RESEARCH ARTICLE

Neuronal activation in zebra finch parents associated with reintroduction of nestlings

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Abstract

Recent studies of the brain mechanisms of parental behaviors have mainly focused on rodents. Using other vertebrate taxa, such as birds, can contribute to a more comprehensive, evolutionary view. In the present study, we investigated a passerine songbird, the zebra finch (*Taeniopygia guttata*), with a biparental caring system. Parenting-related neuronal activation was induced by first temporarily removing the nestlings, and then, either reuniting the focal male or female parent with the nestlings (parental group) or not (control group). To identify activated neurons, the immediate early gene product, Fos protein, was labeled. Both parents showed an increased level of parental behavior following reunion with the nestlings, and no sexual dimorphism occurred in the neuronal activation pattern. Offspring-induced parental behavior-related neuronal activation was found in the preoptic, ventromedial (VMH), paraventricular hypothalamic nuclei, and in the bed nucleus of the stria terminalis. In addition, the number of Fos-immunoreactive (Fos-ir) neurons in the nucleus accumbens predicted the frequency of the feeding of the nestlings. No difference was found in Fos expression when the effect of isolation or the presence of the mate was examined. Thus, our study identified a number of nuclei involved in parental care in birds and suggests similar regulatory mechanisms in caring females and males. The activated brain

regions show similarities to rodents, while a generally lower number of brain regions were activated in the zebra finch. Furthermore, future studies are necessary to establish the role of the apparently avian-specific neuronal activation in the VMH of zebra finch parents.

KEYWORDS

biparental care, bird brain, neuronal activation, offspring provisioning, parental behavior, RRID: AB_2231996, RRID:AB_2340593, RRID:SCR_001905, RRID:SCR_002380, RRID:SCR_003070, RRID:SCR_016041

1 | INTRODUCTION

Parental care includes taxonomically widespread forms of behaviors whereby parents increase the survival chances of their offspring and, therefore, their fitness (Reynolds, Goodwin, & Freckleton, 2002). The underlying brain mechanisms have been extensively studied in rodent species (Numan & Smith, 1984; Rilling & Young, 2014). Although these studies are seminal, a major difficulty in identifying brain centers specifically responsible for parental behaviors is the co-occurrence of lactation, which is a mammalian-specific phenomenon supporting the offspring. In addition, males typically do not take care of the offspring in rodents. Thus, to investigate brain activation in the absence of lactation and to compare brain activation between female and male parents, birds are a promising taxon for use as a vertebrate model for multiple reasons. Unlike in mammals, in which the body and brain of the mother undergo major adaptations, in birds, there are no such confounding alterations, even though some species produce crop milk and feed offspring via regurgitation, which can be associated with parenting-related neuronal activation (Buntin, Berghman, & Buntin, 2006). Moreover, the majority (~90%) of bird species are characterized by the biparental strategy, thus care is provided by both males and females (Cockburn, 2006). In addition, parental care includes diverse behavioral traits in birds, most of which are well-defined and easy to observe and quantify (Morvai et al., 2016; Zann, 1996). Importantly, the regulatory brain centers of social behaviors (e.g., parental behavior) are considered to be evolutionarily conserved (Newman, 1999; O'Connell & Hofmann, 2011a, 2011b; O'Connell & Hofmann, 2012; Young et al., 2019), suggesting that findings revealed in birds might also be indicative of other vertebrate taxa.

A main approach for identifying brain centers involved in parental care is to detect neuronal populations that became active in females due to pup exposure and suckling (Li, Chen, & Smith, 1999). Instead of electrophysiological methods, which are limited to measuring the activity of only a small number of neurons, the c-fos technique uses immunohistochemical detection of the immediate early gene product Fos protein (Herrera & Robertson, 1996), which is widely applied to identify offspring-induced neuronal activation in rodent mothers (Li et al., 1999; Lonstein, Gréco, De Vries, Stern, & Blaustein, 2000; Lonstein, Simmons, Swann, & Stern, 1997). In rodents, it is not possible to establish whether the activation in these brain regions is related to the regulation of

lactation or the behavioral responses of the mothers, which warrants the use of new animal model systems. An experimental paradigm that is particularly fruitful in rodents to establish activated brain regions uses offspring separation from the mothers, causing the Fos protein to disappears from offspring-activated neurons. Thus, brain activation patterns can be examined in such offspring-deprived (control) parents and in mothers who have their offspring returned and re-establish intensive parental care (Lonstein et al., 1997). Using this experimental design in the rat, Fos-labeled neurons were described in several brain regions, with the most intensive signals in the preoptic area of the hypothalamus (POM), the ventrolateral subdivision of the lateral septal nucleus (LSvl), the posterior intralaminar nucleus of the thalamus (PIL), and the ventrolateral subdivision of the periaqueductal gray (PAGvl; Cservenák et al., 2010; Dulac, O'Connell, & Wu, 2014; Fleming & Walsh, 1994; Li et al., 1999; Lonstein et al., 2000; Lonstein, Simmons, & Stern, 1998; Lonstein & Stern, 1998; Wu, Autry, Bergan, Watabe-Uchida, & Dulac, 2014). Recent studies confirmed a similar activation pattern in the brains of parenting mice, as well (Okabe et al., 2013, 2017).

In birds, c-fos activation studies have been performed in relation to nest building(Hall, Bertin, Bailey, Meddle, & Healy, 2014; Hall, Meddle, & Healy, 2015; Klatt & Goodson, 2013) and brooding (Ruscio & Adkins-Regan, 2004). Furthermore, an increasing number of neurobiological studies have investigated the hormonal and neural backgrounds of various social behaviors in zebra finches (Goodson, 2005, 2013; Goodson & Kabelik, 2009; Goodson, Kelly, & Kingsbury, 2012; Goodson, Kelly, Kingsbury, & Thompson, 2012; Goodson, Rinaldi, & Kelly, 2009; Goodson, Schrock, Klatt, Kabelik, & Kingsbury, 2009; Kelly & Goodson, 2014a, 2014b; Klatt & Goodson, 2013). In the present study, we used this small passerine bird, the zebra finch (*Taeniopygia guttata*), which is a widely used model for behavioral studies focusing on mate choice and parental care (Gorman & Nager, 2003; Levréro, Blanc, & Mathevon, 2012; Morvai et al., 2016; Rehling et al., 2012; Rutstein, Brazill-Boast, & Griffith, 2007). Zebra finches breed continuously under laboratory conditions, and methods to monitor parental care in this species are well-established and have been validated previously (Morvai et al., 2016). Importantly, crop milk production does not occur in zebra finches (Griffith & Buchanan, 2010; Immelmann, 1962; Zann, 1996). Using this species, we had the following objectives in the present study: (a) to identify Fos-positive neurons with regard to parental behavior by excluding potentially confounding effects, such as mammalian lactation

or crop milk secretion and (b) to investigate sex-differences in the neuronal regulation of parental behavior in a biparental species. The experiments were performed approximately in the middle of nestling provisioning, when caring behavior is intense (Lemon, 1993). The experimental paradigm was similar to offspring separation paradigms in rodent studies for comparability and generalizability. To exclude potentially interfering adult–adult interactions (Burley & Johnson, 2002; Zann, 1996) and to make the study even more comparable to those in rodents, zebra finch parents were tested in the absence of the mate. A control experiment suggested that social isolation from the mate does not result in increased neuronal activation. The time invested in offspring provisioning was similar in males and females, which was associated with a similar brain activation pattern in the two sexes. Brain activation was generally comparable to but more restricted than that in rodents. We also found a brain area, the ventromedial hypothalamic nucleus, that was activated in zebra finch parents but not in rodents, according to previous studies.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The study was carried out according to the Hungarian Laws for experimenting with animals. Breeding and experimenting was implemented with the permission from the Ethical Board of Eötvös Loránd University (ELTE MÁB 02/2014).

2.2 | Animals

Subjects (n = 24) were randomly selected from a zebra finch (*Taeniopygia guttata*) population kept at the Animal House of Eötvös Loránd University, Hungary. This captive zebra finch population was established from the domesticated stock maintained at Bielefeld University, Germany (Forstmeier, Segelbacher, Mueller, & Kempenaers, 2007). Birds were ringed by a numbered aluminum ring (Principle Kft., Újlengyel, Hungary). A constant light cycle (lights on from 6:00 a.m. to 8:00 p.m.) was kept using full-spectrum light tubes connected to timers. Temperature and humidity in the experimental room were maintained at 20–21°C, and 55–60%, respectively. Adult females and males were paired and couples were housed in separate cages (100 × 30 × 35 cm) for breeding. Wooden nest boxes (12 × 12 × 12 cm) were attached to the cages from outside and coconut fibers were provided as nest material. Food (a seed mixture, supplemental egg-food, and germinated seeds) and water were provided ad libitum as described previously (Morvai et al., 2016). Offspring of the sacrificed focal parent were further raised by the nonfocal parent and recruited to the stock population on posthatching Day 40 (i.e., after becoming independent of parental provisioning).

2.3 | Experimental design

The experiment was designed to examine offspring-related neuronal activation induced by the presence of offspring. Therefore, the parental and control groups were designed to exclude any additional differences. The experimental procedure was implemented on posthatching days 12 and 13 (PHD-12 and PHD-13; counted from the day when the first egg hatched in a given clutch; Morvai et al., 2016). Furthermore, parental behavior was recorded on PHD-10 to collect data for baseline (i.e., unmanipulated) parental behavior (see below) and also on PHD-13 (i.e., after returning the nestlings). On the first day of the manipulation (PHD-12), at 6 p.m., one of the parents (nonfocal) and the nest box with the nestlings were moved to another room to separate them from the focal parent (Figure 1). The focal parent stayed alone in its home cage without disturbance for 16 hr to eliminate (or at least reduce) any mate- and care-related Fos protein in the brain. On the following day (PHD-13) at 8 a.m., the nestlings were separated from the nonfocal parent in the other room for 2 hr to induce a mild offspring-starvation. At 10 a.m., nestlings were reunited with the focal parent in their home cage, and so-called postmanipulation behavioral recording started by a nest camera. In the control condition, the experimental procedure was similar with the only exception that the focal parent did not receive its nestlings back (Figure 1).

To investigate whether the separation of a social pair causes any side effect or stress-induced brain activation, a supplementary experiment was carried out. Social pairs (n = 10) without a nesting opportunity were kept together for 2 weeks to form pair bonds (Aragona et al., 2006; Lei, Liu, Smith, Lonstein, & Wang, 2017). In the separated group, social pairs were isolated from each other for 16 hr (n = 5), while social pairs stayed together with their mate in the paired group (n = 5). In both groups, males were perfused to compare brain activity using Fos immunohistochemistry.

2.4 | Video recordings and behavioral analysis

Recordings of offspring provisioning within the nest were carried out using small Mobius digital cameras (Mobius Action Cam, JooVuu Store, UK) with wide-angle lenses (116° field of view) for 90 min, between 10:00 a.m. and 11:30 a.m. The camera stored video recordings on a microSD card. Video recordings were coded using a Solomon Coder (v 16.06.26, developed by András Péter, Solomon Coder, RRID: SCR_016041; András, 2014). We examined various forms of parental behavior, including brooding, spending time inside the nest (without any apparent action), nest building, feeding, and preening of the nestlings. The phrase "feeding behavior" will refer to feeding of the nestlings later in the text. The behavioral variables were coded individually, but they were combined for the analysis. Thus, nest attendance (total time spent in the nest) was used as a measure of parental behavior (Morvai et al., 2016). Behavioral data were analyzed only for the parenting group because no parenting was possible in the control group.

2.5 | Tissue collection for immunohistochemistry

A reference point was established by the first entrance to the nest by the focal parent after reunion. Ninety minutes after the reference point, when the neuronal activation supposedly reached the maximum level (Bullitt, 1990; Dragunow & Faull, 1989; Hoffman, Smith, & Verbalis, 1993),
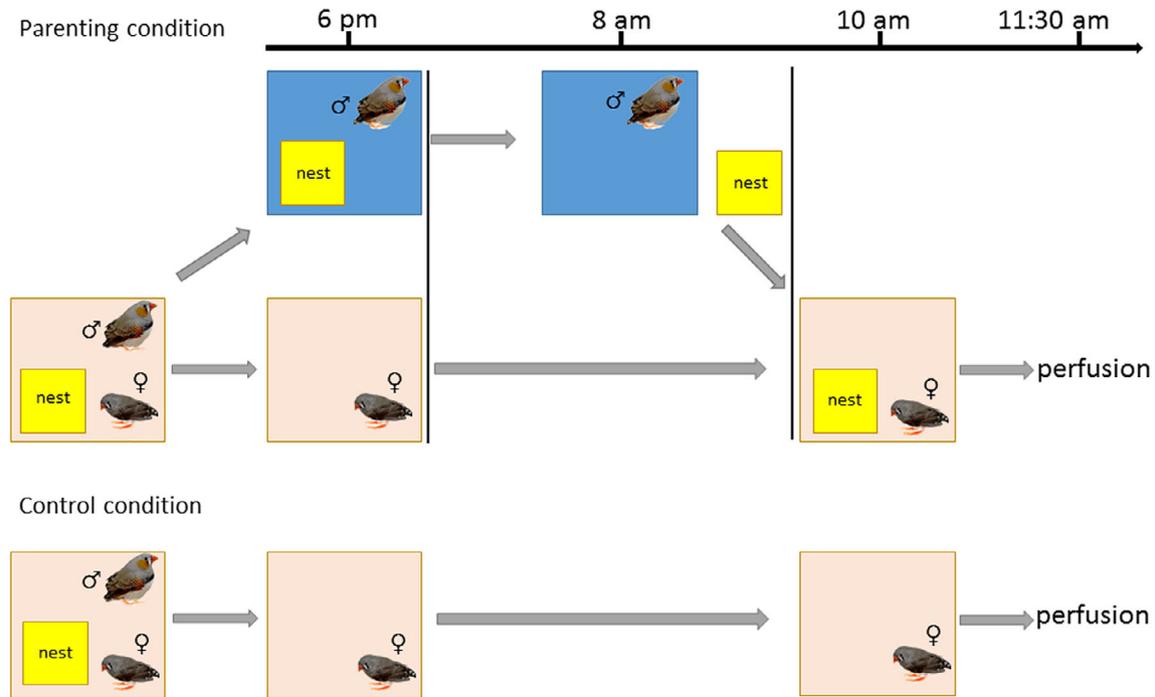
FIGURE 1 Experimental design to investigate neuronal regulatory mechanisms of parental behavior in the zebra finch. Activation changes were investigated both in male and female parents, however, for simplicity, the figure illustrates the experimental protocol only when the female was the focal parent. The male and the offspring were removed from the home cage and taken to another room at 6 p.m. On the following day, the offspring were separated from the male for 2 hr, and then the nestlings were replaced to their home cage i.e. back to the female. Ninety minutes after reunion, the female was sacrificed and perfused. In the control group, the manipulation was the same except for the lack of reunion of the focal parent with its nestlings [Color figure can be viewed at wileyonlinelibrary.com]

transcardial perfusion was performed with 4% paraformaldehyde (PFA) under deep ketamine anesthesia (calypsol:xylazine, 2:1 ratio, injected amount: 0.07 ml/ 10 g). The brains were postfixed for 24 hr in 4% PFA and transferred to a 20% sucrose solution for cryoprotection for 1 day. The brains were sliced at 40 μm into serial coronal sections on a sliding microtome (Frigomobil SM 2000 R, Leica Microsystems, Wetzlar, Germany), and sections were collected in phosphate buffer (PB; pH = 7.4) with 0.05% sodium azide and stored at 4°C until usage.

2.6 | The applied anti-Fos antibody and its validation with western blotting

A rabbit anti-Fos primary antiserum (1:1000, c-Fos Antibody (K-25): sc-253, Santa Cruz Biotechnology, Santa Cruz, USA, RRID:AB_2231996) was applied in the study. This antiserum has been successfully applied previously in studies involving birds (Bolhuis, Zijlstra, den Boer-Visser, & Van der Zee, 2000; Mayer, Watanabe, & Bischof, 2010; Riters, Teague, Schroeder, & Cummings, 2004; Tokarev, Tiunova, Scharff, & Anokhin, 2011). The specificity of the K-25 antibody was previously confirmed in starlings by using blocking peptide (sc-253P, Santa Cruz Biotechnology; Alger, Maasch, & Riters, 2009).

To further confirm the specificity of the antibody in the zebra finch brain, western blotting analysis was performed. For this validation, two male birds were used from our flock living in an indoor aviary. The hypothalamic areas of the brains were dissected and frozen in isopentane kept on dry ice and stored until usage at –80°C. Protein extraction was

performed by lysing the tissue in radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The homogenate was centrifuged at 12,000×g for 30 min at 4 °C, and the supernatant was collected. Protein quantification was carried out using a BCA kit (Sigma-Aldrich, St. Louis, MO, Cat. No. BCA1-1KT). The total protein extract (30 μg per lane) was separated by SDS-PAGE using 15% polyacrylamide gels and electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, Cat. No. 1620112). Nonspecific binding sites were blocked with 5% nonfat dry milk diluted in tris-buffered saline (TBS) Tween buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6). The primary antibody (anti-c-Fos, Santa Cruz Biotechnology, San Diego, CA, Cat. No. sc-253) was used at a dilution of 1:1,000. The membrane was incubated overnight at 4 °C in the primary antibody and then for 2 hr in horseradish peroxidase-conjugated secondary anti-rabbit IgG (1:2000; Jackson ImmunoResearch, West Grove, PA, Cat. No. 711035152). The labeling was visualized using Clarity Western ECL Substrate (BioRad Laboratories, Cat. No. 170–5,060) by the Gel Doc XR+ imaging system (BioRad). To establish the molecular weight of the labeled proteins, PageRuler Prestained Protein Ladder was used (Cat. No. 26616, Thermo Scientific, Waltham, MA).

2.7 | Fos immunohistochemistry

Every third free-floating section of the brains was immunolabeled with the peroxidase method as described previously (Cservenák et al., 2010).

Briefly, hydrogen peroxide (H_2O_2, 1:1,000 dilution, using 3 ml/samples) was used first to eliminate endogenous peroxidase activity. The brain sections were then incubated with bovine serum albumin (BSA) for 1 hr to reduce nonspecific labeling. Then, the rabbit anti-Fos primary antiserum (1:1,000, c-Fos Antibody [K-25]: sc-253, Santa Cruz Biotechnology, Santa Cruz, CA) was applied for 24 hr at room temperature. Sections were incubated in biotinylated donkey antirabbit secondary antibody for 2 hr (1:1,000 dilution, Jackson ImmunoResearch, West Grove, PA, Cat# 711-065-152, RRID:AB_2340593) and further in avidin-biotin-peroxidase complex (ABC; 1:500; Vector Laboratories, Burlingame, CA) for 1 hr. Labeling was visualized using nickel-intensified 3,3-di-aminobenzidine (Ni-DAB). Finally, the sections were washed in 0.01 M PB and then mounted to slides from 0.01 M Tris-solution and coverslipped.

2.8 | Analysis and quantification of Fos immunolabeling

Brain areas were identified using the stereotaxic atlas of the zebra finch and the stereotaxic brain atlas of the canary (*Serinus canaria*; Nixdorf-Bergweiler & Bischof, 2007; Stokes, Leonard, & Nottebohm, 1974) and the revised nomenclatures (Reiner et al., 2004; Reiner, Perkel, Mello, & Jarvis, 2004). In addition, subdivisions of the bed nucleus of the stria terminalis and the septal area involved in the quantitative analyses were identified by a detailed topographical map of the regions (Goodson, Evans, & Lindberg, 2004). The brain areas containing Fos-immunoreactive (Fos-ir) cells were detected and captured with a microscope equipped with a digital camera (Nikon Eclipse Ni, 25.4 2 MP Slider Camera, Spot RT3 software). The densities of Fos-labeled neurons in the different examined brain nuclei/areas were counted in coronal sections at the largest extent of each brain area. Same resolution pictures were taken and analyzed for all subjects for a given brain area.

Based on the available brain atlases mentioned above, the border of each brain area was determined using surrounding characteristic white and gray matter as markers, as shown in diagrams in Figure 2. Briefly, the nucleus accumbens (Acc) was identified in the ventral part of the medial striatum next to the ventricle. The preoptic area (POM) was identified between the tractus septomesencephalicus and the decussatio supraoptica dorsalis. The ventromedial hypothalamic nucleus (VMH) is located above the optic chiasm and below the paraventricular hypothalamic nucleus (PVN). The closely located tractus quintofrontalis and tractus thalamo-frontalis and frontalis-thalamicus medialis also helped us locate these nuclei for the quantification of Fos-ir neurons. The subdivisions of the bed nucleus of the stria terminalis (BST) and the septal regions were analyzed at the level of the anterior commissure: The lateral subdivision of the BST (BSTl) is at the ventral horn of the lateral ventricle, while its medial part is around the anterior commissure. The mediodorsal subdivision (BSTmd) was identified above, while the medioventral subdivision (BSTmv) was below, the commissure. The medial (MS) and lateral septum (LS) and the septohippocampal nucleus (SH) were identified for analysis based on previous descriptions. The ventral tegmental area (VTA) is located in the midbrain, next to the trunk of the oculomotor nerve, which was used as a marker when locating the nucleus for analysis.

The total number of Fos-ir neurons in activated regions was counted using ImageJ software, version 1.50i (ImageJ, RRID:SCR_003070, Wayne Rasband, National Institute of Health, Bethesda, MD) in photomicrographs. The pixel sizes of the examined brain regions used for quantification are given in Table 1. An algorithm was used for quantification based on a combination of intensity, size, and circularity threshold. The following parameters were used as a standardized quantification algorithm to avoid any subjective errors: Brightness intensity was between 13 and 213, size (i.e., the selected spots to be counted) was in the range of 4–22 pixels, and the circularity factor was between 0.7 and 1.0. The corresponding brain areas in the two hemispheres were quantified separately, and the mean value calculated from the two hemispheres was included in the statistical analyses.

2.9 | Statistical analysis

For statistical analysis, linear mixed models (LMMs) were used to analyze behavior and neuronal activation characterized with nonindependent data (Burton, Gurrin, & Sly, 2005; Heckerman et al., 2016; Krueger & Tian, 2004). Calculations were carried out using the R statistical package (R Project for Statistical Computing, RRID: SCR_001905; R Core Team, 2017). LMMs were used to account for nonindependence of brain regions in a given subject. The LMM of the number of Fos-positive cells (response variable) included the experimental group (EG: parenting vs. control group), brain nucleus and sex as fixed factors, and ring number (ID) as a random effect. Initial models included the three-way and all two-way interactions between the fixed factors. We report the final models following stepwise model selection (based on the AIC values), which include only significant effects.

Parental behaviors were analyzed in LMMs including sex, posthatching day (PHD-10, PHD-13), and number of nestlings as fixed effects and bird ID as a random effect. Similar to the activation analysis, we tested for potential effects of interactions between main effects and kept these only if they had significant effects.

In addition, we also investigated how different types of behaviors can be explained by brain activation. In these separate LMMs, neuronal activation was included as an independent predictor and the parental behavior of the focal parent was included as a dependent variable, separately for all identified brain regions.

3 | RESULTS

3.1 | Behavior of experimental parents

Animals in the control group (without reunion with their nestlings) could not perform any parental behaviors, as the nest and the nestlings were absent. Consequently, behaviors such as feeding the young or even staying in the nest were not possible for them. In contrast, parents who received their nestlings back started to perform parental behavior shortly after their reunion. Males spent 52.2
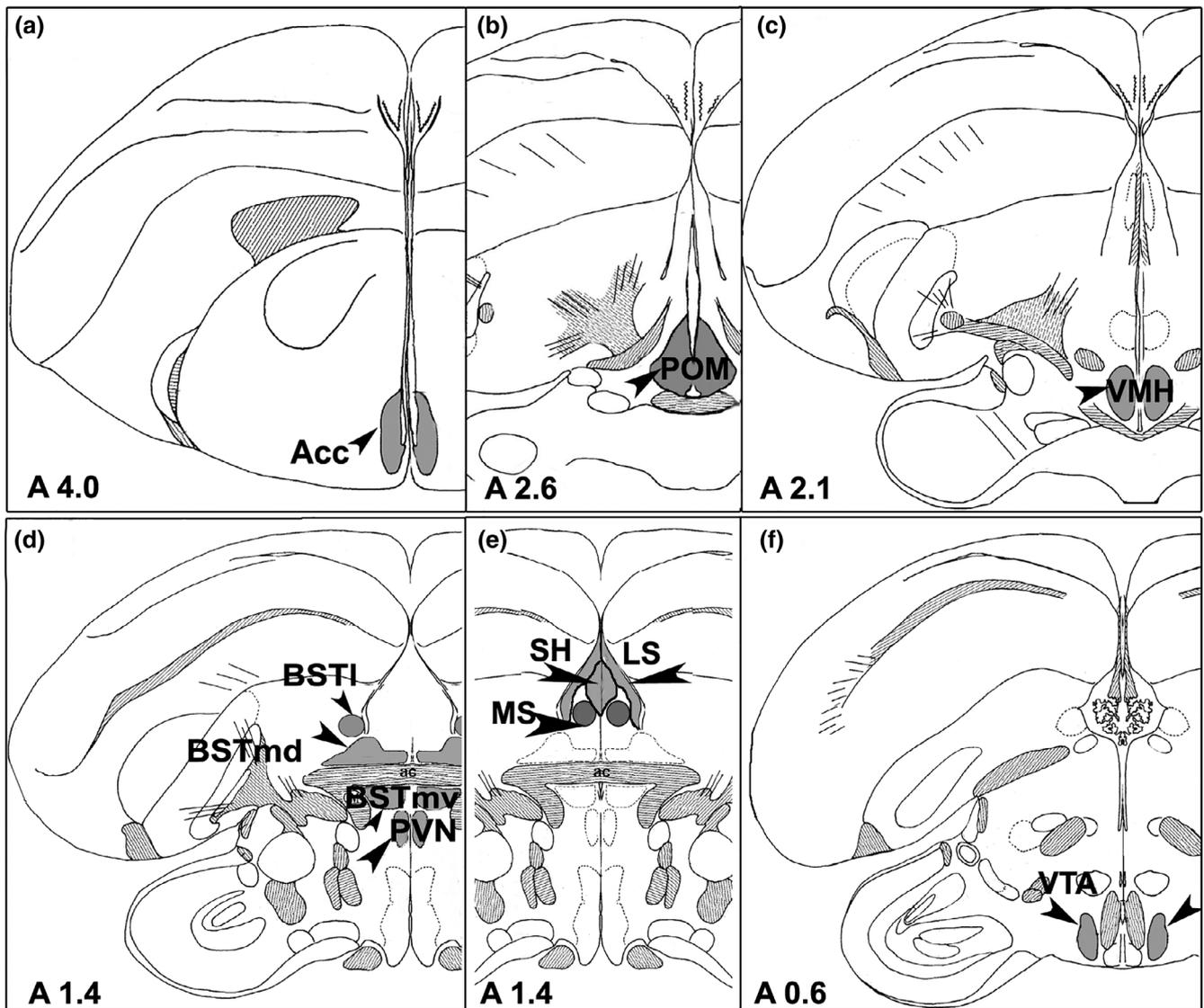
FIGURE 2 Schematic drawings of coronal brain sections indicating the position of the brain regions where Fos expression was quantified. Black arrowheads point to the specific brain regions

± 10.4%, while females spent 75.5 ± 9.4% of their time in the nest with parenting, which did not show a difference between sexes.

3.2 | Parental behavior before and after nestling deprivation

We observed and coded different types of parental behaviors including brooding, staying inside the nest without any parental action, nest building, feeding and preening the nestlings both on Day 10 and Day 13, posthatching (PHD-10 and PHD-13, respectively). We did not observe any behavior in the focal parent on PHD-13 that were not present on PHD-10. Therefore, the same behavioral elements were analyzed on both days. The dominant behaviors were brooding and nonparental activity outside the nest. The proportion of time spent performing parental behavior increased from PHD-10 to PHD-13, both in females and males, and decreased with brood size (Figure 3).

A detailed analysis confirmed no overall differences between the sexes (for all types of parental behaviors [response variables], effect of sex: $p > .148$), as both females and males increased their level of parental behavior between days PHD-10 to PHD-13, with the only exception of preening of the nestlings (males increased preening more from effect of sex × day interaction: $\chi^2 = 7.08$, $p = .008$; PHD-10 → PHD-13 in males vs. females: $1.29 ± 0.46$, $t_{10} = 2.84$, $p = .018$; Table 2.

3.3 | Validation of the anti-c-Fos antibody by western blotting

Western blotting revealed two major bands, suggesting that there were two proteins in the hypothalamic homogenates of the zebra finch that were significantly recognized by the anti-Fos antibody. The more intensely labeled band suggested a protein with a molecular weight of ~39.5 kDa (Figure 4). Based on the UniProt database

TABLE 1 Pixel size of the examined regions, which were included in the analysis

| The pixel sizes of examined brain regions		
Nucleus	**Abbreviation**	**Pixel size**
Nucleus accumbens	Acc	158 × 477
Preoptic area	POM	305 × 405
Ventromedial hypothalamus	VMH	205 × 399
Paraventricular nucles	PVN	230 × 510
Septohippocampal nucleus	SH	198 × 307
Lateral septum	LS	332 × 423
Medial septum	MS	134 × 136
Mediodorsal subdivision of the bed nucleus of stria terminalis	BSTmd	315 × 245
Medioventral subdivision of the bed nucleis of stria terminalis	BSTmv	465 × 205
Lateral subdivision of the bed nucleus of stria terminalis	BSTl	220 × 245
Ventral tegmental area	VTA	205 × 535

(UniProt, RRID:SCR_002380), the mass of the Fos protein (H0ZPP9_TAEGU) in zebra finch is 39.458 kDa, which corresponds to the main band. One additional significant band appeared on the blot, with less intense labeling. This band might represent an alternatively spliced or posttranslationally modified form of Fos protein (Jurado, Fuentes-Almagro, Prieto-Álamo, & Pueyo, 2007). Thus, the western blotting experiment confirmed the specificity of the antibody.

3.4 | Fos activation in different brain regions in response to reunion with the nestlings

In response to reunion with the nestlings, a number of brain regions in the parenting group had higher Fos activation than in the control group (Figure 1). Immediate early gene labeling appeared in the cell nucleus, as expected based on the role of Fos as a transcription factor (Distel & Spiegelman, 1990). The labeled brain areas were included in the quantitative analysis (Figure 2).

Analysis of the number of Fos-positive neurons revealed effects of parenting in several brain regions, as we found a significant experimental group × nucleus interaction (Figure 5). This interaction was driven by a higher number of Fos-activated neurons in reunited parents as opposed to the control parents in some of the nuclei, including the POM (Figure 6), the VMH (Figure 7), the PVN, the BSTmv, and the BSTmd (Figure 8; Table 3). The LS was selected as a baseline reference in the model, as this was a brain area without a change in Fos activity (Table 3). In contrast, we found no significant differences between the experimental groups in the Acc, the septal area (including the MS, LS, and SH), or in the VTA (Table 3).

We found no significant overall differences between the sexes in terms of neuronal activation related to parental behavior; however, sex had a nuclei-specific effect independent of parental behavior (i.e., from experimental treatment, Table 3). This effect was not driven
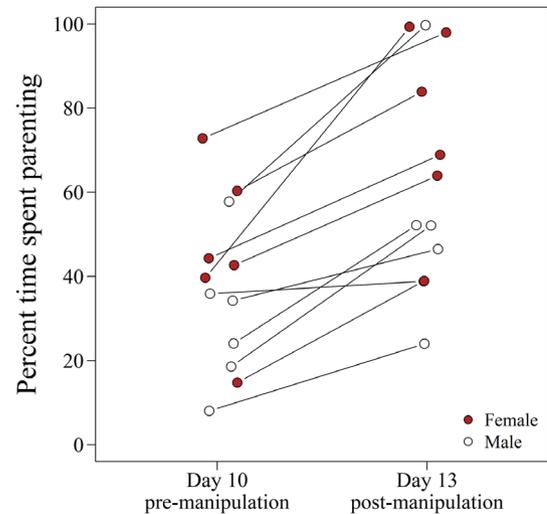
FIGURE 3 Time spent with parental behavior during 90 min observations premanipulation and postmanipulation (i.e., before and after a 16 hr temporal deprivation of the parent from the nestlings). Parental behavior included brooding, spending time inside the nest without any apparent action, nest building, feeding, and preening of the nestlings. Parental behavior increased in all females and males from premanipulation on Day 10, posthatching (PHD-10) to postmanipulation on PHD-13 (in females: from 45.8 ± 8.1% to 75.5 ± 9.4%; in males: from 29.8 ± 7.0% to 52.2 ± 10.4%) and decreased with brood size (LMM, effect of day: χ^2 = 25.14, p < 0.001, PHD-10 → PHD-13: 7.83 ± 1.32%, t_{11} = 5.93, p < .001; and effect of brood size: χ^2 = 27.62, p < .001, 1 nestling → 2 nestlings: −19.34 ± 5.94%, t_8 = −3.25, p = .012; 1 nestling → 3 nestlings: −41.66 ± 4.85%, t_8 = −8.58, p < 0.001; 1 nestling → 5 nestlings: −35.75 ± 5.94%, t_8 = −6.01, p = .001). The times spent with parenting on the PHD13 did not differ between sexes (t_{10} = 1.66, p = .129). In contrast, nest attendance has no significant differences between the sexes (χ^2 = .46, p = .498), and interaction also has no effect (sex × day interaction: χ^2 = .90, p = .344). Sexes are indicated by colors (red—females, white—males) [Color figure can be viewed at wileyonlinelibrary.com]

by marked sex differences in the neural activation of a few nuclei but rather by smaller differences, often in contrasting directions, that added up in this analysis (Figure 5).

3.5 | Fos activation in response to the separation of social pairs

The number of Fos-positive neurons did not differ between socially paired males that stayed with their female partner, and males that were separated from them (Figure 9, Table 4). The density of Fos-positive neurons in these animals was comparable to the density of Fos-positive neurons in the isolated parents.

3.6 | Correlations between brain activation and parental behavior

To increase the statistical power and because our previous behavioral analysis confirmed male and female parental behavior was comparable on PHD-13, the sexes were examined together, only for this analysis.

TABLE 2 Percentage of 90 min observation time that zebra finch parents (n = 12) spent with different parental activities inside the nest on Day 10 and Day 13 posthatching (PHD)

Percentage of time spent with parental behavior (mean ± SE)							
	Females		Males		Effect of variable (*F*-statistics and *p*-value)		
	PHD-10	PHD-13	PHD-10	PHD-13	Sex	Day	Nestlings
Brooding	34.4 ± 5.7	51.8 ± 9.1	20.7 ± 7.1	40.0 ± 11.7	χ^2 = 1.48 p = .224	χ^2 = 15.11 **p < .001**	χ^2 = 27.79 **p < .001**
Inside nest, passively	3.4 ± 1.6	7.8 ± 4.2	3.7 ± 0.8	3.6 ± 1.0	χ^2 = 1.04 p = .307	χ^2 = .96 p = .328	χ^2 = 2.73 p = .436
Nest building	5.9 ± 1.5	12.9 ± 2.5	5.3 ± 2.5	10.3 ± 4.5	χ^2 = 2.09 p = .148	χ^2 = 6.94 **p = .008**	χ^2 = 10.02 **p = .018**
Feeding	3.4 ± 0.8	8.3 ± 1.4	5.3 ± 1.3	8.6 ± 1.4	χ^2 = .09 p = .760	χ^2 = 13.21 **p < .001**	χ^2 = 2.74 p = .433
Preening[a]	4.9 ± 1.8	3.6 ± 2.2	1.1 ± 0.3	4.0 ± 1.3	χ^2 = 1.09 p = .297	χ^2 = .71 p = .398	χ^2 = 5.63 p = .131

Note: Brooding, nest building and feeding increased on PHD-13 following the reunion with the nestlings compared to premanipulation levels on PHD-10. We provide results of likelihood ratio tests (LRT) of LMMs including and excluding the given explanatory variable. In addition to the main effects, day had a sex-specific effect on preening the young (χ^2 = 7.08, p = .008).
[a]Significant two-way interaction between sex and day.
P values of significant changes are highlighted by bold numbers.
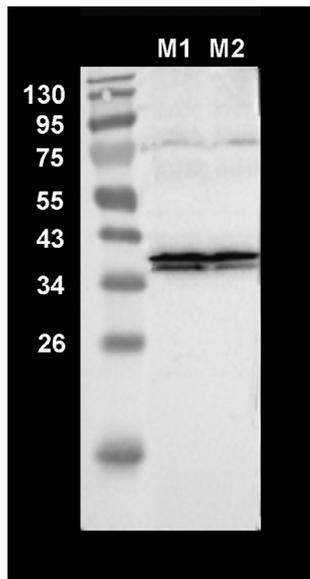
FIGURE 4 Validation of the (K-25, sc-253, Santa Cruz Biotechnology) c-Fos antibody by using hypothalamic area of two male zebra finches. Western blotting revealed two major bands, which were recognized by the anti-Fos antibody. The more intensively labeled band refers to the expected molecular weight of the Fos protein in zebra finch (39.5 kDa). M1 and M2 refers to the hypothalamic samples

Linear regressions of parental behaviors on activation levels in the 11 brain regions that are the focus of our study revealed that the number of active cells in the Acc correlated with the frequency that parents fed the young (Figure 10). We did not identify any other brain regions where the number of Fos-positive cells correlated with the frequency of feeding behavior (although the VMH showed a trend-like relationship in the same direction).

4 | DISCUSSION

This study identified numerous brain nuclei that are involved in off-spring provisioning in biparental songbirds using immunohistochemistry for the early gene product Fos. We start by discussing brain activation and the implications of the results with regard to the possible functions of the nuclei in question. Then, the behavioral findings and their possible correlation with neuronal activation are introduced. Finally, a comparative discussion of the evaluation of the neuronal activation considering previous studies in mammals is provided.

4.1 | Neuronal activation during parenting

We identified five brain regions with significantly elevated numbers of Fos-ir neurons during offspring provisioning: The POM, the VMH, the BSTmv and BSTmd, and the PVN. Furthermore, six brain regions, all members of the social brain network, had some Fos-positive neurons, but the number of activated neurons did not increase in response to nestlings and we did not visually note c-fos increase in any additional brain region. The appearance of Fos can be interpreted as an indicator of increased neuronal activity in response to offspring and the subsequently performed parental behavior. Since the separation of social pairs did not result in any difference, it is unlikely that the absent mate caused neuronal activation in the examined parents. However, the combined removal of the offspring and the mate could be a stressor and may have contributed to the Fos activation, although to a similar degree in both groups. The effect of the reintroduced nest box and nest material was not examined in the present study, but both of these factors could contribute to the measured neuronal activation. Apart from these limitations of our data, the differences between the
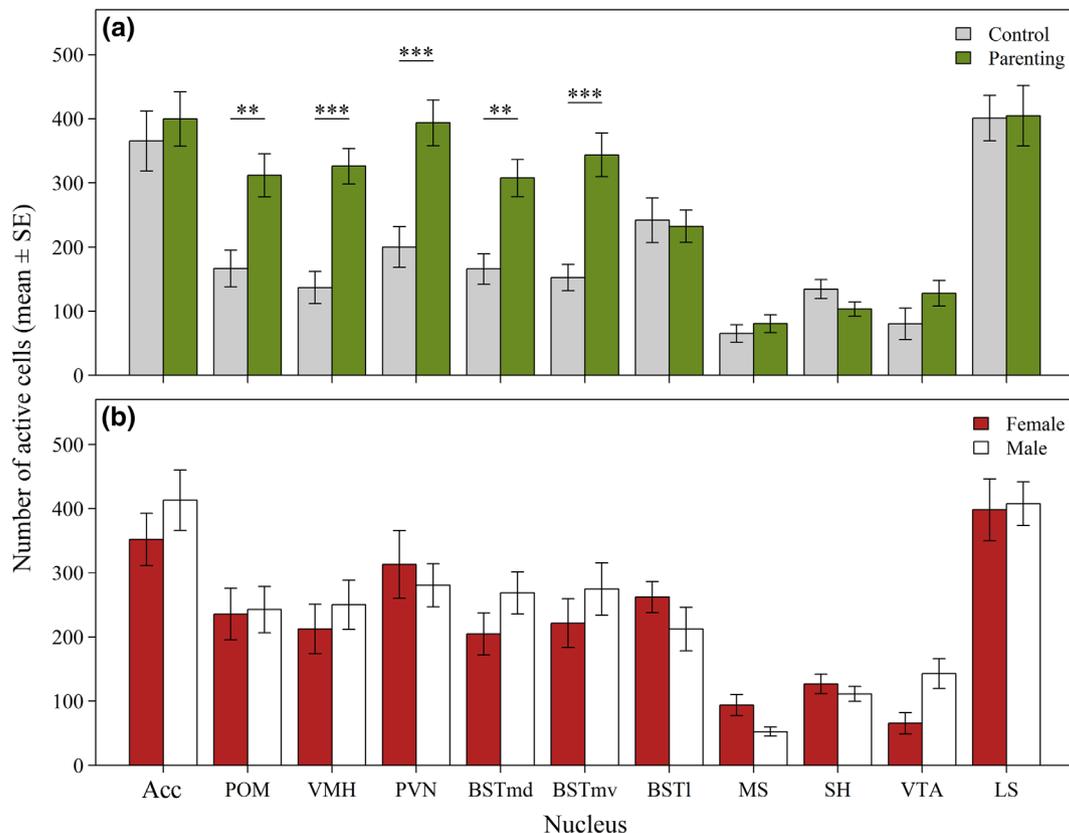
FIGURE 5 The number of Fos-positive neurons in different brain regions in parenting and control zebra finch parents (a) and between the sexes (b). Significant differences in activation levels between the experimental groups were found using linear mixed models (LMM of number of Fos-positive neurons: $\chi^2 = 69.06$, $p < .001$ [**$p < .01$; ***$p < .001$]). The five brain regions with increased activation levels during parenting are the preoptic area (POM), the ventromedial hypothalamic nucleus (VMH), the paraventricular hypothalamic nucleus (PVN), and the mediodorsal (md) and medioventral (mv) subdivisions of the bed nucleus of the stria terminalis (BST). Sex had a nuclei-specific effect independently from the experimental groups (LMM of number of Fos-positive neurons, sex × nuclei interaction: $\chi^2 = 20.51$, $p = .025$) [Color figure can be viewed at wileyonlinelibrary.com]

parenting and control groups supposedly arose from the presence of the offspring.

In the parenting group, caring behavior restored immediately after receiving the nestlings back. The parental effort increased on PHD13 compared to PHD10, which is in contrast with the normal time course of caring behavior (Morvai et al., 2016; Zann, 1996). These suggest increased motivation to care after deprivation, which was also found in prairie vole parents in a similar separation paradigm (Kelly, Hiura, Saunders, & Ophir, 2017). However, the increase in parental care could also reflect compensation because of the absent mate (Royle, Hartley, & Parker, 2002). The returning offspring can also provide a social reward, which can induce c-Fos activation in particular brain regions (Kelly et al., 2017; Lee, Clancy, & Fleming, 2000; Matsushita, Muroi, Kinoshita, & Ishii, 2015). However, none of the reward centers (Acc, VTA) showed elevated neuronal activation in response to nestlings in our study and social pairs did not have higher brain activation than isolated birds in the supplementary experiment, suggesting that offspring-induced activation was primarily elicited not by general social reward or social interactions but rather by parental care or parenting-associated behavior.
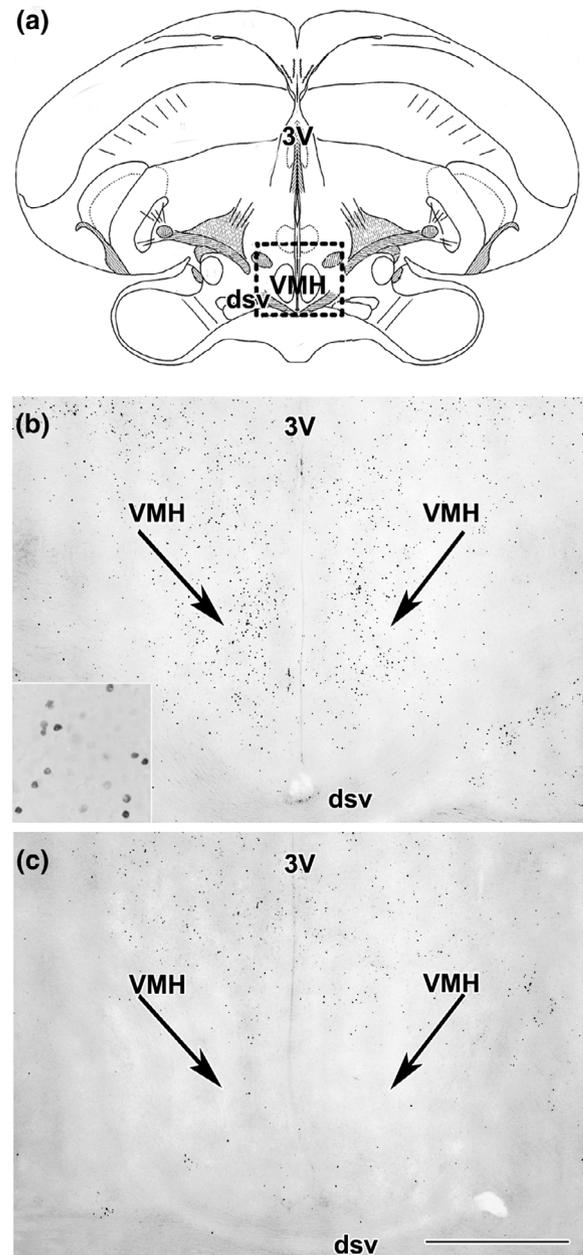
Most importantly, we found overall consistent activation patterns in females and males during parenting, suggesting that similar brain mechanisms control parental behaviors in both sexes. Moreover, chemogenetic activation of neuronal cell populations related to maternal care evoked caring behavior in males, referring to the existence of shared brain networks in the sexes (Dulac et al., 2014; Fischer & O'Connell, 2018; Kohl & Dulac, 2018). However, in biparental prairie voles, hypothalamic cell populations in males and females were found to react differentially to offspring separation. These findings imply that slight differences exist in the modulation of parental behavior between sexes (Kelly et al., 2017). Our findings promote the hypothesis of the shared regulation of parental behavior, with slightly different modulation occurring in males and females.

In line with our results, a relatively low number of brain regions have been reported previously in relation to various forms of parenting in birds (Buntin et al., 2006; Hall et al., 2014; Hall, Healy, & Meddle, 2015; Hall, Meddle, & Healy, 2015; Klatt & Goodson, 2013; Ruscio & Adkins-Regan, 2004; Smiley & Adkins-Regan, 2016, 2018). In relation to nesting behavior, an increase in the number of Fos-ir neurons was found in the POM and BSTmd of zebra finches
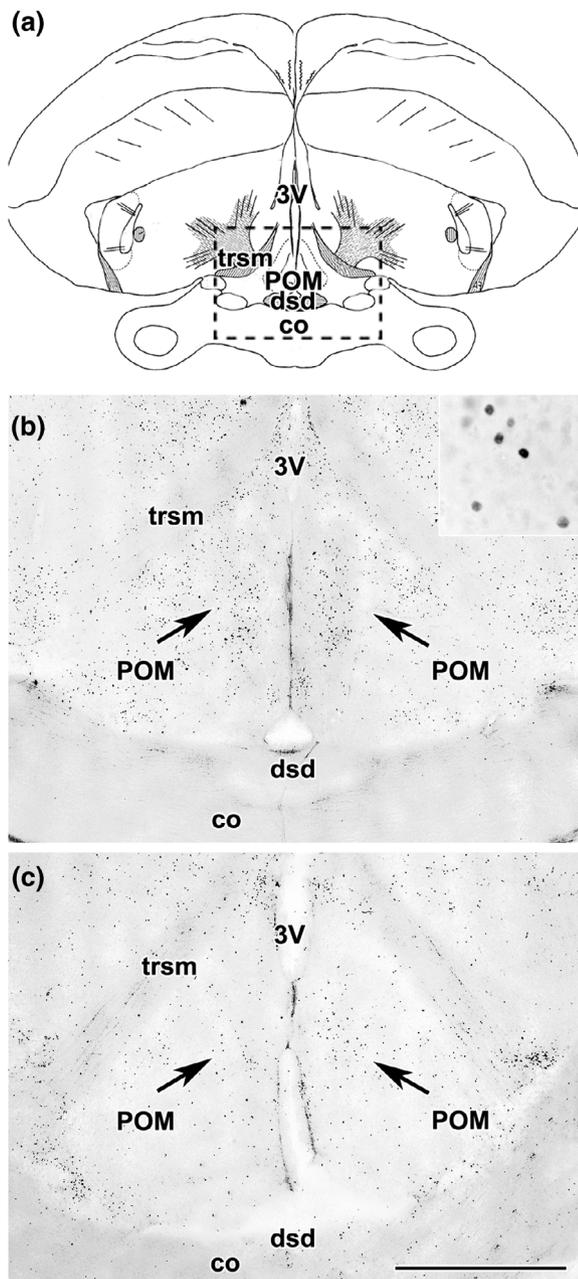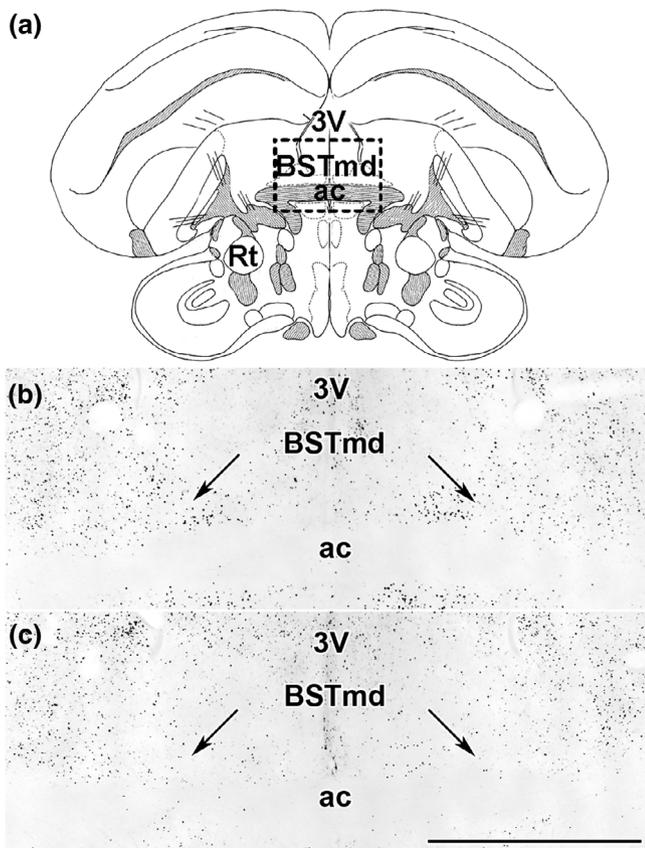
FIGURE 6   Neuronal activation in the medial preoptic area (POM) of parenting and control female zebra finches. (a) Schematic drawing of a coronal section shows the position of the POM. (b) A high density of Fos-ir neurons can be observed in different parts of the preoptic area. The arrows point to the POM. An inlet in the top right corner shows Fos-ir neurons at high magnification. (c) The density of Fos-ir neurons in the POM is low even though Fos-positive neurons are visible in other parts of the preoptic area. Parenting group showed in (b), control group in (c). Scale bar = 1 mm

FIGURE 7   Neuronal activation in the ventromedial hypothalamic nucleus (VMH) of parenting and control female zebra finches. (a) Schematic drawing of a coronal section shows the position of the VMH. (b) The arrows point to the VMH where a high density of Fos-ir neurons can be observed. An inlet in the bottom left corner shows Fos-ir neurons at high magnification. (c) The density of Fos-ir neurons in the VMH is low even though Fos-ir neurons are visible in other parts of the hypothalamus. Parenting group showed in panel (b), control group in (c). Scale bar = 500 μm

(Hall et al., 2014). Similarly, the number of Fos-positive neurons was higher in the BSTm and the entopallium (previously ectostriatum) of Japanese quail (*Coturnix japonica*) in response to brooding in sensitized females compared to females without nestlings or nonmaternal females (Ruscio & Adkins-Regan, 2004). Furthermore, exposure to nestlings in ring dove (*Streptopelia risoria*) parents led to an increase in the number of Fos-positive neurons in the POM and the lateral

hypothalamus (Buntin et al., 2006). The activation pattern found in our study has both similarities to and differences from the above-described findings. The similarities (POM, BSTm) suggest that these brain regions are involved in various types of parental behaviors (i.e., nest building and offspring provisioning, too) and play a general role in parental regulation. In contrast, VMH activation in our study and the activation of the lateral hypothalamus and entopallium in

FIGURE 8 Neuronal activation in the mediodorsal subdivision of the bed nucleus of the stria terminalis (BSTmd) in parenting and control female zebra finches. (a) Schematic drawing of a coronal section shows the position of the BSTmd. (b) A high density of Fos-ir neurons can be observed in the BSTmd. (c) The density of Fos-ir neurons in the BSTmd is low even though Fos-positive neurons are visible in other parts of the brain. Parenting group showed in (a), control group in (b). Scale bar = 1 mm

other studies suggest either species-specific differences (i.e., zebra finch vs. ring dove), or the selective involvement of these regions in different types of parental behaviors (e.g., some of these nuclei might be involved in offspring provisioning but not in brooding, and vice versa). In addition, based on published images, we cannot exclude the alternative explanation that the lateral hypothalamus in a previous article (Buntin et al., 2006) actually corresponds to the VMH in our study.

4.2 | Functional implications of the activated brain regions

In this present study, increased neuronal activity was detected in the POM in parenting zebra finch parents compared to controls, which suggests the role of the POM in offspring provisioning of zebra finches. The above-mentioned studies have suggested the involvement of the POM in the nest building and brooding behavior of bird species (Buntin et al., 2006; Hall et al., 2014). Furthermore, in male European starlings (*Sturnus vulgaris*), the POM was described in

relation to song production (Heimovics & Riters, 2005, 2006), while in Japanese quails (*Coturnix japonica*), the POM was found to control sociosexual behaviors (Iyilikci, Balthazart, & Ball, 2016). All these data suggest a general role of the POM in regulating different parental behaviors. Congruent with this view, the mammalian medial preoptic area (MPOM) was defined to be the main regulatory center of maternal behavior in rodents, as lesion of this area disrupts various forms of maternal behavior (Bridges, 2015; Gray & Brooks, 1984). The MPOM integrates the received neuronal input from pups with inputs from other brain regions and monitors changes in steroid hormone levels (Dobolyi, Grattan, & Stolzenberg, 2014; Rilling & Young, 2014). The POM is also described as being one of the most conserved brain regions with regard to controlling parental behaviors, as it was noted in previously investigated species (Goodson, 2005; O'Connell & Hofmann, 2012). Therefore, similar activation in response to offspring in zebra finch suggests that it may also have a central role in the control of parental responsiveness in birds.

In line with recent studies highlighting the role of the BST in parenting, both the medial ventral and medial dorsal subdivisions of the BST (BSTmv, BSTmd), but not the lateral subdivision, were found to exhibit increased activation during offspring provisioning in the zebra finch. The BSTm has already been characterized as showing increased neuronal activation during nest box possession in male starlings (Heimovics & Riters, 2005, 2007) and during nest building in zebra finch (Hall et al., 2014; Hall, Meddle, & Healy, 2015). It was hypothesized that these regions do not directly regulate nest-building behavior but may be involved in maintaining the reproductive status connected to nest building (Hall et al., 2014). Furthermore, the BST contains nonapeptides, which colocalize with Fos in response to positive social stimuli, thus, this brain area may regulate general social behaviors, including provisioning, nest building, and other prosocial behaviors by expressing nonapeptides (Bharati & Goodson, 2006; Goodson & Kabelik, 2009; Hall, Healy, & Meddle, 2015; Hall, Meddle, & Healy, 2015).

The paraventricular nucleus is well known to contain oxytocin- and vasopressin (AVP)-expressing neurons. These cells were found to be sensitive to separation from pups (Insel & Harbaugh, 1989; Zimmermann-Peruzzatto, Lazzari, de Moura, Almeida, & Giovenardi, 2015), and these hormones are involved in maternal control. Fathers in biparental species showed increased oxytocin expression in the PVN compared to virgin males; therefore, prairie vole males have also been shown to go through physiological and endocrine adaptation (Bales & Saltzman, 2016; Kenkel, Suboc, & Sue Carter, 2014). In biparental rodents, oxytocin and vasopressin cells have a contradictory activation pattern after pup separation (oxytocin cells showed low activation and vasopressin cells showed high activation), although both oxytocin and vasopressin cells were found to increase neuronal activation in response to pup exposure (Kelly et al., 2017; Kelly & Goodson, 2014a, 2014b; Kenkel et al., 2012; Pirnik et al., 2009). The role of the homologues of these hormones is less understood in birds, but limited evidence suggests that mesotocin and vasotocin are located in the avian paraventricular nucleus (Chokchaloemwong et al., 2013), and they were found to modulate behavior (Kelly & Goodson,

2014a; Kelly & Goodson, 2014b). The activation of the PVN in our study suggests its possible role in the regulation of parental behavior in response to offspring in both sexes, although we cannot completely exclude the possibility that offspring removal can evoke stress-related activation. Further investigation of vasotocin and mesotocin coexpression with c-fos in the PVN would be interesting to define their role in parenting.

The ventromedial hypothalamic nucleus (VMH) participates in the regulation of lordosis behavior in rats (Pfaff & Sakuma, 1979a, 1979b). Although the activation of the VMH during parental care has not been reported before, we found a highly significant increase in

TABLE 3 Results from the final linear mixed model of neuronal activation

Explanatory variables in the model	Parameter estimate [95% CI]	t_{10}	p
Nucleus[a]			
Acc	−62 [−137; 14]	−1.50	.135
POM	−234 [−309; −158]	−5.70	.000
VMH	−278 [−354; −203]	−6.79	.000
PVN	−180 [−256; −104]	−4.39	.000
BSTmd	−263 [−338; −187]	−6.40	.000
BSTmv	−271 [−346; −195]	−6.60	.000
BSTl	−130 [−205; −54]	−3.16	.002
MS	−310 [−386; −235]	−7.57	.000
SH	−254 [−330; −179]	−6.20	.000
VTA	−355 [−430; −279]	−8.65	.000
EG[b]			
Parenting	2 [−71; 75]	0.05	.960
Sex[c]			
Male	−32 [−105; 40]	−0.82	.414
EG × nucleus			
Acc × parenting	31 [−57; 118]	0.65	.519
POM × parenting	142 [54; 229]	2.99	**.003**
VMH × parenting	185 [98; 273]	3.92	**<.001**
PVN × parenting	190 [102; 277]	4.01	**<.001**
BSTmd × parenting	138 [51; 225]	2.92	**.004**
BSTmv × parenting	188 [100; 275]	3.96	**<.001**
BSTl × parenting	−13 [−100; 74]	−0.28	.782
MS × parenting	12 [−76; 99]	0.25	.805
SH × parenting	−35 [−122; 53]	−0.73	.466
VTA × parenting	44 [−44; 131]	0.92	.357
Sex × nucleus			
Acc × male	52 [−36; 139]	1.09	.276
POM × male	−2 [−89; 85]	−0.04	.966
VMH × male	28 [−59; 116]	0.60	.549
PVN × male	−42 [−129; 46]	−0.88	.378
BSTmd × male	55 [−33; 142]	1.15	.250

(Continues)

TABLE 3 (Continued)

Explanatory variables in the model	Parameter estimate [95% CI]	t_{10}	p
BSTmv × male	44 [−43; 131]	0.93	.354
BSTl × male	−59 [−146; 28]	−1.25	.214
MS × male	−51 [−138; 37]	−1.07	.287
SH × male	−25 [−112; 62]	−0.53	.599
VTA × male	68 [−19; 155]	1.44	.153

Note: The model included the two-way interactions of experimental group × nucleus and sex × nucleus. Parenting had significant effect in five brain regions while sex differences were not significant for any brain area even though sex had a significant nuclei-specific effect when females were compared to males (refer text).
Abbreviations: Acc, nucleus accumbens; BSTmd/BSTmv/BSTl, mediodorsal/medioventral/lateral subdivisions of the bed nucleus of the stria terminalis; LS, lateral septum; MS, medial septum; POM, preoptic area; PVN, paraventricular hypothalamic nucleus; SH, septohippocampal nucleus; VMH, ventromedial hypothalamic nucleus; VTA, ventral tegmental area.
[a]Nucleus: factor with 11 levels.
[b]EG: experimental group, factor with two levels [control, parenting].
[c]Sex: factor with two levels [female, male].
P values of significant changes are highlighted by bold numbers.

the number of activated neurons after reunion with the nestlings. The activation of neurons in the VMH implies that they may play a role in regulating parental behavior in birds. Alternatively, the VMH could be involved in more general social interactions, as the activation of the VMH was reported during female-directed song production in male zebra finches (Hara, Kubikova, Hessler, & Jarvis, 2007). A further possibility is that as the VMH is involved in the control of food intake, it could be activated because of metabolic changes and because of regurgitation of food to offspring in zebra finch parents. Clarifying the role of the VMH in parenting requires additional experimental evidence to be obtained in future studies.

The reward system of the brain; the VTA-Acc pathway have been reported to show an increased number of Fos-positive neurons in response to pups, which is referring to maternal motivation in rodent mothers (Matsushita et al., 2015). In the zebra finch, high number of Fos-positive neurons were found in the Acc in both experimental groups, suggesting that parents show motivation to provide care, even if their offspring are absent. The high neuronal activation could be the reminiscence of motor-driven gene expression related to feeding behavior, as various movements evoke gene expression in the ventral striatum (Feenders et al., 2008). Our correlative results between neuronal activation of the Acc and feeding behavior suggest a specific role of the Acc in controlling feeding behavior in the zebra finch, however, to explain the function of Acc is difficult as no difference occurred between groups.

Other differences in activation patterns between rodents and zebra finches were also apparent, even though the experimental situation was similar. In rodents, intense activation was also described in the lateral septal nucleus (Li et al., 1999; Lonstein et al., 2000). This result is likely related to pup retrieval behavior and maternal aggression demonstrated by dams (Flannelly, Kemble, Caroline Blanchard, &
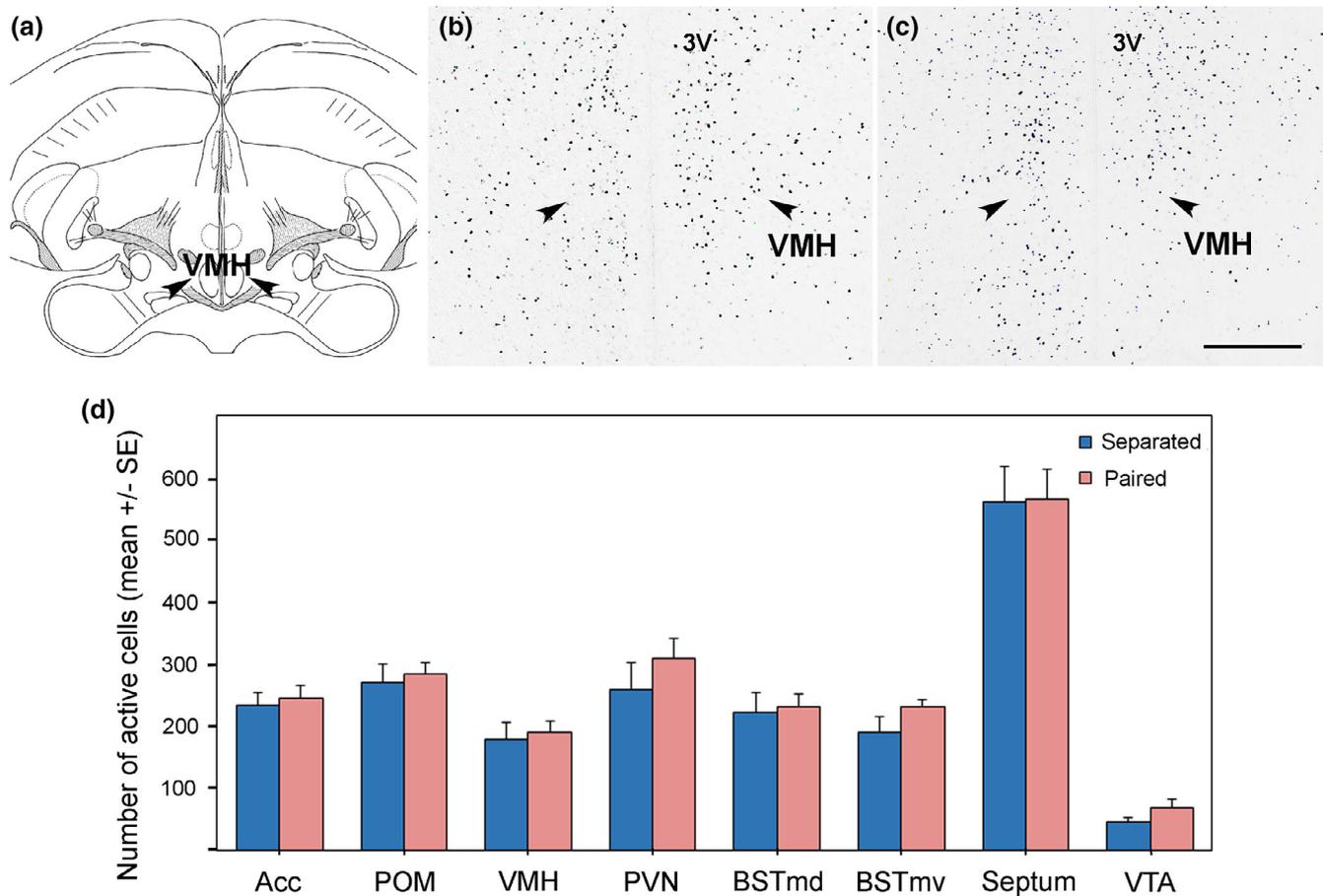
FIGURE 9 The lack of neuronal activation in response to separation of social pairs with the ventromedial hypothalamic nucleus (VMH) of males as a histologically demonstrated example. (a) Schematic drawing of a coronal section shows the position of the VMH. (b) A high density of Fos-ir neurons can be observed in the VMH of socially paired male. (c) A high density of Fos-ir neurons in the VMH of separated male. Scale bar = 500 μm. (d) Quantitative analysis of Fos-ir neurons in different brain regions of socially paired and separated males. None of the nuclei showed significant difference between groups [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 4 The results of neuronal activation social pairs with and without their separation (n = 10)

Number of active cells in brain regions (MEAN ± SE)		
	Experimental group		Student's t-test
Nucleus	**Social pair**	**Separated pair**	**(p-value)**
Acc	246.2 ± 20.5	234.5 ± 20.2	.69
POM	284.5 ± 18.3	270.4 ± 30.3	.69
VMH	190 ± 19.5	179.1 ± 26.4	.74
PVN	309.6 ± 31.9	258.5 ± 44.2	.62
BSTmd	231.6 ± 20.2	222 ± 33	.81
BSTmv	232 ± 10.5	191.1 ± 24.7	.15
Septum	566 ± 48.5	561.3 ± 57.8	.95
VTA	69.4 ± 13.9	45.6 ± 6.4	.14

Note: Each nucleus was compared separately between the groups (mean ± *SE*). No significant difference was detected between socially paired and separated males (p > .05).
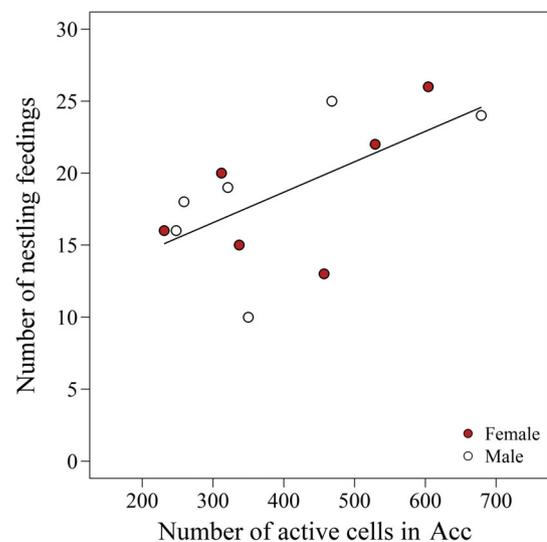
FIGURE 10 Linear regression of feeding visits and neuronal activation in the nucleus accumbens of zebra finch parents. Frequency of feeding increased with neuronal activation in the Acc ($r = .62$, $t_{10} = 2.53$, $p = .030$) and the VMH showed a trend-like relationship ($r = .55$, $t_{10} = 2.11$, $p = .061$) [Color figure can be viewed at wileyonlinelibrary.com]

Blanchard, 1986). We included three subdivisions of the septum in the analysis: The lateral region (LS), the medial region (MS) and the septohippocampal region (SH). We found relatively high neuronal activity in the lateral septum; however, the number of Fos-positive neurons was not different between the experimental groups. Similarly, Fos activation in the PAGvl is well documented in female rodents, which is responsible for the kyphosis posture during nursing (Li et al., 1999; Lonstein et al., 1998; Lonstein & Stern, 1997). Another brain region that shows intense activation in rodents but not in the zebra finch is the PIL. This brain region is thought to play a role in the relay of suckling information to the hypothalamus (Cservenák et al., 2013; Cservenák, Keller, et al., 2017; Cservenák, Kis, et al., 2017). The lack of the above-discussed behaviors in birds may provide an explanation for why these brain regions did not show increased neuronal activation in response to the presence of the nestlings in the zebra finch.

5 | CONCLUSION

We identified five brain regions in both sexes that exhibited activation during offspring provisioning in the zebra finch. The number of activated brain regions was lower and only partially overlapped with those of rodents. The identified brain nuclei, however, are more likely to be responsible for behavioral rather than physiological changes during parenting. Behavioral differences and associated neuronal activation patterns correspond to differences in modulation between vertebrates. Thus, the same circuits may be involved in the regulation of parental behaviors in both sexes, as our results suggest that females and males have similar parental brain activation patterns. Our findings support the existence of evolutionarily conserved neuronal circuits, with slight differences in the modulation of neuronal pathways responsible for parental behavior in different species.

AUTHOR CONTRIBUTIONS

E.A.F. carried out the experiments, the behavioral and histological analyses and drafted the manuscript. B.M. participated in behavioral experimenting. G.Z. participated in designing the experiment and histological analysis. F.D. performed the Western blotting validation of the antibody. T.S. participated in designing the experiment and revised the manuscript. Á.P. participated in the design, the behavioral procedures, the statistical analysis, and the interpretation of the data and revised the manuscript. Á.D. participated in the design, the histological procedures, the analysis, the interpretation of the data, and the revision of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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