Oxytocin variation and brain region-specific gene expression in a domesticated avian species

Yasuko Tobari1,2 | Constantina Theofanopoulou3 | Chihiro Mori4 | Yoshimi Sato1 | Momoka Marutani1 | Sayaka Fujioka1 | Norifumi Konno5 | Kenta Suzuki6 | Akari Furutani4 | Shiomi Hakataya4 | Cheng-Te Yao7 | En-Yun Yang8 | Chia-Ren Tsai8 | Pin-Chi Tang8 | Chih-Feng Chen8 | Cedric Boeckx9,10,11 | Erich D. Jarvis3,12 | Kazuo Okanoya4,13

1Department of Animal Science and Biotechnology, School of Veterinary Medicine, Azabu University, Sagamihara, Japan
2Center for Human and Animal Symbiosis Science, Azabu University, Sagamihara, Japan
3Laboratory of Neurogenetics of Language, Rockefeller University, New York, New York, USA
4Department of Life Sciences, The University of Tokyo, Meguro-ku, Japan
5Department of Biological Science, Graduate School of Science and Engineering, University of Toyama, Toyama, Japan
6Faculty of Health Sciences, Nihon Institute of Medical Science, Moroyama, Japan
7Council of Agriculture, Endemic Species Research Institute (ESRI), Chi-chi, Taiwan
8Department of Animal Science, National Chung Hsing University, Taichung, Taiwan
9Section of General Linguistics, University of Barcelona, Barcelona, Spain
10Institute for Complex Systems, Universitat de Barcelona, Barcelona, Spain
11ICREA, Barcelona, Spain
12Howard Hughes Medical Institute, Chevy Chase, Maryland, USA
13Cognition and Behavior Joint Laboratory, RIKEN Center for Brain Science (Brain Science Institute (BSI) reorganized), Saitama, Japan

Correspondence
Yasuko Tobari, Department of Animal Science and Biotechnology, Azabu University, Fuchinobe 1-17-71, Chuo-ku, Sagamihara 252-5201, Japan.
Email: tobari@azabu-u.ac.jp
Kazuo Okanoya, Department of Life Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan.
Email: okanoya@mail.ecc.u-tokyo.ac.jp

Abstract
The Bengalese finch was domesticated more than 250 years ago from the wild white-rumped munia (WRM). Similar to other domesticated species, Bengalese finches show a reduced fear response and have lower corticosterone levels, compared to WRMs. Bengalese finches and munias also have different song types. Since oxytocin (OT) has been found to be involved in stress coping and auditory processing, we tested whether the OT sequence and brain expression pattern and content differ in wild munias and domesticated Bengalese finches. We sequenced the OT from 10 wild munias and 11 Bengalese finches and identified intra-strain variability in both the untranslated and protein-coding regions of the sequence, with all the latter giving rise to synonymous mutations. Several of these changes fall in specific transcription factor-binding sites, and show either a conserved or a relaxed evolutionary trend in the avian lineage, and in vertebrates in general. Although in situ hybridization in...
INTRODUCTION

Domesticated animals have been long known to exhibit similarities, such as: loss of pigmentation; rounded faces; smaller teeth and weaker biting force; floppy ears; shorter muzzles; curly tails; smaller cranial capacity and brain size; pedomorphosis; neotenous (juvenile) behavior; reduction of sexual dimorphism (feminization); docility; and reduced aggression and glucocorticoid levels. These changes are collectively referred to as “domestication syndrome.” A theoretical analysis by Wilkins et al. proposed that these common changes may be due to reduced numbers and/or delayed migration of neural crest cells during embryogenesis, that end up forming the cells that make up the aforementioned tissues.

White-rumped munia (WRM; Lonchura striata) is a songbird species belonging to Passeriformes, which was imported and brought into captivity from China to Japan around 1762 CE. Munias were initially used to foster exotic birds, which requires calmness under captivity and acceptability of non-kin individuals or species. By artificially selecting against aggressive individuals, Bengalese finches (BF; Lonchura striata var. domestica) evolved as the domesticated strain of munias; they typically have lower levels of the stress hormone corticosterone (CORT), possibly because parenting in small cages requires less handling of the offspring. The assumption that the BF is a domesticated strain of the WRM is supported by the fact that F1 hybrids between BFs and WRMs are fertile, by the similarity of their mostly innate behaviors, including aggression, neophobia, and song learning, with the BF strain. However, the BF strain is generally smaller than its WRM counterpart. One gene that has been proposed to play a role in domestication is oxytocin (OT), which is produced mainly in the supraoptic nucleus (SO) and the paraventricular nucleus (PVN) of the hypothalamus, from where it is released in the capillaries of the posterior pituitary to then be distributed peripherally, acting as a hormone, or from axon terminals of PVN neurons that innervate many other brain regions containing the OT receptor (OTR), acting as a neurotransmitter/neuromodulator. Traditionally, OT has been studied in reproductive contexts, including uterine contractions and milk secretion, while more recently it has been shown to be involved in a wider array of functions, including social bonding and stress suppression. A potential role in domestication has been based on findings that show different brain OT expression patterns in domesticated versus wild mice and rats, strong purifying selection in the OT gene in domesticated placental mammals; single nucleotide polymorphisms in OT in domestic camels; and different urinary OT content, as well as OTR brain gene expression patterns in dogs and wolves.

Further, OT has been shown to be involved in several facets of social cognition, like social communication and social avoidance, in mammals, through innervation of PVN OT neurons in the lateral septum and the nucleus accumbens, among others. Although less extensively studied, OT’s role in social behaviors has been also identified in avian species (we use the term OT for birds, as opposed to the commonly used mesotocin, following the proposal for a universal vertebrate nomenclature for OT, its sister gene vasotocin (VT), and their receptors (OTR and VTR), based on vertebrate-wide synteny results). For example, in zebra finches OT has been shown to regulate aggression, affiliation, allopreening, flocking, and partner preference. Moreover, in a recent study, blocking the OTR in juvenile zebra finches significantly reduced behaviors associated with approach and attention during song tutoring, ultimately affecting the song type the juveniles picked to learn. Several of these behaviors, as highlighted above, have been found to differ in BFs and WRMs, including aggression, neophobia, and song learning, with the domesticated BFs learning syntactically and phonologically more complex songs than those of the WRMs.

We thus hypothesized that OT modifications may have played a role in the domestication of the BFs. To test this hypothesis, we genotyped and compared the OT nucleotide sequences from 10 wild munias and 11 Bengalese finches; we compared hypothalamic OT expression via in situ hybridization (ISH); and compared OT mRNA expression levels in the cerebrum and diencephalon via real-time quantitative PCR (qPCR). We identified variants in both strains that lie in both the untranslated and protein-coding regions of the sequence, with all the latter giving rise to synonymous mutations. Several of...
these variants are located in transcription factor binding sites (TFBS), and show either a conserved or a relaxed evolutionary trend in the avian lineage, and in vertebrates in general. Although ISH in several hypothalamic nuclei did not reveal significant differences, our qPCR analyses showed that OT mRNA expression was significantly higher in the cerebrum, while significantly lower in the diencephalon of the BFs relative to WRMs. The brain region-specific differences in the expression of OT between BFs and WRMs suggest that differences in these central neuromodulatory systems may influence the behaviors that pop up with the process of domestication.

2 | MATERIALS AND METHODS

All procedures were conducted in accordance with: the guidelines and regulations approved by the Ethical committee of Azabu University; the Guidelines for Animal Experiments of Azabu University or of the RIKEN Animal Experiments Committee; the RIKEN BSI guidelines or the guidelines approved by the Animal Experimental Committee at the University of Tokyo, Japan and were around 2 years old when captured and transported to ESRI, were brought into the aviary and were randomly assigned into cages in one room. The birds were kept for at least 1 week after they had been captured from the wild. Because capturing and transporting the birds would impose more stress on wild-caught WRMs, 1 week of accommodation under laboratory conditions was provided to minimize the stress effects. The birds were kept in group housing and on the natural photoperiod (day length was 12 h 23 min; sunrise and sunset occur at 5:24 and 18:11, respectively) and were supplied ad libitum with mixed seeds and water. They sang actively, indicating that they were not over stressed by this time. In order to reduce stimuli disparities between subjects, the day before they were decapitated, their cages were shaded with cardboard from 18:00. Adding shades (cardboard) to the birds’ cages is a common practice in birdsong experiments to ensure that subjects have been exposed to the same social stimuli before decapitation. Additionally, when WRMs and BFs are kept in the dark, they do not sing. In the period between March 20 and 22, all birds were killed by rapid decapitation between 8:00 and 9:00. They weighed from 9.3 to 12 g (mean ± SD: 10 ± 0.64 g), and observation of their large and mature testes after decapitation suggested they were sexually mature males. The brains of the birds were frozen in optimum cutting temperature and embedded in medium (Sakura Finetek) on dry ice. Harvested brains were stored at –80°C until RNA extraction for cDNA cloning. Using mist nets, wild WRMs (WRM3 and WRM4; n = 2) were captured in Huben (23°43'51"N, 120°37'58"E or 23°43'51"N, 120°37'51"E) or Yuchi village (23°53'31"N, 120°53'31"E), in Yunlin, Taiwan between March 12 and 14, 2019. After capture, they were transported to ESRI, were brought into the aviary and were randomly assigned into cages in one room. The birds were decapitated between 9:00 to 12:00 am. The brains of the birds were frozen in optimum cutting temperature, embedded in medium (Sakura Finetek), and frozen on dry ice. Harvested brains were stored at –80°C. Their whole brains were used for ISH. For one WRM (WRM15), the lateral two-thirds of the right hemisphere had already been used for another study; thus, the left hemisphere and the medial one-third of the right hemisphere were used for ISH.

2.2 | Animals used for qPCR and enzyme immunoassay

Thirteen wild male WRMs (WRM5-WRM10, WRM16-WRM22) were captured in Huben (23°43'51"N, 120°37'58"E or 23°43'51"N, 120°37'51"E) or Yuchi village (23°53'31"N, 120°53'31"E), in Yunlin, Taiwan between March 12 and 14, 2019. After capture, they were transported to ESRI, were brought into the aviary and were randomly assigned into cages in one room. The birds were kept for at least 1 week after they had been captured from the wild. Because capturing and transporting the birds would impose more stress on wild-caught WRMs, 1 week of accommodation under laboratory conditions was provided to minimize the stress effects. The birds were kept in group housing and on the natural photoperiod (day length was 12 h 23 min; sunrise and sunset occur at 5:24 and 18:11, respectively) and were supplied ad libitum with mixed seeds and water. They sang actively, indicating that they were not over stressed by this time. In order to reduce stimuli disparities between subjects, the day before they were decapitated, their cages were shaded with cardboard from 18:00. Adding shades (cardboard) to the birds’ cages is a common practice in birdsong experiments to ensure that subjects have been exposed to the same social stimuli before decapitation. Additionally, when WRMs and BFs are kept in the dark, they do not sing. In the period between March 20 and 22, all birds were killed by rapid decapitation between 8:00 and 9:00. They weighed from 9.3 to 12 g (mean ± SD: 10 ± 0.64 g), and observation of their large and mature testes after decapitation suggested they were sexually mature males. The brains of the birds were frozen in optimum cutting temperature and embedded in medium (Sakura Finetek) on dry ice. Harvested brains were stored at –80°C, shipped on dry ice to Azabu University and stored at –80°C until RNA extraction for cDNA cloning. In addition to these birds, ORF cloning was carried out using the total RNA from six WRMs (WRM5-WRM10) and six BFs (BF6-BF11) used in qPCR (Table S1). Six additional BFs (BF12-BF17) and five WRMs (WRM11-WRM15) were used for ISH. Both BFs and WRMs were born at RIKEN and had been kept together with conspecifics until decapitation. The BFs’ approximate ages were 120–240 pdh. The WRMs’ ages were over 90 pdh. Both BFs and WRMs had reached sexual maturity. All the birds were kept under a 12-h light/dark cycle (lights on at 8:00 am) and were supplied ad libitum with mixed seeds and water. The birds were caught by hand and then decapitated between 9:00 to 12:00 am. The brains of the birds were frozen in optimum cutting temperature, embedded in medium (Sakura Finetek), and frozen on dry ice. Harvested brains were stored at –80°C. Their whole brains were used for ISH. For one WRM (WRM15), the lateral two-thirds of the right hemisphere had already been used for another study; thus, the left hemisphere and the medial one-third of the right hemisphere were used for ISH.
They were kept under a 14-h light/10-h dark cycle (lights on at 8:00) and were supplied ad libitum with mixed seeds and water. They also sang actively. In order to keep the same conditions as in Taiwan, the day before they were decapitated, we covered their cages with cardboard from 18:00 onwards. They were killed by rapid decapitation between 8:00 and 9:00. Their brains were frozen on dry ice and stored at −80°C until RNA extraction. Frozen brains were dissected into the cerebrum, diencephalon, midbrain, and cerebellum immediately before RNA extraction. The cerebellum was dissected out. The diencephalon was dissected by two coronal cuts at the level of the tractus septopalliomencesencephalicus (rostral edge of the preoptic area) and the oculomotor nerves (caudal edge of hypothalamus), one parasagittal cut placed 2 mm lateral to the midline and one horizontal cut 5 mm above the floor of the brain, including the hypothalamus. The optic tectum was collected as the midbrain. The posterior telencephalon including the septum and the bed nucleus of the stria terminals (BNST), dorsal to the anterior commissure and was collected as the cerebrum.

Eight BFs were prepared for enzyme immunoassay (EIA). They were kept for 19 days at the aviary of Azabu University, Japan, after they had been purchased from a pet breeder. They were around 2 years old and sang actively. Four birds were placed in one cage, kept under a 12-h light/dark cycle (lights on at 6:00) and were supplied ad libitum with mixed seeds and water. The day before they were decapitated, their cages had been covered with shaded cloth since 18:00. One bird was omitted because of cataracts. Seven birds (BF18-BF24) were killed by rapid decapitation between 8:00 and 9:00. Their brains were dissected by two coronal cuts at the level of the tractus septopalliomencesencephalicus and the oculomotor nerves, one parasagittal cut placed 2 mm lateral to the midline and one horizontal cut 7 mm above the floor of the brain, including the hypothalamus and the BNST. Harvested brain tissue blocks were stored at −80°C until peptide extraction for EIAs.

2.3 Molecular cloning of songbird OT cDNA

Total RNA was extracted from the hypothalamus of the BFs and WRM; it was purified using the RNeasy Lipid Tissue mini kit (Qiagen, Hilden, Germany). The unknown cDNA sequences, including 3′- and 5′-untranslated regions (UTRs) of these two songbirds’ OT genes, were analyzed by PCR amplification of 3′- and 5′-RACE fragments. The first-strand cDNA for 3′-RACE was prepared by incubation of total RNA with adaptor-oligo(dT)18 primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) at 50°C for 5 min. The reaction was performed in a 25-μl mixture containing 0.3 μg cDNA, 0.2 mM dNTPs, 0.4 μM each forward and reverse primers, and 2.5 U Ex Taq polymerase with its buffer (TaKaRa, Shiga, Japan). The template cDNA was reverse transcribed with 5′-RACE RT primer and SuperScript III reverse transcriptase (Invitrogen), followed by poly(A) tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics, Rotkreuz, Switzerland). PCR amplification of 5′-RACE fragments for OT consisted of initial denaturation at 98°C for 5 min; followed by 30 cycles of 30 s at 98°C, 30 s at 55°C, and 1 min at 72°C; and a final elongation at 72°C for 5 min. The reaction was performed in a 25-μl mixture comprising 150 ng cDNA, 0.4 mM dNTPs, 0.4 μM each forward and reverse primers, and 2.5 U Ex Taq polymerase with its buffer.

To confirm the ORF sequence, the PCR reaction was performed in a 25-μl mixture containing 0.4 mM dNTPs, 0.4 μM each forward and reverse primers (Figure S1), and 2.5 U Ex Taq polymerase with its buffer, as well as 250 ng cDNA that had been reverse transcribed with SuperScript IV Vilo Master Mix (Invitrogen). PCR reaction conditions were as follows: 98°C for 1 min; 30 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 5 min.

PCR products were subcloned into pGEM-T easy vectors (Promega, Madison, WI). The resultant plasmids were sequenced commercially (Fasmac, Atsugi, Japan). The ORF sequences obtained were submitted to DDBJ/EMBL/GenBank with accession numbers LC489419 and LC489420. We additionally used the genomic sequences of the OT we found in the publicly available scaffold-level and chromosome-level BF genome assemblies (LonStrDom1; RefSeq assembly accession: GCF_002197715.1; LonStrDom2, GCF_005870125.1; sequences shared in our Github).

2.4 Database analysis

A putative signal peptide was predicted by using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The chromosomal location and strand orientation of the identified BF genes were determined using the Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/). The genomic structure of the BF OT was predicted using the BF genome (LonStrDom2). Genomic regions surrounding OT were compared among human, rat, and BF using the Genome Data Viewer to analyze the synteny relationship.

For the TFBS analysis, zPicture alignments can be automatically submitted to rVista 2.0 to identify conserved TFBS. rVista excludes up to 95% false-positive TFBS predictions, while maintaining a high search sensitivity. In this analysis, we used the OT nucleotide sequences of BF1 and WRM1 (Table S3), as well as the zebra finch (bTaeGut1.4.pri) and chicken OT sequences (GRCg7b) from their publicly available genomes, generated by the Vertebrate Genomes Project.37

To test for possible functional effects of the SNPs we identified, we used the Variant effect predictor tool, available in Ensembl
(v. 100), for the respective sites in both the BF (LonStrDom1) and the chicken genomes (GRCh6a). Further, we used PhylO (phyloP77way), available in the UCSC Browser, that scores the measure of evolutionary conservation at individual sites, by aligning 77 vertebrate species’ genomes. The scores are compared to the evolution that is expected under neutral drift, so that positive scores measure conservation, which is slower evolution than expected, while negative scores measure acceleration, which is faster evolution than expected.38 Using this measure, we can infer whether some elements are functional, based on the rationale used widely in comparative genomics that functional genomic elements evolve more slowly than neutral sequences.39

We aligned our genotyped OT nucleotide sequences from 11 BFs and 10 WRMs using CLUSTAL W (1.81), and visualized via JalView (2.11.1.0) (alignment file in our Github; variant calling results in Table S4). We also aligned the publicly available BF OT sequence (BF 12; LonStrDom2; accession GCF_005870125.1) with the variant sites we identified. We also ran a multialignment, using the same tools, with the OT sequence of the following 29 avian species: BF, Zebra finch, Gouldian finch, Small tree finch, Medium ground-finch, Dark-eyed junco, White-throated sparrow, Blue tit, Rufous-capped babbler, Silver-eye, Flycatcher, Blue-crowned manakin, Eurasian sparrowhawk, Ruff, Spoon-billed sandpiper, Pink-footed goose, Swan goose, Golden pheasant, Ring-necked pheasant, Chicken, Indian peafowl, Turkey, Japanese quail, Helmeted guineafowl, Great spotted kiwi, Little spotted kiwi, Okarito brown kiwi, African ostrich, and Chilean tinamou (for the IDs of the genomes used, Gene-IDs and locations used for the alignment: Table S5; alignment files can be found in Github). We translated all the sequences to their respective protein sequence to decipher if the variation we identified in the exons gives rise to synonymous or non-synonymous mutations using TranslatorX (protein sequences and alignments can be found in Github).40

Further, a Maximum Likelihood phylogetic amino acid tree was constructed for OT in all the avian species available in Ensembl (v.100), using TreeFAM and TreeBeST5 pipeline in the Ensembl “Gene tree” tool package (https://www.ensembl.org/info/genome/compara/homology_method.html).

To test for intraspecies variation of these sites in other avian species, we used the dbSNP (release 150) available for chicken (remapped to GRCh6a), dbSNP (release 139) available for turkey (Turkey_2.01), and dbSNP (release 148) available for zebra finch (bTaeGut1_v1.0).

2.5 | In situ hybridization

We designed antisense probes to bind to different variants of the OT mRNA. Antisense probes for the OT mRNA were synthesized from the BF OT cDNA (Figure S1 and Table S2). Corresponding sense probes were also synthesized for control ISH (Figure S2). BF OT cDNA fragments were inserted into the pGEM-T easy vectors (Promega). The plasmids were digested with restriction enzymes (NcoI or Spel) to release the fragment; probes were synthesized using SP6 or T7 RNA polymerase (Roche Diagnostics) with digoxigenin-labeling mix (Roche Diagnostics).

Frozen brains were cut in 20-μm coronal sections on a cryostat (Leica Microsystems, Wetzlar, Germany). Every 13th section through the hypothalamus from each animal was mounted on 3-aminopropyltriethoxysilane-coated slides and stored at −80°C until use. ISH was performed as described previously,41 except that sections were postfixed in 4% paraformaldehyde for 10 min, proteinase K treatment was omitted and color development of alkaline phosphatase activity was carried out for 1 h with nitro blue tetrazolium chloride (Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) in detection buffer. Images of sections were captured with a LeicaMC170 HD camera attached to a Leica DM5000 microscope (Leica Microsystems).

2.6 | Analysis of OT mRNA expressing cells

The “analyze particle” module of ImageJ (based on size = 50-infinity pixel and threshold = 0–150) was used to count the numbers of OT mRNA-expressing cells in the following regions of interest: the lateral hypothalamus (LHy) on the left side of the brain; the external subgroups of the supraoptic nucleus (SOe) on the left side of the brain; and PVN at the level of the anterior commissure on the both sides (Table S6). This limitation was due to the absence of the lateral two-thirds of the right brain hemisphere of a WRM. The sum of cell numbers in each area was calculated.

2.7 | Statistical analyses for OT mRNA expressing cells

Results are shown as means ± standard errors of the mean and coefficients of variation (Table S6). Statistical analyses were conducted with Prism 4.0 (GraphPad Software, USA), using unpaired Student’s t-tests. P values <0.05 were considered statistically significant.

2.8 | Real-time qPCR

We isolated RNA from four different brain regions: cerebrum, diencephalon, midbrain, and cerebellum. Total RNA was extracted using QIAzol Lysis Reagent and column purified (RNeasy Lipid Tissue Mini Kit; Qiagen). We performed reverse transcription using Superscript First-Strand Synthesis (Invitrogen) with oligo (dT) primer. Primers for OT were designed using sequences downloaded from GenBank or Ensembl (Table S7), and for the OTR were designed based on previous studies.32,42 The control gene was peptidylprolyl isomerase A (PPIA), which has been evaluated in two songbird species (zebra finch and white-throated sparrow) as highly stable in the brain.44 qPCR was performed using Roche LightCycler 96 System with TB Green Premix Ex Taq II (Takara), in triplicate for each sample on 96-well plate. We calculated crossing point values (CT) using the Abs Quant/2nd Derivative Max method using LightCycler 96 SW 1.1 software. CT was used to calculate ΔΔCT (ΔCT target gene−ΔCTcontrol gene) − [CT target gene−CTcontrol gene].
gene] calibrator). We picked the average CT of BF samples as a calibrator. Relative expression levels between the species were calculated using the 2−ΔΔCT method45 (Tables S8 and S9) Using the value of the 2−ΔΔCT, we also analyzed our qPCR results in comparison with each specific variant we identified in our multialignments (Table S10).

2.9 Production of antiserum to the avian OT

The antiserum was raised in a rabbit immunized five times with synthetic polypeptide of the avian OT sequence (CYIQNCPIG-amide) as antigen every 1 week. The specificity of the serum was tested by a dot immuno-blot assay, with the OT and its ortholog and paralog (vasotocin) in other species (OT, isotocin, vasotocin, vasopressin), where we confirmed it binds solely to avian and fish OT (also called, isotocin). Aliquots of the orthologous (oxytocin, mesotocin, isotocin) and paralogous (vasotocin and vasopressin) peptides were spotted onto a 0.2-μm polyvinylidene fluoride membrane (Immun-Blot PVDF Membrane for Protein Blotting, BIO-RAD). The membrane was air dried at room temperature and was washed for 10 min in 0.05 M Tris buffer (pH 7.6) with 0.1% Tween 20 and 0.15 M NaCl (TBS-T) and incubated for 60 min in blocking solution containing 5% skim milk in TBS-T. After blockage, the membrane was exposed for 120 min to oxytocin antiserum (1:1000 dilution in blocking buffer). After the primary immunoreaction, the membrane was further incubated with anti-rabbit biotinylated IgG secondary antibody (Agilent Technologies, Inc., Santa Clara, CA; 1:200 dilution in blocking buffer) for 120 min and avidin-biotin complex reagent (Vector Laboratories, Inc., Burlingame, CA) for 120 min, and subsequently with ImmunPACT DAB peroxidase substrate solution (Vector Laboratories) for 5 min at room temperature.

2.10 OT EIA

The frozen brain tissue blocks of WRM (WRM16-22; n = 7) were dissected as described above immediately before peptide extraction. The frozen brain tissue blocks of WRM and BF were boiled for 8 min and homogenized in 5% acetic acid using a TissueLyser LT (Qiagen) for 6 min at 50 Hz. The homogenate was centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was collected and forced through a disposable C-18 cartridge (Sep-Pak Vac 1 cc; Waters, Milford, MA). The retained material was then eluted with 60% methanol. The pooled eluate was concentrated in a vacuum evaporator, passed through disposable Ultrafree-MC centrifugal filter units (Millipore, Billerica, MA) and dried. The dried material was reconstituted in 220 μL Dilution buffer, and 100 μL of the sample was used for EIA at duplicate.

The samples were subjected to competitive EIA by using the antiserum described above. In brief, different concentrations of OT (0.01–100 pmol) and adjusted tissue and plasma extracts were added with the antiserum against OT (1:1000 dilution) to each antigen-coated well of a 96-well microplate (F96 maxisorp nunc-immuno plate; Thermo Fisher Scientific, Roskilde, Denmark) and incubated overnight at 4°C. After the reaction with alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma; 1:1000 dilution in dilution buffer), immunoreactive products were obtained in a substrate solution of p-nitrophenyl phosphate (SIGMA FAST™ p-nitrophenyl phosphate tablet set) for 90 min, then, 20 μL 5 N NaOH solution was added to and the absorbance was read at 405 nm with a reference filter of 620 nm by iMark Microplate Reader S/N 20255 (Bio-Rad, USA; Table S11).

2.11 Statistical analyses for qPCR and EIA

For analysis of the qPCR results, we performed Steel-Dwass test (nonparametric) for multiple comparison using statistical software R (http://www.r-project.org/; R Core Team, 2013; R Foundation for Statistical Computing) to investigate the difference of relative gene expression levels between BF and WRM. And we performed unpaired Student’s t-tests using Prism 4.0 (GraphPad Software) to examine the effect of the single nucleotide variants we found on OT mRNA expression in the brain of BFs and WRMs. For analysis of the EIA results, statistical analyses were conducted with Prism 4.0 (GraphPad Software), using F-tests, and unpaired Student’s t-tests with Welch’s correction. P values <0.05 were considered statistically significant.

3 RESULTS

3.1 Identification of OT cDNA in Bengalese finches and WRMs

We isolated OT transcripts from diencephalic tissues of 11 BFs and 10 WRMs (Figure 1A; Table S3). To confirm that our identified sequence is the BF OT-ortholog, we BLAST searched the full-length cDNA sequence against a reference BF genome assembly (lonStrDom2; accession GCF_005870125.1).47 Only one locus (BLAST hit) in the genome showed high similarity (E-value <5e−56) that was unnamed (ID: LOC110473283), located on chromosome 4 (68,957,139–68,958,557).

3.2 Synteny and phylogenetic analyses confirm our identified gene in BFs as the OT-ortholog

To further confirm our identified gene in the BF was the true OT-ortholog, we ran synteny analysis on the surrounding territory of the identified gene in BF (LOC110473283). The analysis revealed that this gene is located in a genomic region that is highly conserved in tetrapod vertebrates, exactly where the OT is found in those species (Figure 1B). Conserved syntenic genes in the surrounding territory include mitochondrial antiviral signaling protein (MAVS), protein tyrosine phosphatase receptor type A (PTPRA), mitochondrial ribosomal Protein S26 (MRP26), arginine vasopressin/vasotocin (AVP/VT), FAST kinase domains 5 (FASTKD5), U-box domain containing 5 (UBOX5), and leucine zipper tumor suppressor family member 3 (LZTS3; Figure 1B). Interestingly, in the BF genome (lonStrDom2), AVP/VT was
FIGURE 1  Nucleotide and amino acid sequences of white-rumped munia and Bengalese finch oxytocin (OT) precursor cDNA. (A) Alignment of WRM and BF OT nucleotide sequences. Color-marked nucleotides: sites that show variation in either the BFs or the WRMs sequenced in this study; alternative nucleotides are shown marked up with the same color in close proximity. Predicted protein sequence is shown below the nucleotide sequence alignment in bold. Sequences of putative TFBS in the territory of the variant sites have been underlined with different colors; each color corresponds to the color of the respective transcription factor. ETF: TEA domain family member 2; HIC1: HIC ZBTB transcriptional repressor 1; NERP: Ets-related factor; AP2: activating enhancer binding protein 2; AP4: activating enhancer binding protein 4; AP2GAMMA: activating enhancer binding protein 2 gamma; SP3: Sp3 transcription factor; PAX6: Paired Box 6; CHX10: C. elegans ceh-10 homeodomain-containing homolog; LHX3: LIM homeobox 3. (B) Conserved gene synteny among the human, rat, and Bengalese finch OT loci. Genes are indicated by shaded boxes. OT genes are linked by dotted lines. In rodent genomes, the gonadotropin-releasing hormone 2 gene (GnRH2) is inactivated or deleted.46 (C) Schematic representation of the Bengalese finch OT structure. Exons are boxed and numbered, and introns appear as straight black lines. Shaded and open boxes denote coding and noncoding sequences, respectively. BF, Bengalese finch; WRM, white-rumped munia
also unnamed (Gene ID: LOC110473284). Based on our synteny analysis, and on further synteny and phylogenetic results presented in Theofanopoulou et al.,

\[48\] we propose that LOC110473283 should be named oxytocin (\(\text{OT}\)) and LOC110473284 should be named vasotocin (\(\text{VT}\)) in the BF. Our phylogenetic tree using the BF putative \(\text{OT}\) in LonStrDom1 as a query sequence (ENSLSDG00000001590; unnamed) grouped this sequence with the \(\text{OT}\)-ortholog in all vertebrates (Figure S3 See Gene Tree ID: ENSGT00390000004511 for a capture of the tree only in avian species). These phylogenetic results corroborate with our synteny results in that the gene we identified in the BF is the true \(\text{OT}\)-ortholog.

Using the \(\text{OT}\) sequence from LonStrDom2 (chr4: 68,957,139–68,958,557) and our identified full-length cDNA sequence of BF \(\text{OT}\) (Figure 1A; Figure S1), we further predicted the \(\text{OT}\) genomic structure (Figure 1C). The BF \(\text{OT}\) consists of three exons, spanning 1419 base pairs. exon 1 contains the 5’-UTR, signal peptide, \(\text{OT}\), nonapeptide (CYIQNCPIG), and a portion of the neurophysin peptide sequence. Exon 2 and the first 48 base pairs of exon 3 contain the remaining sequence of the neurophysin peptide; the remainder of exon 3 contains the 3’-UTR (Figure 1C).

3.3 Variant calling in the BF and WRM OT cDNA and comparison with 28 avian species

We aligned our genotyped \(\text{OT}\) sequences from 11 BFs and 10 WRMs using CLUSTAL W (1.81; Figure 1A; alignment file in our Github; variant calling results in Table S4). We also aligned the publicly available BF \(\text{OT}\) sequence (BF12; LonStrDom2; accession GCF_005870125.1) with the variant sites we identified in our multi-alignment. In Figure 1A, for an easier visualization of our results, we show the alignment of two sequences that represent for each strain (WRM and BF) the alleles found in most individuals, and we note the alternative alleles next to the variant sites. We translated all the sequences to their respective protein sequence to decipher if the variation we identified in the exons gives rise to synonymous or non-synonymous mutations. Lastly, to determine if the changes we observed were specific to these strains or shared with other avian species, we aligned their sequences with \(\text{OT}\) sequences from 28 avian species (Figure 2; Figure S3 and Table S5; alignment files in Github).

Several of the \(\text{OT}\) sequences we identified in WRMs and BFs were identical in individuals within and across strains (WRM2, WRM4, WRM6, BF6, BF9, BF10, BF11; Table S4). Variation was found in positions 1–2 (5’-UTR) of the alignment, with only 1 WRM (WRM1; 10% of the WRM sample) having CG, and the rest of the WRMs (90%) showing a deletion on this site; CG was present in five individuals (38% of the BF sample), and the rest of the BFs (62%) also had this site deleted. Interestingly, CG appeared to be the ancestral state of this site (Figure 2), with no other bird showing a similar deletion in our alignment, although the first site (C present in 66% of the birds; Figure 2) was more variable than the second (G present at a 97%; Figure 2). In positions 6–8 (5’-UTR), all WRMs, except WRM1, had GCC, while all three nucleotides were deleted in WRM1; this deletion was WRM-specific, with the BFs showing only variation on the third
site in position 8, which was C/T, with C present in the 62% of our BF sample (Figure 2). GCC was the status shared by most avian species (93%, 100%, and 76%, for each site, respectively; Figure 2). To sum up, both these 5’-UTR sites were variant in both WRMs and BFs.

We identified three exonic sites (positions 62, 101, and 149) that were variant in BFs but invariant in WRMs. On the 62nd locus of the alignment (Figure 1A), all WRMs and BFs had C, except the BF4 that had T (Table S4); this site is variable in the rest of the avian species, with 55% of them having C, and the rest A, T, or G. A similar status was identified in the 101st position, where all WRMs and BFs had C, except two BFs (BF4 and BF12; Table S4) that had T, while this site was found variable in other birds (C in the 52%; Figure 2). In position 149, all WRMs and BFs had C, except for BF2 and BF7 that had G on that site, which represents the plausible ancestral state, since 93% of the avian species had G in our alignment, and C was found to be specific to the WRMs and BFs. We lastly found one exonic site (position 239) that was, in turn, variant in WRMs (G/A; G present in 80% of the WRM sample; Table S4) and invariant in BFs (G), with the majority of the avian species (83%) sharing the same allele (G; Figure 2). All of the exonic mutations identified give rise to synonymous mutations.

We found three sites in the 3’-UTR (positions 389, 400, and 468) where WRMs were variant and BFs invariant, and one site (position 432) that was, instead, variant in BFs and invariant in WRMs (Figure 2; Table S4). In position 389, only one individual (WRM 9) had A (10%), while the rest had G, as was the case for the vast majority of the avian species (97%). WRMs also showed variability (position 400: G/A; G: 80%; A: 20%) on a less conserved site across birds, where 66% of them had G. The WRM-variant site in position 468 (G/C; G: 60%; C: 40%) was, in turn, robustly conserved in the rest of the avian species (G: 93%; Figure 2). The 3’-UTR variant site in BFs (position 432: C/T; C: 62%; T: 38%) is variable across birds, with only 41% having C, and only three species, other than the BF, having T on that site.

Among the avian species we used in our alignment, some of them come from domesticated species (chicken, zebra finch, turkey, Japanese quail, and swan goose), but we were not able to find any strong pattern of convergent evolution among them. For example, although all the domesticated species in our multialignment, including the BF, have G on position 239, this G is present in 83% of the avian species, thus it cannot be considered evidence of convergent evolution.

### 3.4 Conserved/accelerated status of the variant sites in vertebrates

Further, we used PhyloP (phyloP77way) to measure the evolutionary conservation at the variant sites we identified, by aligning 77 vertebrate species’ genomes. The sites in positions 1–2, 6–8, 62, 101, 149, and 239 gave positive scores (Suppl. File S1), which measure conservation, namely slower evolution than expected: the sites in positions 389, 400, 432, and 468 gave negative scores, which measure acceleration, namely faster evolution than expected. Using this measure, we can infer whether some elements are functional, based on the rationale used widely in comparative genomics that functional genomic elements evolve more slowly than neutral sequences.

Interestingly, the conservation status of some of these sites is different on a vertebrate-wide scale than when zoomed in on an avian-specific scale. For example, the sites on positions 62 and 101 are conserved across vertebrates (Suppl. File S1), but appear to be variable in our avian multialignment (Figure 2). These same sites are variant only in BFs and not in WRMs, along with the sites on positions 8, 149, and 432, which are, in turn, conserved in both a vertebrate-wide and an avian-specific scale. These data suggest that these sites, where we found variation in BFs only, are likely to be functional and are bound to shed light on their domestication process. Especially, T in position 8, present in 38% of the BFs (Table S4) is conserved across vertebrates, and at the same time is a rare allele in our avian multialignment (present in 17% of the species; Figure 2); the same holds for T in position 432, which is also present in 38% of the BFs, is conserved in vertebrates, but it is found rarely in birds (10%).

### 3.5 Transcription factor-binding, functional, and variation analyses

Our analysis using the zPicture and rVista softwares revealed that several of the variant sites are located in putative TFBS (Figure 1A). The site on position 62 is located close to the end of a putative binding site of the ETF (TEA domain family member 2) transcription factor, while the variant in position 101 is found in the territory of both the binding sites of the HIC1 (HIC ZBTB transcriptional repressor 1) and E2(F) transcription factors. We found both of the latter TFBS being conserved in the zebra finch. The site in position 239 was found to fall within the NERF (EtS-related factor) binding site, which was also conserved in the chicken. The two last 3’-UTR variants are located within the binding sites of several transcription factors. The site in position 432 is found in the binding sites of the following transcription factors: AP2 (activating enhancer binding protein 2), AP4 (activating enhancer binding protein 4), AP2GAMMA (activating enhancer binding protein 2 gamma), and SP3 (Sp3 transcription factor); the SP3 TFBS was also conserved in the zebra finch. Lastly, the site in position 468 is located in the PAX6 (Paired Box 6), LHX3 (LIM homeobox 3), and CHX10 (C. elegans ceh-10 homeo domain-containing homolog) TFBS, with the latter being conserved in the zebra finch.

We then used variant effect predictor tests Ensembl (v. 100) to further predict the functionality of our identified single nucleotide polymorphisms (SNPs) in the BFs and the WRMs, applied to the respective sites in both the BF (LonStrDom1) and the chicken genomes (GRCg6a), but the tests did not yield any known functional effect. We also searched for possible intraspecies variation of these sites in other avian species, using the dbSNP (release 150) available for the chicken (GRCg6a), dbSNP (release 139) available for the turkey (Turkey_2.01), and dbSNP (release 148) available for the zebra finch (bTaeGut1_v1.p), but we did not find any variation in the sites studied within any of these species.
3.6 Changes in OT mRNA expression in the cerebrum and diencephalon of BFs relative to WRMs

Differential OT gene expression in several diencephalic nuclei has been associated with social behavioral differences in several species. Since OT is expressed primarily in the hypothalamus, we performed ISH for OT mRNA in BF and WRM brain sections containing several hypothalamic nuclei. BFs and WRMs had similar distributions of OT mRNA-expressing cells in the hypothalamus (Figure 3; Figure S2 for ISH with a sense and antisense OT probe), and we did not detect any significant differences in the numbers of cells expressing OT mRNA (Figure 4A–C; Table S6) in any of the nuclei tested between the two strains by ISH. These nuclei included the SOe, LHy, and PVN (Figure 3A–F). We found that the PVN contained a higher average number of OT cells in both strains (48.0 in BF; 46.7 in the WRM), compared to the SOe (7.4 in BF; 11.3 in the WRM) and the LHy (7.0 in BF; 7.6 in the WRM; Table S6).

To compare mRNA expression levels, we performed qPCR to measure relative OT mRNA expression in the BF and WRM diencephalon (which contains the hypothalamus) and cerebrum. We found that OT mRNA expression in the diencephalon and cerebrum of the BFs was significantly different to that of the WRMs (Steel-Dwass test, diencephalon: \( p = 0.016 \), cerebrum: \( p = 0.029 \), Figure 5; Tables S7 and S8). Specifically, we observed that OT mRNA expression was significantly higher in the cerebrum, but significantly lower in the diencephalon of the BFs relative to WRMs. We also conducted qPCR to quantify the levels of OTR mRNA in the cerebrum, diencephalon, midbrain and cerebellum, but we did not detect any significant differences (Steel-Dwass test, \( p > 0.05 \), Table S9; Figure 6).

We then examined if there was any effect of the single nucleotide variants we found on OT mRNA expression in the brain of BFs and WRMs, but we were not able to find any, likely due to the low number of individuals showing the alternative (i.e., less common) variants. For
example, Figure S4 shows the relative expression of OT mRNA in the cerebrum and the diencephalon relative to C or G at the 468th position, with no significant differences between the groups.

We, lastly, generated a specific antiserum against the avian OT to quantify OT mature peptide levels in both strains. Our OT antiserum bound to the avian and fish OT peptide (Figure 7A), but did not cross-react with either the mammalian OT or with VT of any species. We then compared brain OT peptide levels using EIA (Figure 7B; Table S11) in both strains. Although we did not detect significant differences between the groups, we found a significantly higher intra-strain variability in the WRM compared to the BFs ($F = 293.1$, $Dfn = 6$, $Dfd = 6$, $p < 0.0001$). However, consistently with our qPCR results of the diencephalon, OT peptide content levels in the brains of BFs were more clustered at low levels than those of WRM.

4 | DISCUSSION

In this article, we sought to identify whether differences in the sequence and brain expression of OT in the wild WRM and the domesticated BFs would reveal insights in the neuroendocrinological changes that underlie the domestication process of the latter. In this direction, we found sites that are variant in BFs (in positions 8, 62, 149, and 432), and not in the WRM, all of which were shown to be conserved on a vertebrate-wide scale, hence likely functional. Of these, the BF T alleles in positions 8 and 432 were found to be rare in the avian lineage in particular, something that points further to their plausible functional role. Importantly, two of the BF variant sites (positions 62 and 432) are also located within specific TFBS, one of which (SP3) was also conserved in the zebra finch. Although the rest of these TFBS were not conserved in either the chicken or the zebra finch, which would traditionally be viewed as a weaker predictive evidence, an array of recent studies have shown that most TFBS are species-specific, and aligned binding events present in many species are in fact rare.51,52

Since it is known that heterozygous variants can have different impacts on genes and traits than their homozygous counterparts, we suggest that these variants could have a synergistic heterozygous impact on the evolution of domestication in BFs. However, we believe that it may not be a specific allele that is important for specific domesticated traits, but the combination of heterozygous alleles among the BF-population that may influence the interactions differently than a population that were purely homozygous. We also suggest that although this variation can be suspected to come from laboratory population bottlenecks, in fact, we used sequences by individuals coming from different colonies (RIKEN, Japan; and UCSF, USA). Thus, the variation we identified does not seem to be due to laboratory population bottlenecks.

The absence of nucleotide change convergence in BFs and other domesticated avian species should not come by surprise as domestication processes have been shown to not obligatorily work through selection on the same site, but also through selection on the same gene or biological process.53 Nonetheless, we do not exclude the possibility that convergent evolution on different nucleotides in the same gene can occur.

Our ISH findings largely agree with previously identified OT distributions in other avian species (including in chicken,50 zebra finch,36,54 Japanese quail,54,55 domestic mallard,54,55 and starling54,55), in that most of the OT-containing cells we identified were in the PVN.
compared to the other nuclei we tested (SOe, LHy) and the rest of the
brain. Vicario et al.26 found age-dependent downregulation of OT in
the PVN of zebra finches, indicating that expression levels can change
over time within a species. Lastly, it has been shown in chicken50 that
some OT cells in the PVN could also express VT, but in the domestic
duck and the Japanese quail,15 OT neurons are separate from VT neu-
rons in the PVN. Future investigations in the WRMs and BFs will
determine if there is or is not any overlap in the two sister genes.

The brain region-specific differences in the amount of OT mRNA
in the cerebrum and diencephalon of the BFs compared to the WRMs
is intriguing in that it implies that the role of OT in the evolutionary
process of domestication might be more complex than thought.
Although our findings of less OT synthesis in the domesticated BF
diencephalon go in the opposite direction from those reported for
brain OT synthesis in rats and mice (laboratory domestication),16
where they found more OT production for the domesticated strain, as
well from other relevant findings in mammals (e.g., higher urinary OT
levels in dogs vs. wolves19), in fact a closer look at the literature
reveals that a similar brain-region specific pattern has been observed
in the case of rat and mouse domestication.16 In particular, domestici-
mated mice had a significantly higher density of OT-immunoreactive
cells in the anterior hypothalamus (AHy) and medial preoptic area
(MPOA), but a lower density in the lateral hypothalamus (LHy) and
PVN (although not significant). Similarly, domesticated rats showed a
significantly higher density of OT cells in the AHy and the MPOA; a
higher density in the LHy and a lower in the PVN (both were not
significant).

Putting these findings together, we suggest that both higher and
lower OT can be associated with domestication in different species or
lineages, and that the OT brain expression pattern can be region-spe-
cific. Although this region specificity was also found in the case of lab-
atory reared mice and rats,16 our study is the first, to our
knowledge, to observe significantly lower OT in the diencephalon of
a domesticated species (i.e., the BF). This finding can shed light on
differences in the underlying neurobiology between different paths to
domestication (rats and mice vs. BFs). Namely, both rats and mice
underwent a process of human acclimation as companion animals dur-
ing the 17th and 18th century, before they were used as laboratory
animals,56,57 while the BFs did not undergo such an active process of
human acclimation through the process of their domestication.

Concerning the mechanisms through which OT could be acting
differently in the brain circuits of BFs and WRMs, one hypothesis is
that OT from the hypothalamus would impinge differentially on neu-
rons containing OTR in brain regions involved in social cognition and
aggression regulation (e.g., striatum and lateral septum29). Such
changes could account for the decreased aggression and fear seen in
the BFs, compared to the WRMs.1,2 Another possible brain mecha-
nism could be more indirect, through the action of OT to the HPA axis.
OT has been shown to inhibit the general reactivity of the HPA axis,58
and to attenuate ACTH secretion and CORT levels.15 More mechanis-
tically, OT has been found to inhibit synaptic glutamate transmission
onto CRH neurons, suppressing CRH neuron excitability, and stress
axis activity.59 This mechanism could explain the lower CORT levels
seen in the BFs,3 along with the behavioral outcomes that this reduc-
tion implies.

A third possible mechanism, which also takes into account the
reduced OT diencephalic expression in BFs, comes from studies in
other avian species: in male violet-eared waxbills (Uraeginthus
granatina), robust transcriptional activation of OT neurons in the PVN
is observed following pursuits by a human hand.60 OT neurons in the
PVN also promote a passive stress-coping behavior in male zebra
finches.61 If the same mechanism applies in WRM, higher levels of OT
in the PVN can explain why WRMs show a longer tonic immobility
response following rapid inversion and restraint, compared to BFs.21
The tonic immobility response is an innate fear and defensive
response associated with intensely dangerous situations.62 This
response is characterized by a temporary state of profound inactivity
and relative lack of responsiveness to external stimuli.63 The high level
of OT may be needed to induce a longer tonic immobility reaction that
reduces the threat of a potential predator and increases the chances of
survival of WRMs during a predatory attack in the wild. BFs may
no longer require high OT levels in the PVN to cope with predation
because they have adapted to low predation pressures by domestica-
tion. Future experiments of OT manipulations in the PVN would help
test this hypothesis.

In the cerebrum, the OT mRNA expression was significantly
higher in the BFs than the WRMs. This difference might explain some
of the behavioral observations in BFs and WRMs. In a behavioral test-
ing, we showed that aggressive biting was higher in WRMs than in
BFs.1 In another behavioral testing, we showed that neophobic ten-
dency was higher in WRMs than in BFs.64 Oxytocinergic cells in the
avian BSTM are also thought to play a very important role in several
aspects of social behavior, including aggression and gregarious-
ness.28,29,65 OT mRNA expression in the cerebrum includes the
expression of OT mRNA in the medial portion of BSTN (BSTM). The
higher expression of OT mRNA in BFs might be related with lower
aggression as it is known that in the lactating female rats, injection of
OT in the BSTN decreased aggressive behavior.66 Similarly, cerebral
administration of OT reduced neophobia in virgin female rats and
induce parental behavior to alien pups.57 These behavioral modifica-
tions observed in rats are in parallel with our finding of higher mRNA
level in the cerebrum of BFs and lower aggression and lower
neophobia in BFs.

In contrast to OT expression levels, we did not detect any signifi-
cant differences in the expression of OTR mRNA in the cerebrum,
diencephalon, midbrain, and cerebellum. This suggests that down-
regulation of OT in BFs does not affect its receptor expression. OTR
distributions in the telencephalon including the septum correlates
with species-typical group size (gregarious or territorial) in estrildid
finch species.28 WRMs in Taiwan move and forage in flocks of around
4–20 birds.68 Group size of BFs is difficult to define, but in our labora-
tories at the University of Tokyo and RIKEN, birds are kept in cages of
similar flock sizes, and they are socially gregarious. It is possible then
that the similar OTR distributions in both strains correlate with similar
group sizes, as in other finch species, although we cannot rule out the
possibility that strain differences in OTR expression could be detected
using other experimental designs (e.g., larger sample sizes, conditions of breeding and sample collection, or quantifying mRNA level in specific brain regions by ISH or captured by laser microdissection). Other brain regions could include the song learning nuclei. OTR expression has been identified in vocal learning nuclei, like the HVC and RA,\textsuperscript{42,43} and based on different manipulations of the OT system, there is evidence that blocking the OT or the OTR can impact song learning (using the Manning Compound that blocks both OTR-VTRs\textsuperscript{69}) or singing.\textsuperscript{10,31,70} Since BFs sing a more variable song than the WRMs,\textsuperscript{35} it is tempting to hypothesize that the changes we identified in the OT expression levels could partly influence the song nuclei, and thus differences between the BF and the WRM song.

We, lastly, hypothesize that the higher intra-strain variability in brain OT peptide content in WRM, compared to BF, could point to an evolutionary explanation according to which, out of the variable wild WRM-pool, those with low brain OT peptide content were selected for breeding, to eventually make up the domesticated BF. Although such variability could also be due to differential metabolic states,\textsuperscript{71} we believe that our experimental setup minimized the probability, since, before decapitation, all birds were kept in the dark, and did not eat, drink or sing in the hours before.\textsuperscript{72}

In conclusion, our study revealed specific variant sites across the OT 5′-UTR, exons, and 3′-UTR between the BFs and the WRMs and predicted their functionality based on conservation and TFBS analyses. In addition, we found a significantly lower amount of OT mRNA in the diencephalon and a significantly higher amount of OT in the cerebrum of the BFs compared to WRMs. Our results suggest that domestication significantly alters the expression of OT in related brain areas of domesticated BFs and wild WRMs, an observation that could shed light to several of their behavioral differences. Whether these variants and brain region-specific OT expression profile cause increased social behavior, greater song diversity, and/or a diminished stress response in the BF requires further investigation.

ACKNOWLEDGMENTS
We would like to thank all members of Prof. Chen, Tang, and Yao lab groups for their help and support during our stay in Taiwan. We also would like to thank the field workers for their help with mist-netting birds. The present study was supported by Grants-in-Aid for Private University research Branding project and Scientific Research (JP16K18585) from the Ministry of Education, Culture, Sports, Science and Technology Japan (Y.T.) and Scientific Research Grants from the Moritani Scholarship Foundation (Y.T.), in Japan. C.T. recognized funding from the Rockefeller University; E.D.J. from the Howard Hughes Medical Institute and the Rockefeller University. C.B. acknowledges funding from the Spanish Science Ministry (grant PID2019-107042GB-I00). Y.T. is very grateful to Ms. Jung Wang and Yun-Tien Hsu for connecting Y.T. with the professors at National Chung Hsing University.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The authors confirm that all of the data underlying the reported findings are included in the article. All raw data that support the findings of this study are available in the Suppl. Tables and in our publicly available Github repository (https://github.com/constantinatheo/OT-domestication).

ORCID
Yasuko Tobari \(\text{https://orcid.org/0000-0003-4438-2912}\)

REFERENCES
1. Suzuki K, Okanoya K. Domestication effects on aggressiveness: comparison of biting motivation and bite force between wild and domesticated finches. Behav Processes. 2021;193:104503. doi:10.1016/j.beproc.2021.104503
2. Suzuki K, Ikebuchi M, Okanoya K. The impact of domestication on fearfulness: a comparison of tonic immobility reactions in wild and domesticated finches. Behav Processes. 2013;100:58-63. doi:10.1016/j.beproc.2013.08.004
3. Suzuki K, Yamada H, Kobayashi T, Okanoya K. Decreased fecal corticosterone levels due to domestication: a comparison between the white-backed munia (Lonchura striata) and its domesticated strain, the Bengalese finch (Lonchura striata var. domestica) with a suggestion for complex song evolution. J Exp Zool A Ecol Genet Physiol. 2012;317(9):561-570. doi:10.1002/jez.1748
4. Wilkins AS, Wrangham RW, Fitch WT. The “domestication syndrome” in mammals: a unified explanation based on neural crest cell behavior and genetics. Genetics. 2014;197(3):795-808. doi:10.1534/genetics.114.165423
5. Washio K. Enigma of Bengalese finches. Tokyo, Kindai-Bungei-sya; 1996.
6. Taka-Tsukasa N. Companion birds. Tokyo, Shoka-Bo; 1917.
7. Okanoya K. Sexual communication and domestication may give rise to the signal complexity necessary for the emergence of language: an indication from songbird studies. Psychon Bull Rev. 2017;24(1):106-110. doi:10.3758/s13423-016-1165-8
8. Mizuta T, Yamada H, Lin R-S, Yodogawa Y, Okanoya K. Sexing white-rumped Munias in Taiwan, using morphology, DNA and distance calls. Ornithol Sci. 2003;2(2):97-102. doi:10.2326/osj.2.97
9. Restall R. Munias and Mannikins. New Haven and London, Helm; 2010.
10. Theofanopoulou C. Implications of oxytocin in speech. Dissertation, University of Barcelona; 2019.
11. Bakos J, Scancikova A, Havranek T, Bacova Z. Molecular mechanisms of oxytocin signaling at the synaptic connection. Neural Plast. 2018;2018:4864107. doi:10.1155/2018/4864107
12. Robinson ICAF. Oxytocin and the milk-ejection reflex. In: Ganten D, Pfaff D, eds. Neurobiology of Oxytocin. Current Topics in Neuroendocrinology. Vol 6. Berlin: Springer; 1986.
13. Freeman SM, Young LJ. Oxytocin, vasopressin, and the evolution of mating systems in mammals. In: Choleris E, Pfaff DW, Kavaliers M, eds. Oxytocin, Vasopressin and Related Peptides in the Regulation of Behavior. New York, NY: Cambridge University Press; 2013:128-147.
14. Neumann ID. Involvement of the brain oxytocin system in stress coping: interactions with the hypothalamo-pituitary-adrenal axis. Prog Brain Res. 2002;139:147-162. doi:10.1016/s0079-6123(02)39014-9
15. Herbeck YE, Gulevich RG. Neuropeptides as facilitators of domestication. Cell Tissue Res. 2019;375(1):295-307. doi:10.1007/s00441-018-2939-2
16. Ruan C, Zhang Z. Laboratory domestication changed the expression patterns of oxytocin and vasopressin in brains of rats and mice. Anat Sci Int. 2016;91(4):358-370. doi:10.1007/s12565-015-0311-0
17. Fam J, Holmes N, Delaney A, Crane J, Westbrook RF. Oxytocin receptor activation in the basolateral complex of the amygdala enhances discrimination between discrete cues and promotes configur processing of cues. Psychoneuroendocrinology. 2018;96:84-92.

18. Paucluillo A, Ogah DM, Lannaccone M, Erhardt G, Di Stasio L, Cosenza G. Genetic characterization of the oxytocin-neurophysin I gene (OXT) and its regulatory regions analysis in domestic Old and New World camels. PLoS One. 2018;13(4):e0195407. doi:10.1371/journal.pone.0195407

19. Wirobiski G, Range F, Schaefs P, Palme R, Deschner T, Marshall-Pescini S. Endocrine changes related to dog domestication: comparing urinary cortisol and oxytocin in hand-raised, pack-living dogs and wolves. Horm Behav. 2021;128:104901. doi:10.1016/j.yhbeh.2020.104901

20. Nagasawa M, Mitsui S, En S, et al. Oxytocin-gaze positive loop and the coevolution of human-dog bonds. Science. 2015;348(6232):333-336. doi:10.1126/science.1261022

21. Bence M, Marx P, Szantai E, Kubinyi E, Ronai Z, Banlaki Z. Lessons from the canine Oxtr gene: populations, variants and functional aspects. Genes Brain Behav. 2017;16(4):427-438. doi:10.1111/gbb.12356

22. Donaldson ZR, Young LJ. Oxytocin, vasopressin, and the neu-rogenetics of的社会. Science. 2008;322(5903):900-904.

23. Tang Y, Benusiglio D, Lefevre A, et al. Social touch promotes inter- female communication via activation of parvocellular oxytocin neu- rons. Nat Neurosci. 2020;23(9):1125-1137. doi:10.1038/s41593-020-0674-y

24. Lukas M, Toth I, Reber SO, Slattery DA, Veemena AH, Neumann ID. The neuropeptide oxytocin facilitates pro-social behavior and pre- vents social avoidance in rats and mice. Neuropsychopharmacology. 2011;36(11):2159-2168. doi:10.1038/npp.2011.95

25. Zoicas I, Slattery DA, Neumann ID. Brain oxytocin in social fear conditioning and its extinction: involvement of the lateral septum. Neuropsychopharmacology. 2014;39(13):3027-3035. doi:10.1038/npp.2014.156

26. Menon R, Grund T, Zoicas I, et al. Oxytocin signaling in the lateral septum prevents social fear during lactation. Curr Biol. 2018;28(7): 1066-1076.e6. doi:10.1016/j.cub.2018.02.044

27. Johnson ZV, Walum H, Xiao Y, Riefkohl PC, Young LJ. Oxytocin receptors modulate a social salience neural network in male prairie voles. Horm Behav. 2017;87:16-24. doi:10.1016/j.yhbeh.2016.10.009

28. Goodson JL, Schrock SE, Klatt JD, Kabekol D, Kingsbury MA. Mes- otocin and nonapeptide receptors promote estrildid flocking behavior. Science. 2009;325(5942):862-866. doi:10.1126/science.1174929

29. Goodson JL, Lindberg L, Johnson P. Effects of central vasotocin and mesotocin manipulations on social behavior in male and female zebra finches. Horm Behav. 2004;45(2):136-143. doi:10.1016/j.yhbeh.2003.08.006

30. Warren WC, Clayton DF, Ellegren H, et al. The genome of a songbird. Nature. 2010;464:757-762. doi:10.1038/nature08819

31. Pedersen A, Tomaszycki ML. Oxytocin antagonist treatments alter the formation of pair relationships in zebra finches of both sexes. Horm Behav. 2012;62(2):113-119. doi:10.1016/j.yhbeh.2012.05.009

32. Klatt JD, Goodson JL. Oxytocin-like receptors mediate pair bonding in a socially monogamous songbird. Proc R Soc B. 2013;280:20122396. doi:10.1098/rspb.2012.2396

33. Pilgeram NR, Baran NM, Bhise A, et al. Oxytocin receptor antagonism during song tutoring in zebra finches reduces preference for and learning of the tutor’s song. bioRxiv. 2021. doi:10.1101/2021.06.16.448133

34. Suzuki K, Ikeuchi M, Kagawa H, Koike T, Okanoya K. Effects of domestication on neophobia: a comparison between the domesticated Bengalese finch (Lonchura striata var. domestica) and its wild ancestor, the white-rumped munia (Lonchura striata). bioRxiv. 2021. doi:10.1101/2021.03.23.436696
Insights from comparative genomics. PLOS ONE. 2017;12(10):e0185306. https://doi.org/10.1371/journal.pone.0185306

54. Goossens N, Blähser S, Oksche A, Vandesande F, Dierickx K. Immunocytochemical investigation of the hypothalamo-neurohypophysial system in birds. Cell Tissue Res. 1977;184:1-13. https://doi.org/10.1007/BF00220523

55. Bons N. The topography of mesotocin and vasotocin systems in the brain of the domestic mallard and Japanese quail: Immunocytochemical identification. Cell Tissue Res. 1980;213(1):37-51. doi:10.1007/BF00236919

56. Kuramoto T, Nakanishi S, Ochiai M, Nakagama H, Voigt B, Serikawa T. Origins of albino and hooded rats: implications from molecular genetic analysis across modern laboratory rat strains. PLoS One. 2012;7(8):e43059. doi:10.1371/journal.pone.0043059

57. Reuveni E. The genetic background effect on domesticated species: a mouse evolutionary perspective. Sci World J. 2011;11:429-436. doi:10.1100/tsw.2011.32

58. Neumann ID, Wigger A, Torner L, Holsboer F, Landgraf R. Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. J Neuroendocrinol. 2000;12(3):235-243. doi:10.1046/j.1365-2826.2000.00442.x

59. Jamieson BB, Nair BB, Iremonger KJ. Regulation of hypothalamic corticotropin-releasing hormone neurone excitability by oxytocin. J Neuroendocrinol. 2017;29(11):e12532. doi:10.1111/jne.12532

60. Goodson JL, Schrock SE, Kingsbury MA. Oxytocin mechanisms of stress response and aggression in a territorial finch. Physiol Behav. 2015;141:154-163. doi:10.1016/j.physbeh.2015.01.016

61. Kelly AM, Goodson JL. Hypothalamic oxytocin and vasopressin neurons exert sex-specific effects on pair bonding, gregariousness, and aggression in finches. Proc Natl Acad Sci U S A. 2014;111(16):6069-6074. doi:10.1073/pnas.1322554111

62. Klemm WR. Neurophysiologic studies of the immobility reflex (‘animal hypnosis’). In: Ehrenpreis S, Solnitcky OC, eds. Neurosciences Research. Vol 4. New York, NY: Academic Press; 1971:165-212.

63. Ratner SC. Comparative aspects of hypnosis. In: Gordon JE, ed. Clinical and Experimental Hypnosis. New York, NY: MacMillan; 1967:550-587.

64. Suzuki K, Ikeuchi M, Kagawa H, Koike T, Okanoya K. Effects of domestication on neophobia: a comparison between the domesticated Bengalese finch (Lonchura striata var. domestica) and its wild ancestor, the white-rumped munia (Lonchura striata). Behav Processes. 2021;193:104502. doi:10.1016/j.beproc.2021.104502

65. Maekawa F, Nagino K, Yanga J, et al. Strain differences in intermale aggression and possible factors regulating increased aggression in Japanese quail. Gen Comp Endocrinol. 2018;256:63-70.

66. Consiglio AR, Borsoi A, Pereira GAM, Lucion AB. Effects of oxytocin microinjected into the central amygdaloid nucleus and bed nucleus of stria terminalis on maternal aggressive behavior in rats. Physiol Behav. 2005;85(3):354-362. doi:10.1016/j.physbeh.2005.05.002

67. Pedersen CA, Prange AJ Jr. Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin. Proc Natl Acad Sci U S A. 1979;76(12):6661-6665. doi:10.1073/pnas.76.12.6661

68. Kagawa H, Yamada H, Lin RS, Mizuta T, Hasegawa T, Okanoya K. Ecological correlates of song complexity in white-rumped munias: the implication of relaxation of selection as a cause for signal variation in birdsong. Interact Stud. 2012;13:263-284. doi:10.1075/is.13.2.05kag

69. Baran NM, Peck SC, Kim TH, Goldstein MH, Adkins-Regan E. Early life manipulations of vasopressin-family peptides alter vocal learning. Proc Biol Sci. 2017;284(1859):20171114.

70. Theofanopoulou C, Boeckx C, Jarvis ED. A hypothesis on a role of oxytocin in the social mechanisms of speech and vocal learning. Proc Biol Sci. 2017;284(1861):20170988.

71. Sherman TG, Day R, Civelli O, et al. Regulation of hypothalamic magnocellular neuropeptides and their mRNAs in the Brattleboro rat: coordinate responses to further osmotic challenge. J Neurosci. 1988;8(10):3785.

72. Prabhat A, Batra T, Kumar V. Effects of timed food availability on reproduction and metabolism in zebra finches: molecular insights into homeostatic adaptation to food-restriction in diurnal vertebrates. Horm Behav. 2020;125:104820. doi:10.1016/j.ybbeh.2020.104820

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

**How to cite this article:** Tobari Y, Theofanopoulou C, Mori C, et al. Oxytocin variation and brain region-specific gene expression in a domesticated avian species. Genes, Brain and Behavior. 2022;21(2):e12780. doi:10.1111/gbb.12780