Brain transcriptomic changes in Japanese quail reveal a role for neurotensin in 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

While parental care is rare in many vertebrate taxa, it is essential for survival in the majority of avian species, with biparental care being the most frequent modality observed [1]. As a group, birds show an incredible diversity in forms of parental care, from brood parasitism, where care is lost, to cooperative breeding, where non-reproductive individuals provide care to offspring that are not their own [1]. The neural underpinnings of young-directed parental care in birds are, however, severely understudied. In terms of neuroendocrine mechanisms associated with incubation and caring for young, the majority of attention has been paid to a single pituitary hormone: prolactin [2,3]. In contrast to birds, uniparental maternal care is the most frequently observed mode of care in mammals and, therefore, the neuroendocrine basis of this behaviour is more well studied and understood than any other form of parental care across vertebrate taxa.

Using a sensitization procedure developed by [4], a recent experiment by our group confirmed that it is possible to induce parental behaviours in Japanese quail through a single overnight exposure to chicks [5]. After the sensitization procedure, both sexes display chick brooding behaviour and reduced aggression towards chicks (pecking behaviour). While the change in behaviour after sensitization is drastic, it is not observed in all animals, i.e., some animals fail to respond to the sensitization. Conversely, a few animals not exposed to the sensitization spontaneously show some parental care when paired with chicks.

In the current study, we take advantage of this variation in responses to the sensitization treatment to understand, from a neural molecular perspective, a) what modifications occur due to the sensitization treatment, b) how animals that are unable to show care after this treatment differ from parental ones, and c) how some animals are capable of spontaneously showing care during their first encounter with chicks. We focused our analysis on the hypothalamus, the bed nucleus of the stria terminalis and the nucleus taeniae. We targeted these brain regions because they contain nuclei

important for parental care, affiliative interactions, and agonistic interactions in birds and other vertebrates [6–11]. Our experiments highlight the importance of using intra-species variation in parental responses to elucidate neural mechanisms of parental behaviour and to discover new molecular pathways involved in avian parental care, several of which we believe are conserved across other vertebrate taxa.

2. Materials and methods

(a) Parental care induction procedure and behaviour quantification

Japanese quail (*Cortunix japonica*) were raised in the lab from eggs obtained from AA Lab Eggs, Inc. (Westminster, CA) following the procedures described in [5]. Adult birds were kept in an 8L:16D light cycle and maintained in groups of 4-5 same sex animals in cages (100 × 40 × 50 cm). Two days prior to carrying out the experiments, a replica of the wooden box (18 × 18 × 18 cm) used in the parental care sensitization procedure was added to those cages. The day prior to the procedure, animals were separated individually into cages identical to the group cages, which also contained a wooden box. The wooden box was always open during these days, allowing the birds to inspect it and go in and out. Food and water were provided *ad libitum* during the entire experiment.

The day of the parental care sensitization procedure, each adult bird was locked inside the wooden box in their individual cage starting at one hour before lights were off. Under the parental induction treatment, two chicks (1-3 days old) were added to this box just before lights off. Under the control treatment, no chicks were added to the box. Animals were left undisturbed overnight. The morning after, the wooden box was opened, the overnight chicks were removed and two new chicks (1-3 days old) were added to the cage. This swap of chicks ensured that birds in both treatments (controls and sensitized) were being tested for their behaviours towards novel chicks (in case familiarity influenced the outcome). The novel chicks were allowed to stay in the cage for 20 min, during which videos were continuously recorded (Axis M1065L network

camera by Axis Communications). At the end of the 20 min, adults were euthanized by isoflurane inhalation and decapitation. The brain was removed from the skull, flash frozen and then stored at -80 °C until further processing. Chicks were returned to their cages. In total, 50 adults (ages 90-110 days old) were subjected to the experimental procedures, of which 11 females and 13 males were randomly assigned to the sensitization treatment and 19 females and 7 males to the control treatment. Two control females were removed from the study due to methodological failures during the experiments, bringing the final number of control females to 17. In total, 77 chicks were used. Behaviours towards chicks within the 20 min period of observation were scored by observers blind to the treatment, as described in De Bruijn et al. (2020). We used these behavioural data to place birds in different categories for the subsequent transcriptomics analysis. In particular, we used the duration of parental behaviour (chick brooding, measured as time spent sitting on top of chicks) to define whether sensitized birds were responders (>= 180 sec of parental behaviour) or non-responders (< 60 sec of parental behaviour) to this treatment. Similarly, we classified controls birds as showing spontaneous parental behaviour (>= 180 sec of parental behaviour) or no parental behaviour (< 60 sec of parental behaviour). Animals with chick brooding duration between 60 and 180 sec were removed from the data for the responsiveness comparisons, but not for the treatment comparisons, during the transcriptomic analysis.

(b) Brain dissection

Brains were coronally sectioned on a Leica CM1860UV cryostat, at -18 °C. We used separate surgical micropunches (EMS Rapid Core Instruments) to punch out the three brain regions of interest from 100 µm slices, spaced apart by three 30 µm slices collected onto microscope slides (Fisherbrand, item 12–550-15) for future use. The brain regions collected were the entire hypothalamus, the nucleus taeniae (Tn) and the bed nucleus of the stria terminalis (BnST). These regions were identified based on use of both the quail and the chicken brain atlases [12,13]. Punches from each of these brain regions were placed in individual tubes containing 2 mm size beads (ZR BashingBeads Lysis Tubes, Zymo Research, item S6003-50) and 1 mL of QIAzol lysis

reagent (Qiagen, item #79306). Tissue homogenization was done by agitating the tubes for 20 s at a 7 m s^{-1} speed (Beadbug 6 homogenizer, Benchmark Scientific), followed by a 5 min rest period. The homogenate was then transferred into a new tube and preserved at −80 °C until RNA isolation.

(c) RNA isolation, library preparation and sequencing

Total RNA was extracted from the aqueous layer formed after chloroform precipitation, using the RNA Clean & Concentrator Kit-5 (Zymo Research, item # R1013) following manufacturer's instructions and including the DNase I in-column treatment step. These samples were then shipped on dry-ice to Novogene Corporation Inc. (Chula Vista, CA, USA), where RNA quantity, purity and integrity were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), cDNA libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) and cDNA fragments (150 ~ 200 bp in length) were then purified using the AMPure XP System (Beckman Coulter, Beverly, USA). Paired-end sequencing of libraries (PE150; Illumina Novaseq 6000) was performed according to standard protocols. An average of 52 million paired-end raw reads were obtained for each sample.

(d) Mapping and differential gene expression analysis

An average of 85.3% of clean (post adapter removal and quality filtering) reads were mapped to the Japanese quail reference genome (Coturnix_japonica_2.0, INSDC Assembly Mar 2016, downloaded from Ensembl), representing an average of 43.7 million mapped reads per sample (Table S1 contains information on mapping statistics per sample). Mapping was done using HISAT2 [14], and HTSeq was used to count the number of mapped reads to each gene [15]. Differential gene expression analysis was performed using the DESeq2 R package [16,17].

For each sex and for each brain region, the following pairwise comparisons were performed: birds subjected to the sensitization treatment relative to control treated birds;

birds responsive to the sensitization treatment (responders) versus non-responsive birds (non-responders); and birds subjected to the control treatment that showed spontaneous parental care (spontaneous) relative to birds in the same treatment that showed no care (no-care). As only one control male showed spontaneous parental behaviour, comparisons of this male relative to the non-parental males in the control group were not performed. To control for the false discovery rate due to multiple testing, *p*-values were adjusted using the Benjamini-Hochberg procedure. Genes were considered as statistically differentially expressed when adjusted *p*-values were < 0.05. Boxplots for a subset of the differentially expressed genes were prepared using the ggplot2 R package [18], using DESeq2 normalized counts. A heatmap was produced for the differentially expressed genes in the hypothalamus of control females using the pheatmap package (version 1.0.12; [19]) in R, using variance stabilized DESeq2 counts.

(e) Gene ontology (GO) enrichment analysis and KEGG pathway enrichment analysis of differentially expressed genes

GO enrichment analysis was done only for the differentially expressed genes (DEGs) found in the hypothalamus of control females with spontaneous versus control females with no parental care. The analysis was done separately for up- and down-regulated DEGs, using ShinyGO (v0.61; [20]) with the Japanese quail as the species, and FDR cut-off of 0.05. ShinyGO can also group genes by functional categories defined by high-level GO terms, which are often more easily interpretable and therefore, also reported. KEGG pathway enrichment analysis was also performed using ShinyGO with an FDR cut-off of 0.05, but this time with mouse as the reference species, as this analysis tool is not well developed for Japanese quail.

3. Results

(a) Effect of sensitization treatment

Behavioural response. In response to spending an overnight enclosed in a box with two chicks (sensitization treatment), 72.7 % of females responded with parental behaviours (responders) and all others were non-responders. Concerning the males, 69.2 % males were responders, 23.1% non-responders and one male undefined (> 60 sec of parental behaviour but < 180 sec). Out of control treated animals (no chicks overnight), 25 % of the females showed spontaneous parental behaviour, 62.5% of females showed no parental behaviour and two females were undefined. The duration of parental behaviour in control animals is, however, shorter (average ± s.e.m = 396 ± 119 sec) than that of parental females in the sensitization treatment (744 ± 54 sec). Of the control males, only 12.5 % showed spontaneous parental behaviour and all others were non-parental.

Transcriptomic response. To our surprise, very few genes were differentially expressed in the brains of animals that underwent the sensitization treatment relative to controls during interaction with chicks (Table S2). Neurotensin (NTS) expression was significantly higher in the hypothalamus of sensitized females and trended in that direction in males (figure 1). In both sexes, Urocortin-3 (UCN3) was upregulated in the hypothalamus of sensitized relative to control animals (figure 1). In females, one additional gene was upregulated in sensitized animals: a zinc finger protein (ZFN385C). In males, one different additional gene was upregulated in sensitized animals, calbindin 1 (CALB1), and a different gene downregulated, BTG anti-proliferation factor 2 (BTG2) (figure 1). Patterns of BTG2 expression were similar in females (figure 1). In the bed nucleus of the stria terminalis (BnST), two DEGs were upregulated in sensitized females (pentatricopeptide repeat domain 1 or PTCD1, and an unidentified gene) and two were downregulated (FOS, and salt inducible kinase 1, SIK1). In males, only one DEG was found in this region (Kruppel like factor 2, KLF2) and it was downregulated in sensitized relative to control animals. No genes were differentially expressed due to treatment in either sex in the Nucleus Taeniae (Tn).

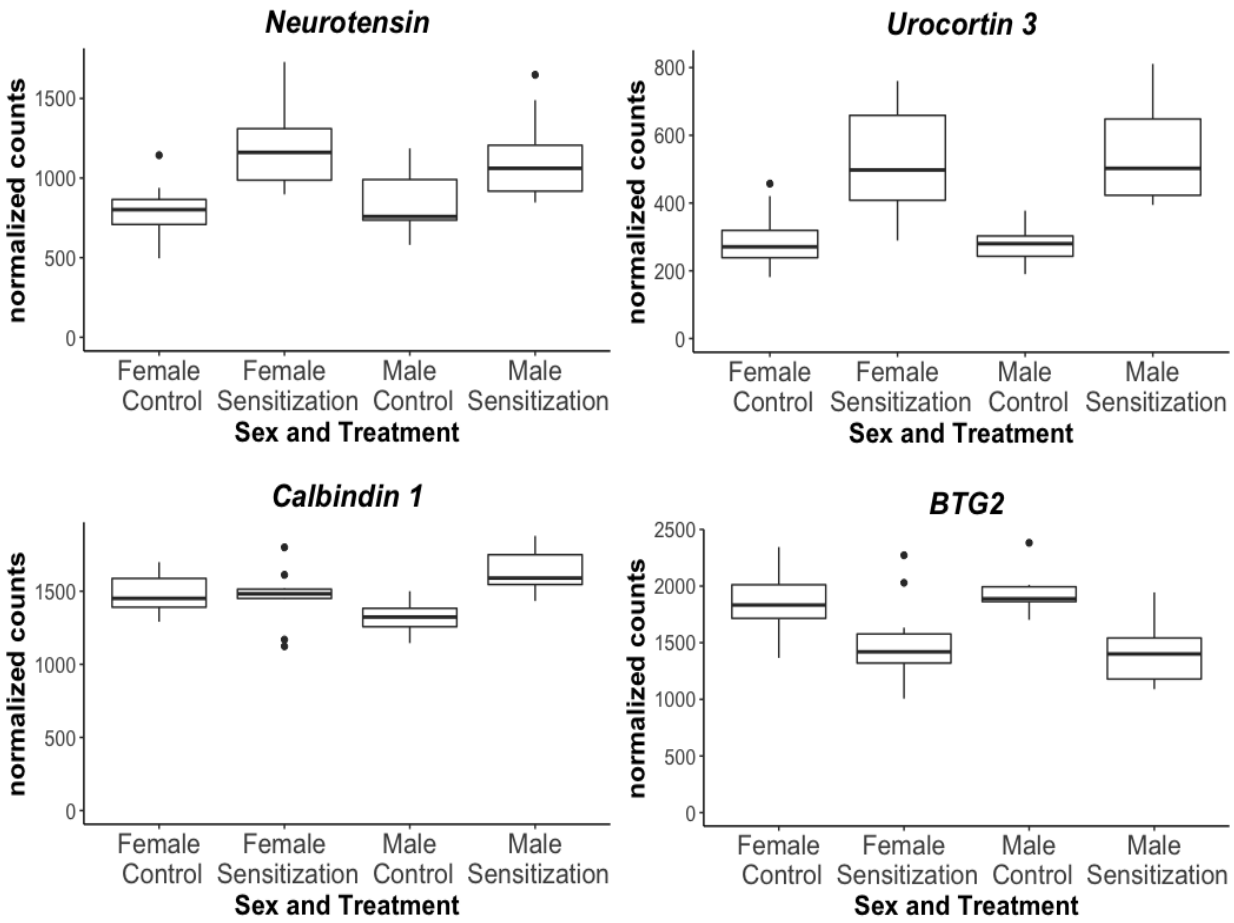


Figure 1. Expression levels of selected genes differentially expressed in the hypothalamus of animals exposed or not exposed to the sensitization treatment. Males and females are represented in all graphs for purposes of comparison.

(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

DEGs upregulated in female responders were all unidentified. In males, there were seven DEGs upregulated in responders in this brain region, and one downregulated. Of the upregulated genes, one was also AMH, as found in the hypothalamus. In the Tn, four DEGs were upregulated in female responders, two of which being unidentified and the other two being target of EGR1 (TOE1) and t-complex-associated-testis-expressed 3 (TCT3). In males, two DEGs were upregulated in this region responders, transthyretin (TTR) and solute carrier family 13 member 4 (SLC13A4).

(c) Differences between females showing spontaneous parental behaviour relative to non-parental females

As four females in the control treatment showed parental behaviour when presented with chicks (spontaneous), we compared the transcriptomic responses of these relative to control females showing no parental behaviour (non-spontaneous). One gene, IRF5 (interferon regulatory factor 5) was upregulated in spontaneous females in all three brain regions analysed. While in the BnST, no genes were downregulated, in the Tn three genes were (sulfatase 1, SULF1; farnesyl diphosphate synthase, FDPS; and tachykinin precursor 1, TAC1). Meanwhile, three thousand genes were differentially expressed in the hypothalamus (Table S4; figure 2). Given the large number of DEGs in this region, we used GO enrichment analysis and KEGG pathway enrichment analysis for data reduction. The GO enrichment analysis revealed enrichment in several GO categories related with metabolism, immune system function/immune response, reproduction, circadian biology and behaviour (including reproductive behaviour, locomotor behaviour, feeding behaviour, and behavioural defence response) (Table S5 and Table S6). In addition to also identifying several pathways involved in metabolism (e.g., insulin resistance and fatty acid metabolism pathway) and immune system responses (e.g., platelet activation and TNF signalling pathway) and one pathway related to circadian biology (circadian rhythm), enrichment analysis of KEGG pathways identified important hormone signalling (e.g., thyroid hormone signalling, GnRH signalling pathway, oxytocin signalling pathway and prolactin signalling pathway) and synaptic function pathways (e.g., cholinergic synapse, GABAergic synapse,

dopaminergic synapse, glutamatergic synapse) that are involved in reproduction and behaviour (Figure 3; Table S7).

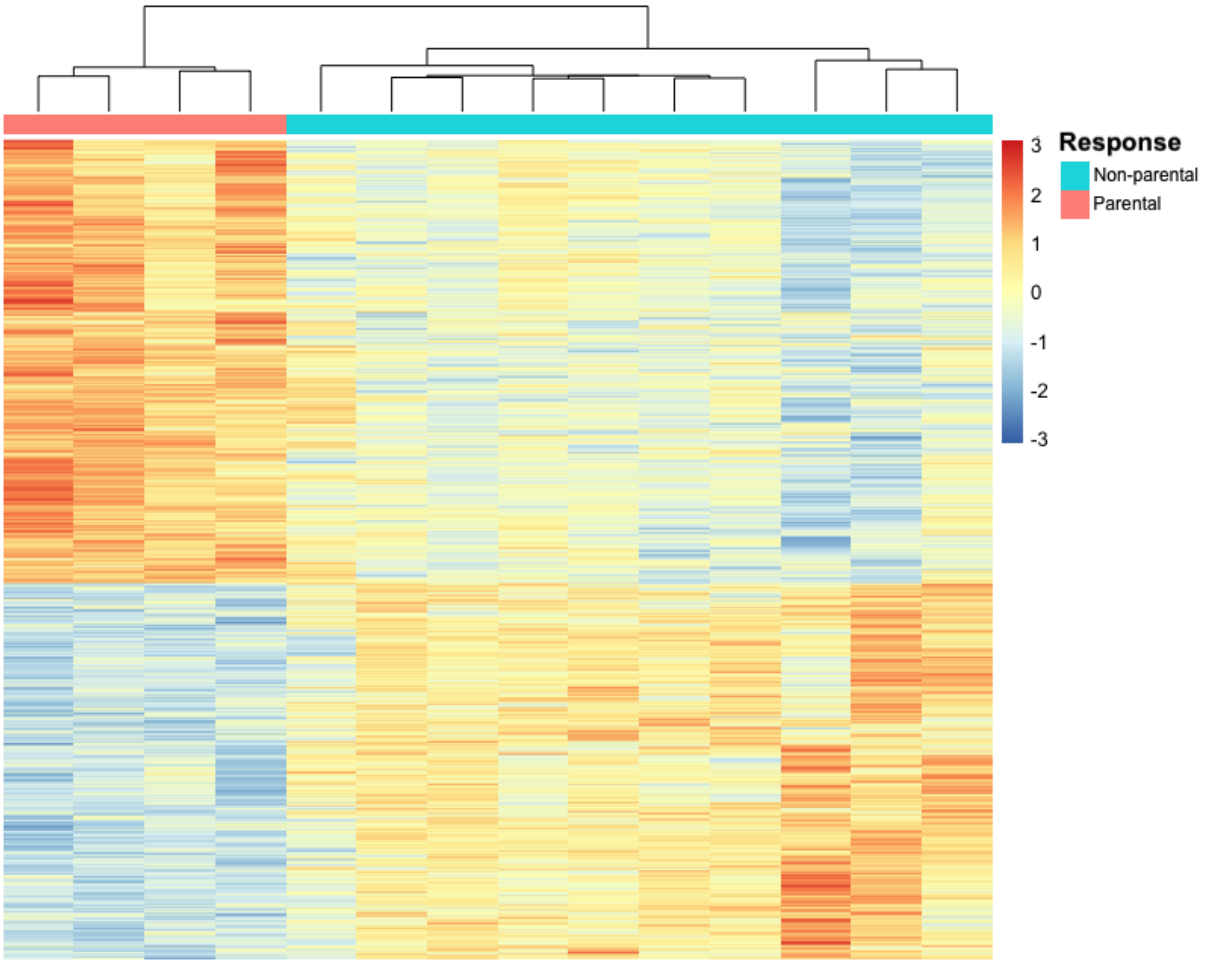
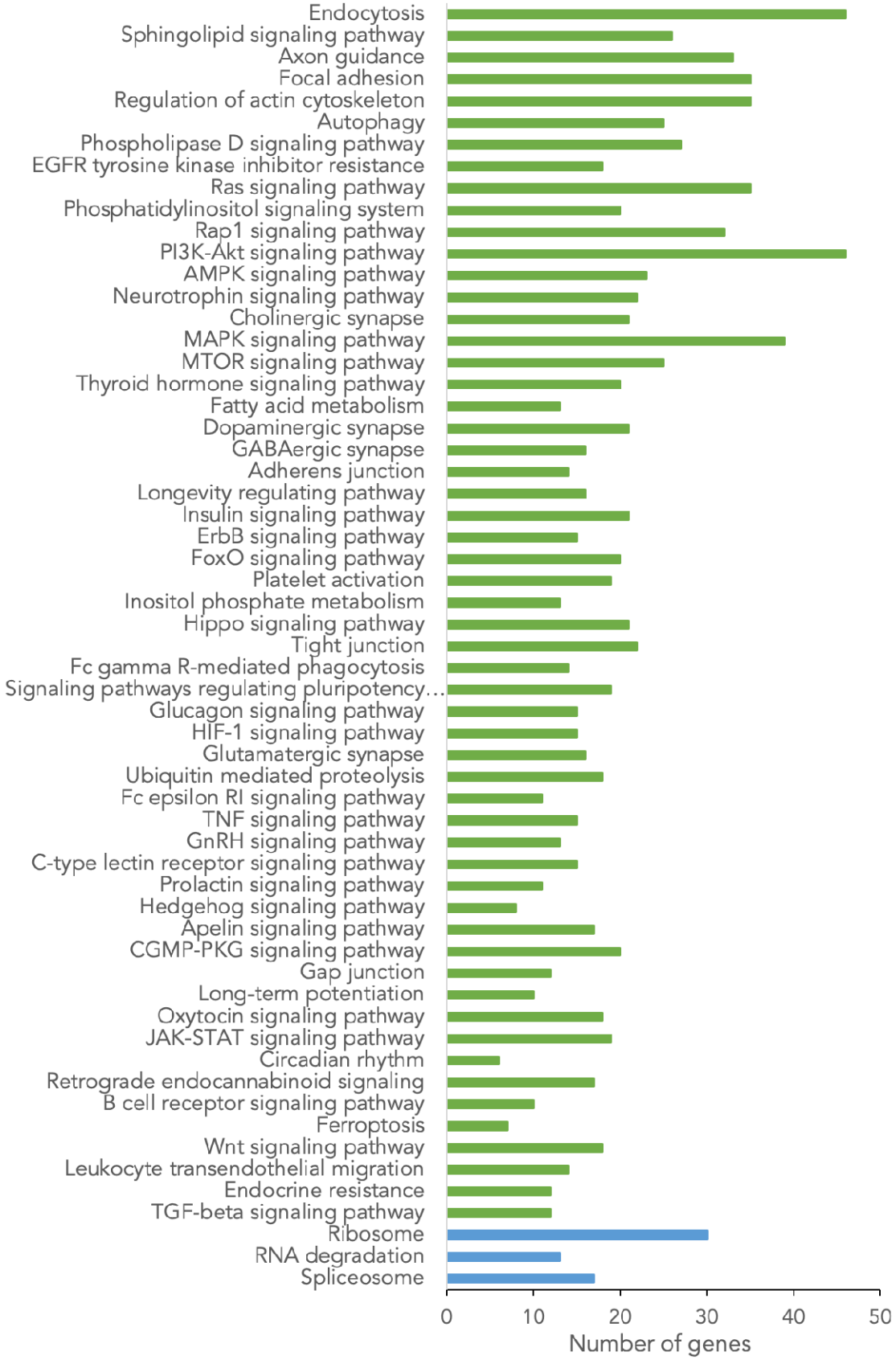


Figure 2. Heatmap of hypothalamic genes differentially expressed between control females that showed spontaneous parental behaviour when placed with chicks, relative to females that did not. Blue colours indicate lower expression and red colours higher expression levels in parental relative to non-parental controls.


Endocytosis
Sphingolipid signaling pathway
Axon guidance
Focal adhesion
Regulation of actin cytoskeleton
Autophagy
Phospholipase D signaling pathway
EGFR tyrosine kinase inhibitor resistance
Ras signaling pathway
Phosphatidylinositol signaling system
Rap1 signaling pathway
PI3K-Akt signaling pathway
AMPK signaling pathway
Neurotrophin signaling pathway
Cholinergic synapse
MAPK signaling pathway
MTOR signaling pathway
Thyroid hormone signaling pathway
Fatty acid metabolism
Dopaminergic synapse
GABAergic synapse
Adherens junction
Longevity regulating pathway
Insulin signaling pathway
ErbB signaling pathway
FoxO signaling pathway
Platelet activation
Inositol phosphate metabolism
Hippo signaling pathway
Tight junction
Fc gamma R-mediated phagocytosis
Signaling pathways regulating pluripotency…
Glucagon signaling pathway
HIF-1 signaling pathway
Glutamatergic synapse
Ubiquitin mediated proteolysis
Fc epsilon RI signaling pathway
TNF signaling pathway
GnRH signaling pathway
C-type lectin receptor signaling pathway
Prolactin signaling pathway
Hedgehog signaling pathway
Apelin signaling pathway
CGMP-PKG signaling pathway
Gap junction
Long-term potentiation
Oxytocin signaling pathway
JAK-STAT signaling pathway
Circadian rhythm
Retrograde endocannabinoid signaling
B cell receptor signaling pathway
Ferroptosis
Wnt signaling pathway
Leukocyte transendothelial migration
Endocrine resistance
TGF-beta signaling pathway
Ribosome
RNA degradation
Spliceosome
0
10
20
30
40
50
Number of genes

271

Figure 3. Number of hypothalamic downregulated DEGs (blue) or upregulated DEGs (green) in significantly enriched KEGG pathways in control females showing spontaneous parental behaviour compared to non-parental females. Pathways that were redundant, related to infectious diseases or cancer, or not brain-specific were excluded from this figure for simplicity (see Table S7 for full list).

4. Discussion

The neural mechanisms mediating parental care remain poorly understood. We took advantage of a domesticated species, the Japanese quail, for which parental behaviour towards chicks does not tend to occur spontaneously in captivity. Parental behaviour in these animals can be induced through a sensitization procedure, but the process is not 100 % effective. Using the variation in parental responses, we asked what neural molecular mechanisms are: a) altered upon sensitization, b) different between birds that show chick care behaviours in response to the sensitization relative to non-responders, and c) different between untreated animals that spontaneously show parental care relative to those that do not.

Out of the three brain regions studied, we found that most gene expression differences were located in the hypothalamus. We identified a gene, neurotensin, that was previously only demonstrated to be causally associated with parental care in mammals. Transcriptomic studies focused on animals that fail to show parental care under care-promoting circumstances, or studies on those animals that show care unprompted provide great opportunities to understand the neural basis of variation in parental care.

(a) How does sensitization modify neural responses?

By comparing the neural transcriptomic responses of adult quail that had undergone a sensitization procedure to those that did not undergo this process, we were able to identify a small number of differentially expressed genes that may be involved in avian

parental care. One of those genes, neurotensin, was expressed at higher levels in the hypothalamus of both females and males (albeit only significantly so in females) after sensitization. This finding is significant given that neurotensin levels in the hypothalamus of postpartum female mice were also observed to be elevated relative to that of virgin mice [21]. Neurotensin gene expression was also recently shown to be elevated in the preoptic area of males of one species of poison frog (*Dendrobates tinctorius*) when performing parental care relative to males not performing care [22]. Furthermore, intracerebroventricular injections of neurotensin significantly decreased maternal aggression in female mice [23], indicating the neurotensin participates in at least one component of maternal responses in rodents. Previously, the only reports of behavioural effects of neurotensin on birds suggested a possible relationship between neurotensin expression in the medial preoptic nucleus (POM) and non-vocal courtship behaviour and agonistic behaviour in male European starlings (*Sturnus vulgaris*) [24]. Interestingly, that study also reported that male starlings that had acquired a nest box also had higher neurotensin expression in the POM relative to males that did not acquire boxes and the measures of non-vocal courtship behaviour were mostly related to nest building (e.g., such as number of times entering or landing on the nest and gathering nesting material). European starlings show biparental care and the increase in neurotensin in a hypothalamic nucleus in that study could have been related to nest-building activity, which, for many species, is a component of parental care.

Out of the hormones associated with avian parental care to date, prolactin is by far the one that has receive the most attention [3]. In precocial avian species, as is the case with Japanese quail, prolactin tends to be elevated in females during the incubation period and to decline when chicks hatch [2]. Prolactin release is known to be under the control of dopamine, which can have both inhibitory and stimulatory effects depending on the dopamine receptor subtype it acts on [3]. Neurotensin could impact parental behaviour by playing a role in controlling prolactin secretion, through its action in enhancing dopamine release [25]. Neurotensin could also affect care through its involvement in reward processing [26].

Another interesting gene that was upregulated following the sensitization procedure in both sexes was urocortin-3. In rodents, central administration or overexpression of urocortin-3 increases stress-induced anxiety and suppresses ingestive behaviour [27,28]. We are only aware of two reports relating urocortin-3 to young-directed parental care. One is the poison frog paper mentioned above [22] where urocortin-3 was upregulated in the preotic area of frogs performing parental care relative to non-parental one. The other one revealed that urocortin-3 expressing neurons in the perifornical area of the hypothalamus are activated specifically during infant-directed aggression in both male and female mice and that optogenetic activation of these neurons triggers infant-directed aggression and neglect [29]. Given that we observed a reduction in aggression towards chicks after the sensitization procedure [5], urocortin-3 may have an opposing effect on infant-directed aggressive behaviour in our system or serve different functions that support parental care, such as altered feeding patterns [30,31].

Calbindin 1 was only upregulated in the hypothalamus of sensitized males. Calbindin 1 has a sexually dimorphic distribution in certain brain regions, including the hypothalamic nuclei in mice [32,33] and in regions of the song control system and related areas in certain bird species [34–36]. In mice, calbindin 1 appears to also have sexually dimorphic effects on behaviour, including effects on fear conditioning and on social preferences in males [37]. Calbindin 1 has been found to limit dopamine release in certain brain regions, which, as mentioned previously, can affect prolactin secretion [38]. Also exclusive to sensitized males was the downregulation of BTG2. One study in mice and zebrafish found increased neural expression of this gene in males exposed to aggressive social encounters [39]. As we do observe aggression towards chicks in control males [5], differences in BTG2 may be linked to aggressive interactions in our study.

FOS, an immediate early gene downregulated in the BnST in sensitized females relative to controls, is widely used as a marker of neuronal activation of specific brain regions [40] and the difference between sensitized and control females may imply that the BnST is activated in response to chicks only in control females. Interestingly, sensitized

parental female quail separated from their chicks show increased fos immunoreactivity in this region [11], which may indicate an involvement of BnST in maternal separation. As the sensitization elicited no significant gene expression changes in the Tn in neither sex, this region is likely less critically involved during the expression of parental care in birds.

(b) What makes a non-responder?

One important aspect of our study is that rather than excluding animals that did not respond to the sensitization treatment, we used them to understand what neural differences there are in animals that fail to show parental behaviours under conditions that induce these behaviours. Female responders showed lower expression of several immediate early genes (EGR1, EGR2, FOS, ARC) in the hypothalamus, which could be indicative of lower hypothalamic activity after effective sensitization. Several of the downregulated genes in responders are also associated with the modulation of synaptic signalling and synapse reorganization (CNKSR2, NPAS4, ARC, GRIN2B, EGR2) and with nervous system development (CPNE5, MEF2D, NR4A3, EGR2, FOS, NPAS4, ARC, CDHR1, GRIN2B). It has been suggested that maternal experience can alter both the functioning and the structure of the medial preoptic area (a hypothalamic nucleus) in a way that increases neuronal responsiveness to young-related cues (reviewed in [41]). It is therefore possible that, in females that failed to show parental behaviours, the sensitization procedure was ineffective in triggering the necessary re-structuring of hypothalamic nuclei. Other brain regions studied had fewer differences between female responders and non-responders.

Contrary to results in females, in males, most differences between responders and non-responders were seen in the BnST. Of particular interest are two genes that had higher expression in responders: Anti-Müllerian Hormone (AMH) and Butyrylcholinesterase (BCHE). Increased expression of AMH was also found in the hypothalamus of male responders. While the most familiar role of AMH is in the regression of Müllerian ducts in male embryos during development, AMH is also produced in the brain [42,43].

Emerging actions of AMH are being found in the control of hypothalamic function, in particular, in increasing GnRH neuron excitability and GnRH secretion [42,43]. BChE has an interesting role in inactivating ghrelin (a hormone that affects hunger, feeding and stress), and mice with overexpression of BCHE show decreased fighting [44,45]. A reduction in aggression towards young is essential for proper parental care and BChE might facilitate this in responders. The two DEGs in the Tn (Transthyretin, TTR, and the sulfate transporter SLC13A4) showed some of the greatest fold changes observed in responders relative to non-responders. TTR expression has been shown to change in mammals in ways that depend on the manipulation and brain region. For instance, TTR expression decreases in the cortex of mice exposed to chronic immobilization stress [46] but increases in the amygdala during the formation of fear memory [47]. One interpretation is, therefore, that the presence of chicks may be perceived as a stressor by non-responders. Regarding SLC13A4, a recent study in mice showed that Slc13a4 haploinsufficiency (Slc13a4$^{+/-}$) results in atypical social behaviours, including longer time to retrieve pups and reduced nest-building activity in females, and reduced social exploration and impaired social memory in both sexes [48]. While parental behaviours were only tested in females in that study (given that male mice do not display those), a Slc13a4 increase may be required for proper parental behaviours in other species.

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

A number of females in our experiment that had no previous experiences with chicks showed parental behaviour in the absence of sensitization. It was in the hypothalamus of these females that we found the largest number of differentially expressed genes out of all comparisons performed in our study. While these control females showed some parental behaviour, the time spent engaging in those behaviours was less than those observed in sensitized animals. The changes in gene expression in parental controls may therefore represent processes associated with the *onset* of the modifications that will be needed for a more complete parental response. For example, enrichment analysis highlighted several pathways associated with synaptic function, including

cholinergic, GABAergic, dopaminergic, and glutamatergic synapse, which may again indicate the necessity for a hypothalamic re-structuring needed for parental care.

Many of the pathways detected through enrichment analysis have been linked to parental care in mammals and other species, including thyroid hormone, GnRH, oxytocin and prolactin signalling pathways [7,10,41,49]. The onset of parental care is associated with reduced reproductive behaviour, reduced young-directed aggression, and with changes in metabolism, locomotion and feeding patterns [10,49]. Pathways associated with this array of behavioural and metabolic changes were highlighted through enrichment analysis. Interestingly, recent work proposes that molecules related to feeding behaviour may also be involved in complex social behaviours, such as parental care [22,50].

Changes in immune function were also detected through enrichment analysis. In fact, one DEG, IRF5, was upregulated in parental females controls across all three brain regions analysed. IRF5 is produced in the brain by microglia (resident immune cells in the brain) and it is known for its pro-inflammatory functions [51,52]. Extensive neuroimmune changes occur in rodents postpartum (reviewed in [53]) and it is likely that some of these extend to other vertebrate species (e.g., [54]).

5. Conclusion

Recent studies have used transcriptomic approaches to compare gene expression in specific brain areas as female birds transition from territorial defence to egg incubation [55], or from egg-laying to incubation related behaviours [56]. Still, the range of neural mechanisms involved in post-hatching care in birds is virtually unknown. Our findings revealed new potential modulators of avian young-directed parental care, which deserve to be examined through further mechanistic studies and explored in other bird species and other taxonomic groups. Particularly exciting are neurotensin and urocortin-3, which potentially show conserved roles in parental care in mammals, amphibians, and birds, but are extremely under-explored in the context of parental behaviour. Natural variation

in avian parental behaviour in response to young extends from abandoning the nest to alloparenting or even adoption of non-offspring deposited by brood parasites. The pathways and genes found to be associated with spontaneous parental care, with lack of parental responsiveness, and with parental sensitization in our study serve as a foundation for future studies aimed at investigating the neural underpinnings of variation in parental care within and across species.

Ethics

Animal use and experimental design were approved by the Chapman University Institutional Animal Care and Use Committee (protocol # 2019-01).

Data accessibility

RNA-seq mapping statistics, lists of DEGs and lists of enrichment terms have been uploaded as part of the supplementary material. Sequencing datasets generated and analysed during the current study are deposited in the NCBI Gene Expression Omnibus (GEO) repository [dataset will be made publicly available once manuscript is accepted for publication].

Authors' contributions

PCL conceived the study, helped RDB carry out the animal experiments, collected the brain samples, performed the molecular work, the data analysis and wrote the manuscript. RDB carried out the animal experiments. All authors read and edited the manuscript.

Competing interests

We declare we have no competing interests.

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References

1. Cockburn A. 2006 Prevalence of different modes of parental care in birds. *Proc. R. Soc. B* **273**, 1375–1383. (doi:10.1098/rspb.2005.3458)

2. Ohkubo T. 2017 Neuroendocrine Control of Broodiness. *Adv. Exp. Med. Biol.* **1001**, 151–171. (doi:10.1007/978-981-10-3975-1_10)

3. Smiley KO. 2019 Prolactin and avian parental care: New insights and unanswered questions. *Horm. Behav.* **111**, 114–130. (doi:10.1016/j.yhbeh.2019.02.012)

4. Richard-Yris M-A. 1994 Comportement parental chez les gallinacés□: importance du facteur émotivité sur la vitesse d'émergence des réponses parentales□: apport du modèle caille japonaise. In *Comportement et adaptation des animaux domestiques aux contraintes de l'élevage□: bases techniques du bien-être animal* (eds M Picard, R Porter, & JP Signoret), pp. 61–76. Paris: INRA Editions.

5. de Bruijn R, Wright-Lichter JX, Khoshaba E, Holloway F, Lopes PC. 2020 Baseline corticosterone is associated with parental care in virgin Japanese quail (Coturnix japonica). *Horm. Behav.* **124**, 104781. (doi:10.1016/j.yhbeh.2020.104781)

6. Cheng M-F, Chaiken M, Zuo M, Miller H. 1999 Nucleus Taenia of the Amygdala of Birds: Anatomical and Functional Studies in Ring Doves (Streptopelia risoria) and European Starlings (Sturnus vulgaris). *Brain Behav. Evol.* **53**, 243–270. (doi:10.1159/000006597)

7. Dulac C, O'Connell LA, Wu Z. 2014 Neural control of maternal and paternal behaviors. *Science* **345**, 765–770. (doi:10.1126/science.1253291)

8. Nelson RJ, Trainor BC. 2007 Neural mechanisms of aggression. *Nat. Rev. Neurosci.* **8**, 536–546. (doi:10.1038/nrn2174)

9. O'Connell LA, Hofmann HA. 2011 The Vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. *J. Comp. Neurol.* **519**, 3599–3639. (doi:10.1002/cne.22735)

10. Rogers FD, Bales KL. 2019 Mothers, Fathers, and Others: Neural Substrates of Parental Care. *Trends Neurosci.* **42**, 552–562. (doi:10.1016/j.tins.2019.05.008)

11. Ruscio MG, Adkins-Regan E. 2004 Immediate early gene expression associated with induction of brooding behavior in Japanese quail. *Horm. Behav.* **46**, 19–29. (doi:10.1016/j.yhbeh.2004.02.002)

12. Baylé JD, Ramade F, Oliver J. 1974 Stereotaxic topography of the brain of the quail (Coturnix coturnix japonica). *J. Physiol. (Paris)* **68**, 219–241.

13. Kuenzel WJ, Masson M. 1988 *A Stereotaxic Atlas of the Brain of the Chick (Gallus domesticus).* Poultry Science Faculty Publications and Presentations. See https://scholarworks.uark.edu/poscpub/1.

14. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. 2019 Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotech.* **37**, 907–915. (doi:10.1038/s41587-019-0201-4)

15. Anders S, Pyl PT, Huber W. 2015 HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169. (doi:10.1093/bioinformatics/btu638)

16. Anders S, Huber W. 2010 Differential expression analysis for sequence count data. *Genome Biology* **11**, R106. (doi:10.1186/gb-2010-11-10-r106)

17. Love MI, Huber W, Anders S. 2014 Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550. (doi:10.1186/s13059-014-0550-8)

18. Wickham H. 2016 *ggplot2: Elegant Graphics for Data Analysis*. 2nd edn. Springer International Publishing. See https://www.springer.com/gp/book/9783319242750.

19. Kolde R. 2019 pheatmap: Pretty Heatmaps. R package version 1.0.12.

20. Ge SX, Jung D, Yao R. 2020 ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* **36**, 2628–2629. (doi:10.1093/bioinformatics/btz931)

21. Driessen TM, Zhao C, Whittlinger A, Williams H, Gammie SC. 2014 Endogenous CNS Expression of Neurotensin and Neurotensin Receptors Is Altered during the Postpartum Period in Outbred Mice. *PLOS ONE* **9**, e83098. (doi:10.1371/journal.pone.0083098)

22. Fischer EK, Roland AB, Moskowitz NA, Tapia EE, Summers K, Coloma LA, O'Connell LA. 2019 The neural basis of tadpole transport in poison frogs. *Proc. R. Soc. B* **286**. (doi:10.1098/rspb.2019.1084)

23. Gammie SC, D'Anna KL, Gerstein H, Stevenson SA. 2009 Neurotensin inversely modulates maternal aggression. *Neuroscience* **158**, 1215–1223. (doi:10.1016/j.neuroscience.2008.11.045)

24. Merullo DP, Cordes MA, Susan DeVries M, Stevenson SA, Riters LV. 2015 Neurotensin neural mRNA expression correlates with vocal communication and other highly-motivated social behaviors in male European starlings. *Physiol. Behav.* **151**, 155–161. (doi:10.1016/j.physbeh.2015.07.019)

25. Kasckow J, Nemeroff CB. 1991 The neurobiology of neurotensin: focus on neurotensin-dopamine interactions. *Regul. Pept.* **36**, 153–164. (doi:10.1016/0167-0115(91)90053-J)

26. Torruella-Suárez ML, McElligott ZA. 2020 Neurotensin in reward processes. *Neuropharmacol.* **167**, 108005. (doi:10.1016/j.neuropharm.2020.108005)

27. Kuperman Y, Issler O, Regev L, Musseri I, Navon I, Neufeld-Cohen A, Gil S, Chen A. 2010 Perifornical Urocortin-3 mediates the link between stress-induced anxiety and energy homeostasis. *Proc. Natl Acad. Sci. USA* **107**, 8393–8398. (doi:10.1073/pnas.1003969107)

28. Skelton KH, Owens MJ, Nemeroff CB. 2000 The neurobiology of urocortin. *Regul. Pept.* **93**, 85–92. (doi:10.1016/S0167-0115(00)00180-4)

29. Autry AE, Wu Z, Kohl J, Bambah-Mukku D, Rubinstein ND, Marin-Rodriguez B, Carta I, Sedwick V, Dulac C. 2019 Perifornical Area Urocortin-3 Neurons Promote Infant-directed Neglect and Aggression. *bioRxiv* , 697334. (doi:10.1101/697334)

30. Ogino M, Okumura A, Khan MSI, Cline MA, Tachibana T. 2014 Comparison of brain urocortin-3 and corticotrophin-releasing factor for physiological responses in chicks. *Physiol. Behav.* **125**, 57–61. (doi:10.1016/j.physbeh.2013.11.006)

31. Wang J, Gilbert ER, Cline MA. 2019 Hypothalamic mechanisms associated with neuropeptide K-induced anorexia in Japanese quail (Coturnix japonica). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **237**, 110539. (doi:10.1016/j.cbpa.2019.110539)

32. Edelmann M, Wolfe C, Scordalakes EM, Rissman EF, Tobet S. 2007 Neuronal nitric oxide synthase and calbindin delineate sex differences in the developing hypothalamus and preoptic area. *Dev. Neurobiol* **67**, 1371–1381. (doi:10.1002/dneu.20507)

33. Gilmore RF, Varnum MM, Forger NG. 2012 Effects of blocking developmental cell death on sexually dimorphic calbindin cell groups in the preoptic area and bed nucleus of the stria terminalis. *Biol. Sex Differ.* **3**, 5. (doi:10.1186/2042-6410-3-5)

34. Garcia-Calero E, Scharff C. 2013 Calbindin expression in developing striatum of zebra finches and its relation to the formation of area X. *J. Comp. Neurol.* **521**, 326–341. (doi:https://doi.org/10.1002/cne.23174)

35. Moe Y *et al.* 2016 A comparative study of sex difference in calbindin neurons among mice, musk shrews, and Japanese quails. *Neurosci. Lett.* **631**, 63–69. (doi:10.1016/j.neulet.2016.08.018)

36. Pinaud R, Fortes AF, Lovell P, Mello CV. 2006 Calbindin-positive neurons reveal a sexual dimorphism within the songbird analogue of the mammalian auditory cortex. *J. Neurol.* **66**, 182–195. (doi:https://doi.org/10.1002/neu.20211)

37. Harris EP, Abel JM, Tejada LD, Rissman EF. 2016 Calbindin Knockout Alters Sex-Specific Regulation of Behavior and Gene Expression in Amygdala and Prefrontal Cortex. *Endocrinology* **157**, 1967–1979. (doi:10.1210/en.2016-1055)

38. Brimblecombe KR, Vietti-Michelina S, Platt NJ, Kastli R, Hnieno A, Gracie CJ, Cragg SJ. 2019 Calbindin-D28K Limits Dopamine Release in Ventral but Not Dorsal Striatum by Regulating Ca2+ Availability and Dopamine Transporter Function. *ACS Chem. Neurosci.* **10**, 3419–3426. (doi:10.1021/acschemneuro.9b00325)

39. Malki K *et al.* 2016 Transcriptome analysis of genes and gene networks involved in aggressive behavior in mouse and zebrafish. *Am. J Med. Genet. B Neuropsychiatr. Genet.* **171**, 827–838. (doi:https://doi.org/10.1002/ajmg.b.32451)

40. Sheng M, Greenberg ME. 1990 The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477–485. (doi:10.1016/0896-6273(90)90106-P)

41. Michael Numan. 2020 *The Parental Brain: Mechanisms, Development, and Evolution*. Oxford, New York: Oxford University Press.

42. Barbotin A-L, Peigné M, Malone SA, Giacobini P. 2019 Emerging Roles of Anti-Müllerian Hormone in Hypothalamic-Pituitary Function. *Neuroendocrinology* **109**, 218–229. (doi:10.1159/000500689)

43. Silva MSB, Giacobini P. 2020 New insights into anti-Müllerian hormone role in the hypothalamic–pituitary–gonadal axis and neuroendocrine development. *Cell. Mol. Life Sci.* (doi:10.1007/s00018-020-03576-x)

44. Brimijoin S, Chen VP, Pang Y-P, Geng L, Gao Y. 2016 Physiological Roles for Butyrylcholinesterase: A BChE-Ghrelin Axis. *Chem. Biol. Interact.* **259**, 271–275. (doi:10.1016/j.cbi.2016.02.013)

45. Chen VP, Gao Y, Geng L, Parks RJ, Pang Y-P, Brimijoin S. 2015 Plasma butyrylcholinesterase regulates ghrelin to control aggression. *Proc. Natl Acad. Sci. USA* **112**, 2251–2256. (doi:10.1073/pnas.1421536112)

46. Joo Y *et al.* 2009 Chronic immobilization stress induces anxiety- and depression-like behaviors and decreases transthyretin in the mouse cortex. *Neurosci. Lett.* **461**, 121–125. (doi:10.1016/j.neulet.2009.06.025)

47. Stork O, Stork S, Pape H-C, Obata K. 2001 Identification of Genes Expressed in the Amygdala During the Formation of Fear Memory. *Learn. Mem.* **8**, 209–219. (doi:10.1101/lm.39401)

48. Zhang Z, Dawson PA, Piper M, Simmons DG. 2019 Postnatal N-acetylcysteine administration rescues impaired social behaviors and neurogenesis in Slc13a4 haploinsufficient mice. *EBioMedicine* **43**, 435–446. (doi:10.1016/j.ebiom.2019.03.081)

49. Stolzenberg DS, Hernandez-D'Anna KL, Bosch OJ, Lonstein JS. 2019 Maternal Behavior From a Neuroendocrine Perspective. *Oxford Research Encyclopedia of Neuroscience*. (doi:10.1093/acrefore/9780190264086.013.237)

50. Fischer EK, O'Connell LA. 2017 Modification of feeding circuits in the evolution of social behavior. *Journal of Experimental Biology* **220**, 92–102. (doi:10.1242/jeb.143859)

51. Almuttaqi H, Udalova IA. 2019 Advances and challenges in targeting IRF5, a key regulator of inflammation. *The FEBS Journal* **286**, 1624–1637. (doi:https://doi.org/10.1111/febs.14654)

52. Mamun AA *et al.* 2020 Microglial IRF5-IRF4 regulatory axis regulates neuroinflammation after cerebral ischemia and impacts stroke outcomes. *Proc. Natl Acad. Sci. USA* **117**, 1742–1752. (doi:10.1073/pnas.1914742117)

53. Haim A, Julian D, Albin-Brooks C, Brothers HM, Lenz KM, Leuner B. 2017 A survey of neuroimmune changes in pregnant and postpartum female rats. *Brain Behav. Immun.* **59**, 67–78. (doi:10.1016/j.bbi.2016.09.026)

54. Bukhari SA, Saul MC, James N, Bensky MK, Stein LR, Trapp R, Bell AM. 2019 Neurogenomic insights into paternal care and its relation to territorial aggression. *Nat. Commun.* **10**, 4437. (doi:10.1038/s41467-019-12212-7)

55. Bentz AB, Rusch DB, Buechlein A, Rosvall KA. 2019 The neurogenomic transition from territory establishment to parenting in a territorial female songbird. *BMC Genomics* **20**, 819. (doi:10.1186/s12864-019-6202-3)

56. Ye P, Li M, Liao W, Ge K, Jin S, Zhang C, Chen X, Geng Z. 2019 Hypothalamic transcriptome analysis reveals the neuroendocrine mechanisms in controlling

broodiness of Muscovy duck (Cairina moschata). *PLOS ONE* **14**, e0207050. (doi:10.1371/journal.pone.0207050)