non-parental.

199

200   ***Transcriptomic response.*** To our surprise, very few genes were differentially

201   expressed in the brains of animals that underwent the sensitization treatment relative to

202   controls during interaction with chicks (Table S2). Neurotensin (NTS) expression was

203   significantly higher in the hypothalamus of sensitized females and trended in that

204   direction in males (figure 1). In both sexes, Urocortin-3 (UCN3) was upregulated in the

205   hypothalamus of sensitized relative to control animals (figure 1). In females, one

206   additional gene was upregulated in sensitized animals: a zinc finger protein (ZFN385C).

207   In males, one different additional gene was upregulated in sensitized animals, calbindin

208   1 (CALB1), and a different gene downregulated, BTG anti-proliferation factor 2 (BTG2)

209   (figure 1). Patterns of BTG2 expression were similar in females (figure 1). In the bed

210   nucleus of the stria terminalis (BnST), two DEGs were upregulated in sensitized

211   females (pentatricopeptide repeat domain 1 or PTCD1, and an unidentified gene) and

212   two were downregulated (FOS, and salt inducible kinase 1, SIK1). In males, only one

213   DEG was found in this region (Kruppel like factor 2, KLF2) and it was downregulated in

214   sensitized relative to control animals. No genes were differentially expressed due to

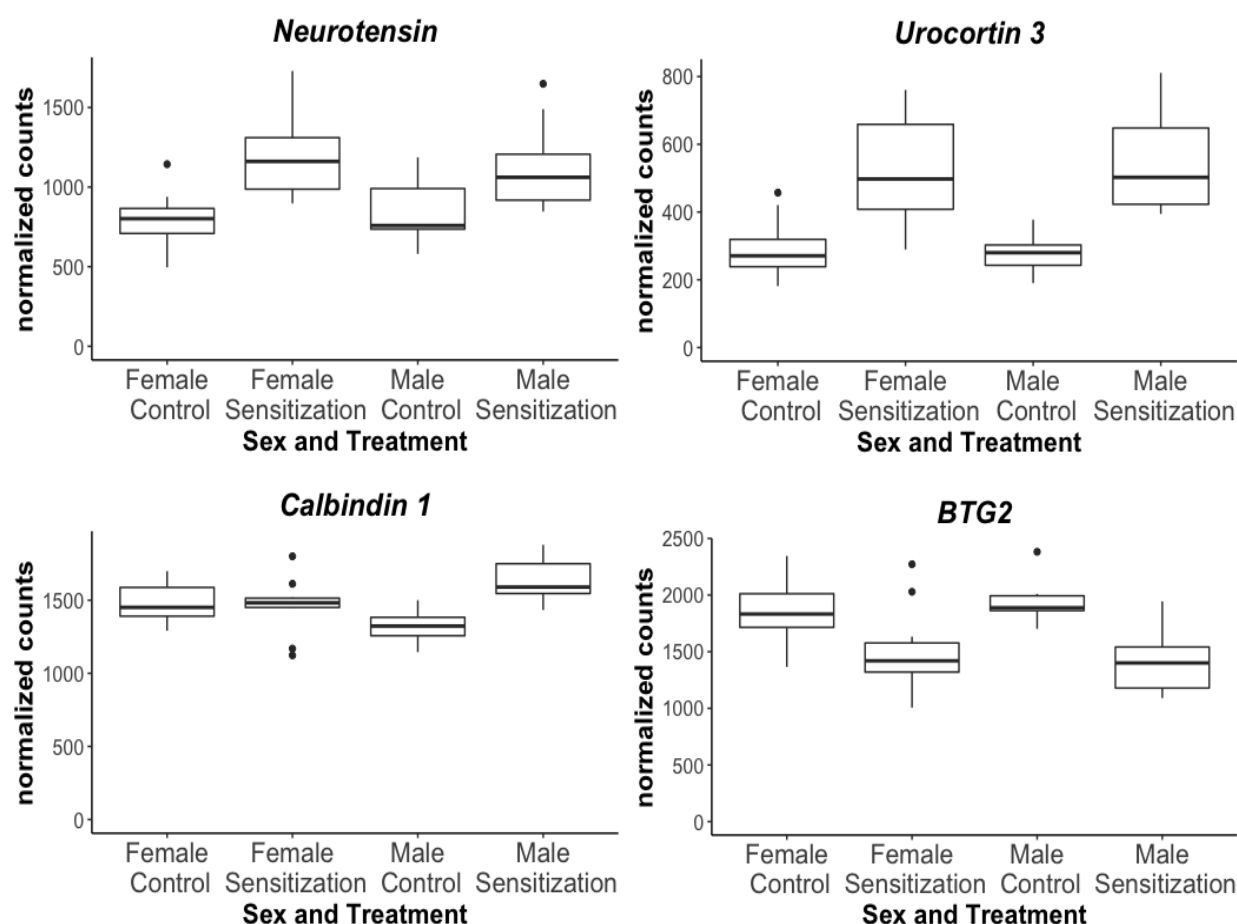215   treatment in either sex in the Nucleus Taeniae (Tn).

Figure 1. Expression levels of selected genes differentially expressed in the hypothalamus of animals exposed or not exposed to the sensitization treatment. Males and females are represented in all graphs for purposes of comparison.

(b) Differences between responders and non-responders

We compared the transcriptomic responses of the animals that responded to the sensitization treatment by showing parental behaviours (> 180 sec of chick brooding response; responders) to those of animals with no clear signs of parental behaviours (< 60 sec of chick brooding response; non-responders). In the hypothalamus of females, 32 genes were differentially expressed between responders and non-responders (Table S3), several of which were either immediate early genes (EGR1, EGR2, FOS, ARC). In males, there were only 3 hypothalamic DEGs, including COL28A1 and COL20A1, both related to collagen formation, and anti-Mullerian hormone (AMH). The three BnST

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232   DEGs upregulated in female responders were all unidentified. In males, there were

233   seven DEGs upregulated in responders in this brain region, and one downregulated. Of

234   the upregulated genes, one was also AMH, as found in the hypothalamus. In the Tn,

235   four DEGs were upregulated in female responders, two of which being unidentified and

236   the other two being target of EGR1 (TOE1) and t-complex-associated-testis-expressed

237   3 (TCT3). In males, two DEGs were upregulated in this region responders, transthyretin

238   (TTR) and solute carrier family 13 member 4 (SLC13A4).

239

240   **(c) Differences between females showing spontaneous parental behaviour**

241   **relative to non-parental females**

242

243   As four females in the control treatment showed parental behaviour when presented

244   with chicks (spontaneous), we compared the transcriptomic responses of these relative

245   to control females showing no parental behaviour (non-spontaneous). One gene, IRF5

246   (interferon regulatory factor 5) was upregulated in spontaneous females in all three

247   brain regions analysed. While in the BnST, no genes were downregulated, in the Tn

248   three genes were (sulfatase 1, SULF1; farnesyl diphosphate synthase, FDPS; and

249   tachykinin precursor 1, TAC1). Meanwhile, three thousand genes were differentially

250   expressed in the hypothalamus (Table S4; figure 2). Given the large number of DEGs in

251   this region, we used GO enrichment analysis and KEGG pathway enrichment analysis

252   for data reduction. The GO enrichment analysis revealed enrichment in several GO

253   categories related with metabolism, immune system function/immune response,

254   reproduction, circadian biology and behaviour (including reproductive behaviour,

255   locomotor behaviour, feeding behaviour, and behavioural defence response) (Table S5

256   and Table S6). In addition to also identifying several pathways involved in metabolism

257   (e.g., insulin resistance and fatty acid metabolism pathway) and immune system

258   responses (e.g., platelet activation and TNF signalling pathway) and one pathway

259   related to circadian biology (circadian rhythm), enrichment analysis of KEGG pathways

260   identified important hormone signalling (e.g., thyroid hormone signalling, GnRH

261   signalling pathway, oxytocin signalling pathway and prolactin signalling pathway) and

262   synaptic function pathways (e.g., cholinergic synapse, GABAergic synapse,

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263   dopaminergic synapse, glutamatergic synapse) that are involved in reproduction and

264   behaviour (Figure 3; Table S7).
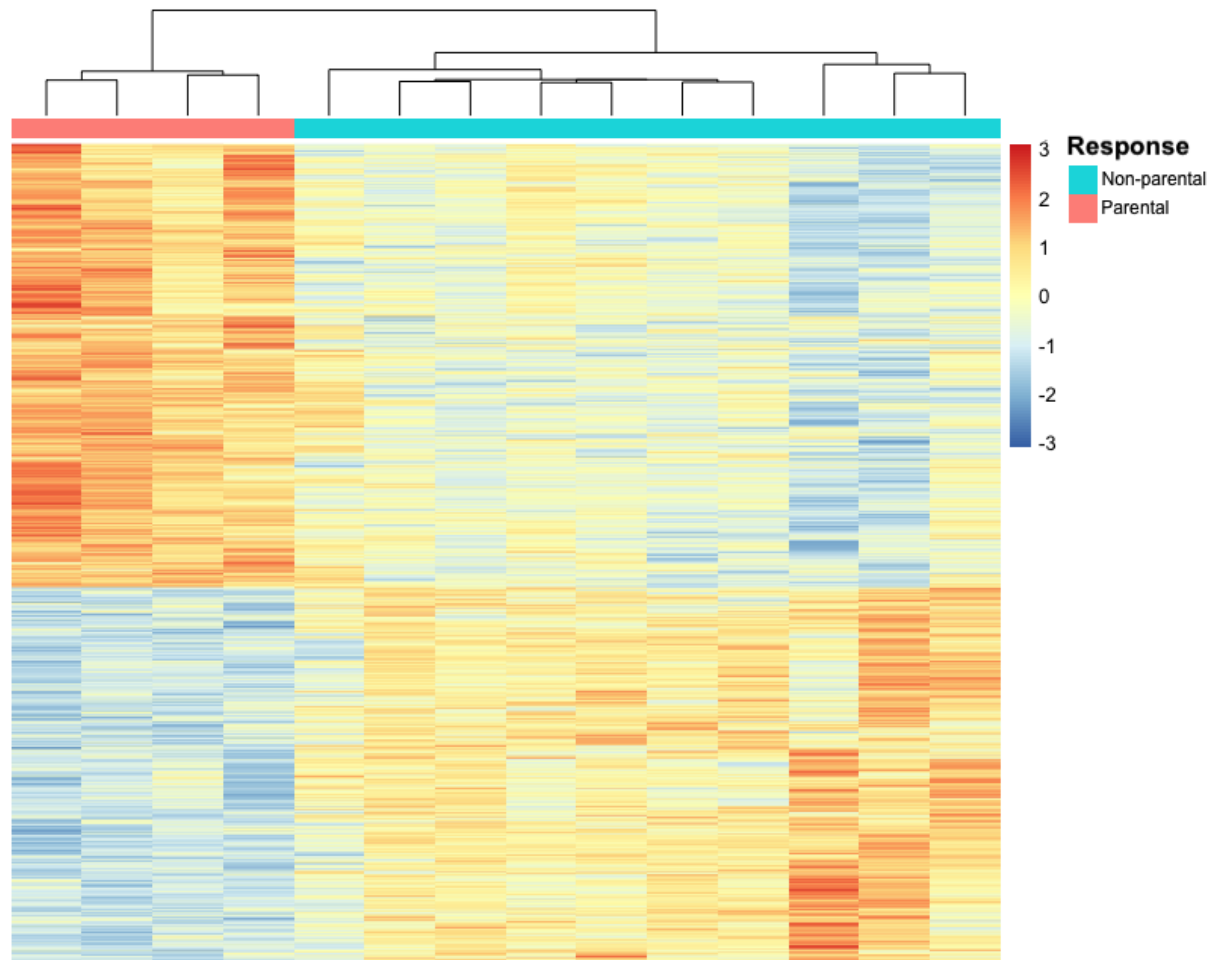
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266

267   **Figure 2.** Heatmap of hypothalamic genes differentially expressed between control

268   females that showed spontaneous parental behaviour when placed with chicks, relative

269   to females that did not. Blue colours indicate lower expression and red colours higher

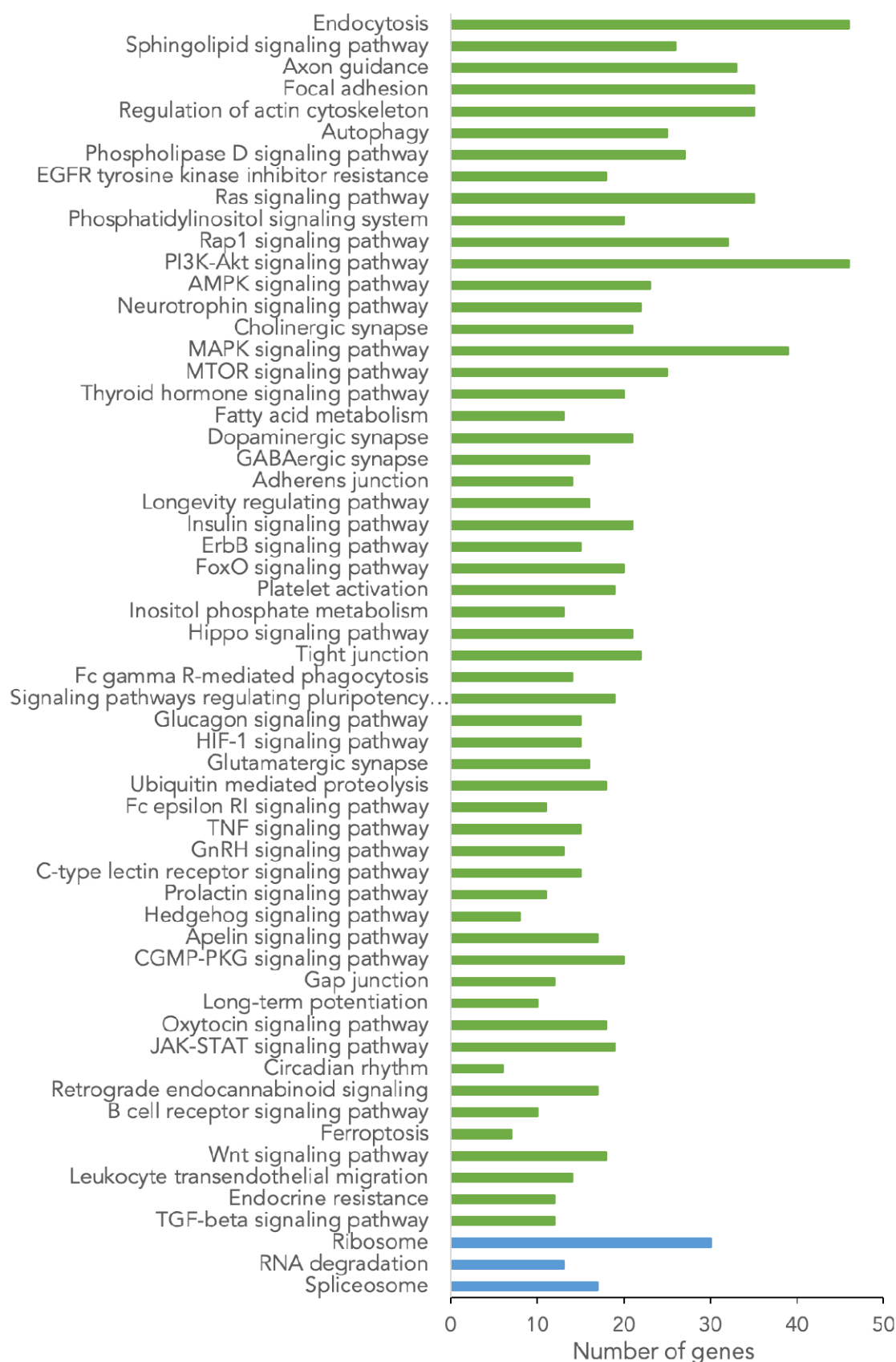270   expression levels in parental relative to non-parental controls.

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271

272 **Figure 3.** Number of hypothalamic downregulated DEGs (blue) or upregulated DEGs

273 (green) in significantly enriched KEGG pathways in control females showing

274 spontaneous parental behaviour compared to non-parental females. Pathways that

275 were redundant, related to infectious diseases or cancer, or not brain-specific were

276 excluded from this figure for simplicity (see Table S7 for full list).

277

278

279 **4. Discussion**

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281 The neural mechanisms mediating parental care remain poorly understood. We took

282 advantage of a domesticated species, the Japanese quail, for which parental behaviour

283 towards chicks does not tend to occur spontaneously in captivity. Parental behaviour in

284 these animals can be induced through a sensitization procedure, but the process is not

285 100 % effective. Using the variation in parental responses, we asked what neural

286 molecular mechanisms are: a) altered upon sensitization, b) different between birds that

287 show chick care behaviours in response to the sensitization relative to non-responders,

288 and c) different between untreated animals that spontaneously show parental care

289 relative to those that do not.

290

291 Out of the three brain regions studied, we found that most gene expression differences

292 were located in the hypothalamus. We identified a gene, neurotensin, that was

293 previously only demonstrated to be causally associated with parental care in mammals.

294 Transcriptomic studies focused on animals that fail to show parental care under care-

295 promoting circumstances, or studies on those animals that show care unprompted

296 provide great opportunities to understand the neural basis of variation in parental care.

297

298 **(a) How does sensitization modify neural responses?**

299

300 By comparing the neural transcriptomic responses of adult quail that had undergone a

301 sensitization procedure to those that did not undergo this process, we were able to

302 identify a small number of differentially expressed genes that may be involved in avian

13

303   parental care. One of those genes, neurotensin, was expressed at higher levels in the

304   hypothalamus of both females and males (albeit only significantly so in females) after

305   sensitization. This finding is significant given that neurotensin levels in the

306   hypothalamus of postpartum female mice were also observed to be elevated relative to

307   that of virgin mice [21]. Neurotensin gene expression was also recently shown to be

308   elevated in the preoptic area of males of one species of poison frog (*Dendrobates*

309   *tinctorius*) when performing parental care relative to males not performing care [22].

310   Furthermore, intracerebroventricular injections of neurotensin significantly decreased

311   maternal aggression in female mice [23], indicating the neurotensin participates in at

312   least one component of maternal responses in rodents. Previously, the only reports of

313   behavioural effects of neurotensin on birds suggested a possible relationship between

314   neurotensin expression in the medial preoptic nucleus (POM) and non-vocal courtship

315   behaviour and agonistic behaviour in male European starlings (*Sturnus vulgaris*) [24].

316   Interestingly, that study also reported that male starlings that had acquired a nest box

317   also had higher neurotensin expression in the POM relative to males that did not

318   acquire boxes and the measures of non-vocal courtship behaviour were mostly related

319   to nest building (e.g., such as number of times entering or landing on the nest and

320   gathering nesting material). European starlings show biparental care and the increase in

321   neurotensin in a hypothalamic nucleus in that study could have been related to nest-

322   building activity, which, for many species, is a component of parental care.

323

324   Out of the hormones associated with avian parental care to date, prolactin is by far the

325   one that has receive the most attention [3]. In precocial avian species, as is the case

326   with Japanese quail, prolactin tends to be elevated in females during the incubation

327   period and to decline when chicks hatch [2]. Prolactin release is known to be under the

328   control of dopamine, which can have both inhibitory and stimulatory effects depending

329   on the dopamine receptor subtype it acts on [3]. Neurotensin could impact parental

330   behaviour by playing a role in controlling prolactin secretion, through its action in

331   enhancing dopamine release [25]. Neurotensin could also affect care through its

332   involvement in reward processing [26].

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14

334   Another interesting gene that was upregulated following the sensitization procedure in

335   both sexes was urocortin-3. In rodents, central administration or overexpression of

336   urocortin-3 increases stress-induced anxiety and suppresses ingestive behaviour

337   [27,28]. We are only aware of two reports relating urocortin-3 to young-directed parental

338   care. One is the poison frog paper mentioned above [22] where urocortin-3 was

339   upregulated in the preotic area of frogs performing parental care relative to non-parental

340   one. The other one revealed that urocortin-3 expressing neurons in the perifornical area

341   of the hypothalamus are activated specifically during infant-directed aggression in both

342   male and female mice and that optogenetic activation of these neurons triggers infant-

343   directed aggression and neglect [29]. Given that we observed a reduction in aggression

344   towards chicks after the sensitization procedure [5], urocortin-3 may have an opposing

345   effect on infant-directed aggressive behaviour in our system or serve different functions

346   that support parental care, such as altered feeding patterns [30,31].

347

348   Calbindin 1 was only upregulated in the hypothalamus of sensitized males. Calbindin 1

349   has a sexually dimorphic distribution in certain brain regions, including the hypothalamic

350   nuclei in mice [32,33] and in regions of the song control system and related areas in

351   certain bird species [34–36]. In mice, calbindin 1 appears to also have sexually

352   dimorphic effects on behaviour, including effects on fear conditioning and on social

353   preferences in males [37]. Calbindin 1 has been found to limit dopamine release in

354   certain brain regions, which, as mentioned previously, can affect prolactin secretion

355   [38]. Also exclusive to sensitized males was the downregulation of BTG2. One study in

356   mice and zebrafish found increased neural expression of this gene in males exposed to

357   aggressive social encounters [39]. As we do observe aggression towards chicks in

358   control males [5], differences in BTG2 may be linked to aggressive interactions in our

359   study.

360

361   FOS, an immediate early gene downregulated in the BnST in sensitized females relative

362   to controls, is widely used as a marker of neuronal activation of specific brain regions

363   [40] and the difference between sensitized and control females may imply that the BnST

364   is activated in response to chicks only in control females. Interestingly, sensitized

15

365   parental female quail separated from their chicks show increased fos immunoreactivity

366   in this region [11], which may indicate an involvement of BnST in maternal separation.

367   As the sensitization elicited no significant gene expression changes in the Tn in neither

368   sex, this region is likely less critically involved during the expression of parental care in

369   birds.

370

371   **(b) What makes a non-responder?**

372

373   One important aspect of our study is that rather than excluding animals that did not

374   respond to the sensitization treatment, we used them to understand what neural

375   differences there are in animals that fail to show parental behaviours under conditions

376   that induce these behaviours. Female responders showed lower expression of several

377   immediate early genes (EGR1, EGR2, FOS, ARC) in the hypothalamus, which could be

378   indicative of lower hypothalamic activity after effective sensitization. Several of the

379   downregulated genes in responders are also associated with the modulation of synaptic

380   signalling and synapse reorganization (CNKSR2, NPAS4, ARC, GRIN2B, EGR2) and

381   with nervous system development (CPNE5, MEF2D, NR4A3, EGR2, FOS, NPAS4,

382   ARC, CDHR1, GRIN2B). It has been suggested that maternal experience can alter both

383   the functioning and the structure of the medial preoptic area (a hypothalamic nucleus) in

384   a way that increases neuronal responsiveness to young-related cues (reviewed in [41]).

385   It is therefore possible that, in females that failed to show parental behaviours, the

386   sensitization procedure was ineffective in triggering the necessary re-structuring of

387   hypothalamic nuclei. Other brain regions studied had fewer differences between female

388   responders and non-responders.

389

390   Contrary to results in females, in males, most differences between responders and non-

391   responders were seen in the BnST. Of particular interest are two genes that had higher

392   expression in responders: Anti-Müllerian Hormone (AMH) and Butyrylcholinesterase

393   (BCHE). Increased expression of AMH was also found in the hypothalamus of male

394   responders. While the most familiar role of AMH is in the regression of Müllerian ducts

395   in male embryos during development, AMH is also produced in the brain [42,43].

16

396 Emerging actions of AMH are being found in the control of hypothalamic function, in

397 particular, in increasing GnRH neuron excitability and GnRH secretion [42,43]. BChE

398 has an interesting role in inactivating ghrelin (a hormone that affects hunger, feeding

399 and stress), and mice with overexpression of BCHE show decreased fighting [44,45]. A

400 reduction in aggression towards young is essential for proper parental care and BChE

401 might facilitate this in responders. The two DEGs in the Tn (Transthyretin, TTR, and the

402 sulfate transporter SLC13A4) showed some of the greatest fold changes observed in

403 responders relative to non-responders. TTR expression has been shown to change in

404 mammals in ways that depend on the manipulation and brain region. For instance, TTR

405 expression decreases in the cortex of mice exposed to chronic immobilization stress

406 [46] but increases in the amygdala during the formation of fear memory [47]. One

407 interpretation is, therefore, that the presence of chicks may be perceived as a stressor

408 by non-responders. Regarding SLC13A4, a recent study in mice showed that Slc13a4

409 haploinsufficiency (Slc13a4$^{+/-}$) results in atypical social behaviours, including longer

410 time to retrieve pups and reduced nest-building activity in females, and reduced social

411 exploration and impaired social memory in both sexes [48]. While parental behaviours

412 were only tested in females in that study (given that male mice do not display those), a

413 Slc13a4 increase may be required for proper parental behaviours in other species.

414

415 **(c) What neural changes are associated with spontaneous care?**

416

417 A number of females in our experiment that had no previous experiences with chicks

418 showed parental behaviour in the absence of sensitization. It was in the hypothalamus

419 of these females that we found the largest number of differentially expressed genes out

420 of all comparisons performed in our study. While these control females showed some

421 parental behaviour, the time spent engaging in those behaviours was less than those

422 observed in sensitized animals. The changes in gene expression in parental controls

423 may therefore represent processes associated with the *onset* of the modifications that

424 will be needed for a more complete parental response. For example, enrichment

425 analysis highlighted several pathways associated with synaptic function, including

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426   cholinergic, GABAergic, dopaminergic, and glutamatergic synapse, which may again

427   indicate the necessity for a hypothalamic re-structuring needed for parental care.

428

429   Many of the pathways detected through enrichment analysis have been linked to

430   parental care in mammals and other species, including thyroid hormone, GnRH,

431   oxytocin and prolactin signalling pathways [7,10,41,49]. The onset of parental care is

432   associated with reduced reproductive behaviour, reduced young-directed aggression,

433   and with changes in metabolism, locomotion and feeding patterns [10,49]. Pathways

434   associated with this array of behavioural and metabolic changes were highlighted

435   through enrichment analysis. Interestingly, recent work proposes that molecules related

436   to feeding behaviour may also be involved in complex social behaviours, such as

437   parental care [22,50].

438

439   Changes in immune function were also detected through enrichment analysis. In fact,

440   one DEG, IRF5, was upregulated in parental females controls across all three brain

441   regions analysed. IRF5 is produced in the brain by microglia (resident immune cells in

442   the brain) and it is known for its pro-inflammatory functions [51,52]. Extensive

443   neuroimmune changes occur in rodents postpartum (reviewed in [53]) and it is likely that

444   some of these extend to other vertebrate species (e.g., [54]).

445

446   **5. Conclusion**

447

448   Recent studies have used transcriptomic approaches to compare gene expression in

449   specific brain areas as female birds transition from territorial defence to egg incubation

450   [55], or from egg-laying to incubation related behaviours [56]. Still, the range of neural

451   mechanisms involved in post-hatching care in birds is virtually unknown. Our findings

452   revealed new potential modulators of avian young-directed parental care, which deserve

453   to be examined through further mechanistic studies and explored in other bird species

454   and other taxonomic groups. Particularly exciting are neurotensin and urocortin-3, which

455   potentially show conserved roles in parental care in mammals, amphibians, and birds,

456   but are extremely under-explored in the context of parental behaviour. Natural variation

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457   in avian parental behaviour in response to young extends from abandoning the nest to

458   alloparenting or even adoption of non-offspring deposited by brood parasites. The

459   pathways and genes found to be associated with spontaneous parental care, with lack

460   of parental responsiveness, and with parental sensitization in our study serve as a

461   foundation for future studies aimed at investigating the neural underpinnings of variation

462   in parental care within and across species.

463

464   **Ethics**

465   Animal use and experimental design were approved by the Chapman University

466   Institutional Animal Care and Use Committee (protocol # 2019-01).

467

468   **Data accessibility**

469   RNA-seq mapping statistics, lists of DEGs and lists of enrichment terms have been

470   uploaded as part of the supplementary material. Sequencing datasets generated and

471   analysed during the current study are deposited in the NCBI Gene Expression Omnibus

472   (GEO) repository [dataset will be made publicly available once manuscript is accepted

473   for publication].

474

475   **Authors' contributions**

476   PCL conceived the study, helped RDB carry out the animal experiments, collected the

477   brain samples, performed the molecular work, the data analysis and wrote the

478   manuscript. RDB carried out the animal experiments. All authors read and edited the

479   manuscript.

480

481   **Competing interests**

482   We declare we have no competing interests.

483   **Funding**

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487   **Acknowledgements**

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493   **References**

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