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Distinct neuroendocrine mechanisms control neural activity underlying sex differences in sexual motivation and performance

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Abstract

Sexual behavior can be usefully parsed into an appetitive and a consummatory component. Both appetitive and consummatory male-typical sexual behaviors (respectively ASB and CSB) are activated in male Japanese quail by testosterone (T) acting in the medial preoptic nucleus (POM) but never observed in females. This sex difference is based on a demasculinization (= organizational effect) by estradiol during embryonic life for CSB, but a differential activation by T in adulthood for ASB. Males expressing rhythmic cloacal sphincter movements (RCSM, a form of ASB) or allowed to copulate display increased Fos expression in POM. We investigated Fos brain responses in females exposed to behavioral tests after various endocrine treatments. T-treated females displayed RCSM but never copulated when exposed to another female. Accordingly they showed an increased Fos expression in POM after ASB but not CSB tests. Females treated with the aromatase inhibitor Vorozole *in ovo* and T in adulthood displayed both male-typical ASB and CSB and Fos expression in POM was increased after both types of tests. Thus the neural circuit mediating ASB is present or can develop in both sexes but is inactive in females unless they are exposed to exogenous T. In contrast, the neural mechanism mediating CSB is not normally present in females but can be preserved by blocking the embryonic production of estrogens. Overall these data confirm the difference in endocrine controls and probably neural mechanisms supporting ASB and CSB in quail and highlight the complexity of mechanisms underlying sexual differentiation of behavior.

Keywords

Appetitive sexual behavior; Consumatory sexual behavior; Fos expression; Sexual differentiation; Japanese quail

INTRODUCTION

The relationships between sex differences in brain structure and behavior among vertebrates have been the subject of extensive and at times controversial research during the last few decades (Becker *et al.*, 2008). When the seminal paper of Phoenix *et al.* (1959) proposed a theory of sexual differentiation based on organizational (i.e., ontogenetic) and activational

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(i.e., adult) effects of steroid hormones, it was generally thought that microscopic morphological brain sex differences or differences in peripheral physiology were responsible for behavioral variation. Enough prominent neuroanatomical sex differences have now been identified to support the notion that brain sex differences do underlie behavioral differences (Cahill, 2006; Becker *et al.*, 2008; Ngun *et al.*, 2011). However, the general applicability of this link has been questioned (Kimchi *et al.*, 2007; Jordan-Young, 2010).

Japanese quail (*Coturnix japonica*) are a useful species to analyze sex differences in brain and behavior (Ball & Balthazart, 2010). Male-typical consummatory (i.e., copulatory) sexual behaviors (CSB) and rhythmic cloacal sphincter movements (RCSM), a part of the appetitive sexual behavior (ASB) repertoire, are never produced naturally by female quail (Adkins, 1975; Balthazart *et al.*, 1983; Balthazart *et al.*, 2009). The neuroendocrine sex difference regulating RCSM is activational in nature: testosterone (T)-treated females produce RCSM in a male-like fashion and absence of this behavior in untreated females only reflects their lower plasma T concentrations (Adkins-Regan & Leung, 2006; Cornil *et al.*, 2011). In contrast, sex differences in copulatory behaviors are organizational: females never exhibit this male-typical behavior even after adult treatment with T because they were exposed during embryonic life to ovarian estrogens (Adkins, 1975; Balthazart *et al.*, 1992; Balthazart *et al.*, 2009).

Parts of the neural circuit mediating male-typical ASB and CSB were identified in male quail based on a variety of studies (Ball & Balthazart, 2010), including increased expression of the immediate early gene Fos after expression of these behaviors (Meddle *et al.*, 1997; Taziaux *et al.*, 2006). In particular, expression of ASB (RCSM) or CSB by males induces Fos expression in the POM, the bed nucleus of the stria terminalis (BST) and other forebrain nuclei (Meddle *et al.*, 1997; Taziaux *et al.*, 2006). Females exposed to a male that will mount and copulate with them have a substantially different pattern of c-fos expression and enhanced expression of this immediate early gene extends through the entire hypothalamus including the ventromedial nucleus but not the preoptic area (Meddle *et al.*, 1999).

We investigated here based on the expression of the immediate early gene c-Fos via immunohistochemistry, brain areas activated in females that express male-typical behaviors because they were either treated with T in adulthood and/or their demasculinization was blocked by an aromatase inhibitor injected during embryonic life. We demonstrate that the neural circuit mediating male-typical ASB is present or can develop in females and exhibits activity only after they are exposed to T. In contrast, the neural substrate of CSB is not normally present in females but can be preserved in genetic females by blocking their endogenous production of estrogens during embryonic life.

MATERIAL AND METHODS

Experimental subjects

Two separate experiments were carried out with a total of 93 female quail (*Coturnix japonica*; n=48 in experiment 1, n=45 in experiment 2) that had been raised from eggs laid and incubated in our colony at the GIGA Neurosciences in the University of Liege. Throughout their life in the laboratory, birds were maintained on a photoperiod simulating long summer days (16h light/8h dark). They were raised in mixed-sex groups until 4 weeks of age and then isolated in individual cages until the end of the experiment. Birds were continuously provided with food and water *ad libitum*. All experimental procedures were in agreement with the Belgian laws on the « Protection and Welfare of Animals » and on the « Protection of Experimental Animals » and the International Guiding Principles for Biomedical Research involving Animals published by the Council for International

Organizations of Medical Sciences. The protocol was approved by the Ethics Committee for the Use of Animals at the University of Liege.

Experiment 1: Effects of exogenous testosterone on male-typical sexual behavior in females

Subjects, endocrine treatments and behavioral habituation: A total of 48 females were raised until six weeks of age and then were implanted under the skin of the neck with two 20 mm long SilasticTM capsules (Silclear Tubing, Degania Silicone Ltd, Degania Israel; 1.57 mm i.d.; 2.41 mm o.d.). Implants were filled with crystalline T for half of the females or were left empty for the non-treated control females group. These T implants mimic male quail physiological steroid levels, which produce a full activation of male sexual behavior (Balthazart *et al.*, 1983).

One to two weeks after T implantation, all experimental females (control or T-treated) were tested four times (once a day) for male-typical consummatory sexual behavior (CSB, i.e. copulation) in order to potentially provide them with sexual experience. For each test, the female was introduced in a test arena (60×40×50 cm) containing a sexually mature stimulus female and both birds were allowed to interact freely for 5 min (see (Adkins & Adler, 1972; Hutchison, 1978) for detail of behavior quantification). During the same period, all animals were also habituated to the test aquarium used for quantification of Rhythmic Cloacal Sphincter Movements (RCSM) a measure of appetitive sexual behavior (ASB) (Balthazart *et al.*, 1998; Seiwert & Adkins-Regan, 1998; Adkins-Regan & Leung, 2006). They were placed five times (once a day) in an empty aquarium (40×20×25 cm) for five minutes.

Final behavior test and brain collection: Females in the control (Fem) and T (Fem+T) groups were then assigned to three sub-groups (No sex as a control=C, ASB or CSB) matched on the basis of behavioral data collected during habituation tests, on their cloacal gland area and body mass. This generated six final experimental groups (Fem-C, Fem-ASB, Fem-CSB, Fem+T-C, Fem+T-ASB, Fem+T-CSB; see figure 2 for final number of subjects in each group). Four days after the last habituation test, all birds were killed and their brain collected for histological analyses 90 min after a final behavior test that was different in each of the three sub-groups. Birds were either held in the experimenter's hands for 10 seconds and then put back in their home cage as a control (No sex group=C) or tested for the expression of RCSM in response to the view of an unfamiliar sexually mature female (ASB group) or for the expression of male-typical copulatory behavior (CSB group) and returned to their home cage. These behavioral tests and the associated procedures are described in the following sections.

Experiment 2: Effects of *in ovo* aromatase inhibition on male-typical sexual behavior in females

Subjects, endocrine treatments and behavioral habituation: A group of 45 female Japanese quail was used in this experiment. They were hatched from eggs laid in our colony that had been injected on embryonic day 9 (E9) with the aromatase inhibitor VorozoleTM (R83842, generously provided by Dr De Coster, Janssen Research Foundation, Beerse, Belgium; see (Wouters *et al.*, 1989; De Coster *et al.*, 1990)) or with its solvent propylene glycol as a control. For injection, a hole was drilled in the small end of the eggshell with a needle and 50 µl of VorozoleTM (VOR; 10 µg/egg in 50 µl propylene glycol, PG) or its vehicle PG (Control group) were injected under sterile conditions. The hole in the shell was then sealed with paraffin (see (Balthazart *et al.*, 1992; Cornil *et al.*, 2011) for previous experiments in quail based on the same procedures). After hatching birds were maintained in groups in the same conditions as in experiment 1. On post-natal day 42, all birds injected with VorozoleTM and half of the birds injected with PG received two SilasticTM implants

filled with crystalline T as described in experiment 1. The other half of propylene glycol injected females received empty implants of the same size as a control (C). This combination of treatments thus generated 3 experimental groups: females (Fem-C), females +T (Fem+T), and females+Vorzole+T (Fem+VOR+T). All birds were then isolated in individual cages. Two weeks later, all subjects were then habituated to the ASB and CSB test procedures as described in experiment 1.

Final behavior test and brain collection: Four days after the last habituation test, birds from these three groups were assigned to three subgroups each and tested for ASB or CSB or just handled as a control procedure (C). This generated nine final experimental groups (3 endocrine treatments by 3 behavior test conditions; see figure 2 for final number of subjects in each group). They were then killed 90 min later and their brain was collected for analysis. All procedures were the same as described in experiment 1. The sex of all subjects was confirmed at autopsy by inspection of the gonads. It is well established that the morphological aspect of the ovary is not affected in females by injections of an aromatase inhibitor on day 9 of incubation (Balthazart *et al.*, 1992; Cornil *et al.*, 2011) contrary to what is observed after such a treatment earlier in incubation (development of a testis or ovo-testis; see Wade and Arnold, 1996 in zebra finch or Elbrecht and Smith, 1992 in chicken). The morphological sexing was thus never ambiguous.

Quantification of appetitive sexual behavior (ASB): the rhythmic cloacal sphincter movements (RCSM)

Male quail possess a large external protuberance of the caudal lip of the cloaca, the cloacal gland. This sexually dimorphic, androgen-dependent structure produces, by repeated rhythmic contractions, a meringue-like foam that is transferred into the female's cloaca during copulation (Sachs, 1967; Seiwert & Adkins-Regan, 1998) and enhances male fertilization success (Cheng *et al.*, 1989a; Cheng *et al.*, 1989b). Rhythmic cloacal sphincter muscles movements (RCSM) are elicited in males by the view of a female (Balthazart *et al.*, 1998; Seiwert & Adkins-Regan, 1998; Absil *et al.*, 2002). The expression of RCSM strongly decreases after castration, but increases following a systemic treatment with exogenous T in males but also in females (Balthazart *et al.*, 1998; Adkins-Regan & Leung, 2006; Cornil *et al.*, 2011). The frequency of RCSM is a reliable measure of male appetitive sexual behavior; they are produced in anticipation of copulation when the male comes into the visual contact with the female (Ball & Balthazart, 2010; 2011).

During both experiments, females in the two ASB groups were tested for RCSM frequency in response to the visual presentation of a female. The aquarium (40×20×25 cm) used to quantify RCSM is divided into two equal chambers by a glass partition. A mirror was located under the aquarium at an angle of 45° providing the observer with an unobstructed view of the test female's cloacal gland (see (Absil *et al.*, 2002) for description of the procedure and test chamber). The experimental female was placed for two minutes and 30 sec in one of the chamber while the other chamber was kept empty and the baseline RCSM frequency was recorded during this period. A stimulus female was then placed into the empty chamber and the RCSM frequency was recorded during five minutes during which the experimental bird had visual access to a female but could not physically interact with her.

Quantification of consummatory sexual behavior (CSB)

Experimental females were placed in the test arena that contained a sexually mature stimulus female with which the subject could freely interact for five minutes. The frequency of the different behavior patterns of the male-typical copulatory sequence were recorded during the whole test time that lasted 5 min. These behaviors include neck grabs (NG), mount attempts

(MA), mounts (M) and cloacal contact movements (CCM) (see (Adkins & Adler, 1972; Hutchison, 1978) for description).

Brain collection and Fos-immunohistochemistry

Ninety minutes after the final behavior test, birds were killed by decapitation. Their brain was quickly dissected out of the skull, placed in acrolein fixative (5% in phosphate buffer 0.1 M-Saline 0.9%, pH 7.2, PBS) for two and a half hour, washed twice during thirty min in Tris buffer and cryoprotected in 30% sucrose for 48 h at 4°C. Brains were then frozen on dry ice and stored at -80°C until cut in the coronal plane from the tractus septopallio-mesencephalicus (TSM) to the third nerve level with a cryostat at -20°C in four series of 30 µm thick sections. One series was used for immunostaining. A rabbit polyclonal antibody raised against a chicken protein sequence (Fujiwara et al., 1987) prepared and validated by D'Hondt and colleagues was used to label the protein product of the *c-fos* gene (Fos) (D'Hondt *et al.*, 1999). Immunohistochemical labeling was performed using the avidin-biotin technique on free-floating sections as previously described by Taziaux et al. (Taziaux *et al.*, 2006). Sections for a given experiment were stained together in one or several batches that contained matched numbers of sections from the different experimental groups. No between group difference could therefore be induced by procedural variations in staining intensity but these variations probably explain the larger number of Fos-positive cells detected in the first experiment as compared to the second one. Sections were then mounted on microscope slides in a gelatin-based medium and coverslipped.

Fos quantification

Sections were scanned to identify brain regions containing Fos-immunoreactive (Fos-ir) cells where qualitative examination suggested the presence of a differential expression between experimental groups. Sections were then coded and the numbers of Fos-ir cells were counted in selected brain regions by computer-assisted image analysis by an observer who was blind to the experimental groups and subjects' identity. Selected fields within regions of interest were selected in a standardized manner based on predefined anatomical landmarks and were digitized by a CCD camera attached to a microscope (10x objective). Fos-ir cells were counted in the entire images (File covered= 920×700 µm) with the ImageJ software (<http://rsbweb.nih.gov/ij/index.html>). Images were made binary and manual threshold was used to discriminate the labeled cells from the background. Exclusion size was set at 10 and 100 pixels to remove the dark objects that did not have the size of a cell nucleus. Brain structures were identified based on the atlases of the quail and chicken brain (Baylé *et al.*, 1974; Kuenzel & Masson, 1988).

Fos-ir cells were quantified in seven different nuclei as described previously (Taziaux *et al.*, 2006; Taziaux *et al.*, 2008a): the medial preoptic nucleus (POM), the bed nucleus of the stria terminalis medial part (BSTM), the periaqueductal central gray (PAG), the lateral septum (SL), the nucleus taeniae of the amygdala (TnA), the ventrolateral nucleus of the thalamus (VLT) and the ventromedial nucleus of the hypothalamus (VMN). Fos-ir cells were counted at three separate rostro-caudal levels of the POM separated by 240 µm (anterior, medial and caudal i.e. at the level of the anterior commissure), two levels in the BSTM (above the anterior commissure and 240 µm more caudally where BSTM assumes a characteristic V-shape) and at one single rostro-caudal level in other nuclei. For each nucleus or field, the number of Fos-ir cells was quantified on both the left and right side of the brain and data reported are the means of these results. In POM and BSTM, analyses took into account data at each separate rostro-caudal level as well as the total of the corresponding results. The specific landmarks that were used to locate these fields have been described in detail in several previous publications from our lab (Taziaux *et al.*, 2006; Taziaux *et al.*, 2008a).

Data Analysis

Frequencies of consummatory sexual behaviors (NG, MA, M and CCM) contained a lot of zeros and therefore were compared between groups by non-parametrical statistical tests (Mann Whitney tests and Kruskal Wallis analysis of variance [ANOVA]). RSCM frequencies and numbers of Fos-ir cells were analyzed by two- or three way ANOVAs depending on the experiments (see results). When significant, ANOVAs were followed by post-hoc analyses with the Fisher protected least significant difference (PLSD) test or t tests adapted for post-hoc analyses run after repeated-measure ANOVAs. Differences were considered significant for $p < 0.05$.

RESULTS

Experiment 1

Behavior

Appetitive sexual behavior: The frequencies of RSCM produced during the 2.5 min pre-test period and then during the 5 min when the female had visual access to a another female were analyzed by a repeated measures two-way ANOVA. This analysis showed a significant effect of the test condition ($F_{1,14} = 11.27$, $p = 0.005$), the endocrine treatment ($F_{1,14} = 6.48$; $p = 0.023$) and an interaction between both factors ($F_{1,14} = 6.26$; $p = 0.026$; see Fig. 1A). Post-hoc tests demonstrated a significant difference in RSCM frequency measured in the absence vs. presence of the female in the T-treated ($p < 0.05$) but not in control birds. The RSCM frequency in the presence of a stimulus female was also significantly higher ($p < 0.05$) in the T-treated females than in control birds.

Consummatory sexual behavior: As expected based on previous work, no consummatory sexual behavior was observed when intact females or females treated with T were allowed to freely interact with a stimulus female.

Fos-ir cells—Fos-ir cells characterized by a dense nuclear staining with a clear cytoplasm were observed in a large number of brain regions. Separate two-way ANOVAs (endocrine conditions [2 levels] and type of behavior test before brain collection [3 levels] as factors) were used to assess changes in the numbers of Fos-ir cells in each of the seven nuclei considered (total POM, total BSTM, PAG, SL, TnA, VLT, VMN).

In the POM (sum of results for 3 sections), this analysis identified a significant effect of the type of behavior test ($F_{2,33} = 3.94$, $p = 0.030$) and a nearly significant interaction between behavior test and endocrine condition ($F_{2,33} = 2.86$, $p = 0.072$) but no overall effect of the endocrine condition ($F_{1,33} = 1.41$, $p = 0.234$; see Fig. 2A). Although the interaction did not fully reach the classical criterion for statistical significance, visual inspection of the average data clearly indicated that the significant test condition effect was almost exclusively related to changes in the T-treated group. Accordingly, the separate analysis of data by one-way ANOVA in the two groups of females identified no effect of test conditions in the control female group ($F_{2,17} = 0.27$, $p = 0.799$) but a fully significant effect in the T-treated group ($F_{2,16} = 3.78$, $p = 0.045$). Post-hoc Fisher PLSD test indicated that this effect resulted mostly from a significant increase in the number of Fos-ir cells in the ASB group as compared to birds in the control condition ($p = 0.017$).

The same overall pattern of results was observed in the separate analysis of Fos-ir cells in the 3 separate sections of POM (CA, CA-2 and CA-4) that were analyzed (increased average number of positive cells in the T-treated female tested in the ASB condition as compared with all other groups) but this difference never reached statistical significance even if trends were observed at the CA and CA-2 levels (CA: test: $F_{2,39} = 1.18$, $p = 0.317$, endocrine

condition: $F_{1,39} = 1.71$, $p = 0.199$; interaction: $F_{2,39} = 2.65$, $p = 0.083$; CA-2: test: $F_{2,36} = 3.18$, $p = 0.054$, endocrine condition: $F_{1,36} = 0.874$, $p = 0.356$; interaction: $F_{2,36} = 2.14$, $p = 0.132$; CA-4: test: $F_{2,37} = 2.02$, $p = 0.147$, endocrine condition: $F_{1,37} = 0.09$, $p = 0.759$; interaction: $F_{2,37} = 0.73$, $p = 0.492$.

In the 6 other nuclei, the two-way ANOVAs identified no significant effects of endocrine or test conditions nor of their interaction (all $p > 0.05$, see Table 1) with the exception of the VMN where a significant interaction was detected ($F_{2,38} = 4.59$, $p = 0.016$). Here also this effect resulted mostly from an increased number of Fos-ir cells in the ASB condition for T treated females. However, the one-way ANOVA of data in this group of females failed to indicate significant differences between test conditions ($F_{2,19} = 3.48$, $p = 0.051$) even if the post-hoc tests suggested the existence of these differences (ASB vs. C: $p = 0.074$, ASB vs. CSB: $p = 0.019$).

Experiment 2

Behavior

Appetitive sexual behavior: As expected all females treated with exogenous T in adulthood (Fem+T and Fem+VOR+T) produced a large number of RSCM when visually exposed to another female. These contractions were almost absent in the Fem group (see Fig 1B, top panel). The analysis of these frequencies by two-way ANOVA with the three endocrine conditions as independent factor and the two test conditions (with and without female) as a repeated factor identified a significant effect of endocrine treatment ($F_{2,12} = 7.32$, $p = 0.008$) and of test conditions ($F_{1,12} = 30.66$, $p < 0.001$) together with a significant interaction between these factors ($F_{2,12} = 5.89$, $p = 0.016$). The origin of this interaction is quite obvious when looking at figure 1B: RSCM frequency markedly increased in the groups Fem+T and Fem+VOR+T when they saw a female but not in the Fem group. These differences were confirmed by post-hoc tests (see figure for detail).

Consummatory sexual behavior: Consummatory sexual (copulatory) behaviors were observed exclusively in the Fem+VOR+T group as expected based on our previous experiments (Balthazart *et al.*, 1992; Cornil *et al.*, 2011). During the final test before brain collection, all females in this group that were tested for CSB displayed neck grabs (NG), mount attempts (MA) and mounts (M) and two of them even achieved cloacal contact movements (CCM). Note, however, that all females in the Fem+VOR+T group had displayed the full copulatory sequence including CCM during at least one of the pre-tests performed in the preceding days. All these behaviors were completely absent in the Fem-C and Fem+T groups both during pre-test (not shown) and the final test (average behavior shown Fig. 2B, bottom panel).

Given the small numbers of behaviors observed and their complete absence in two groups, these behavioral frequencies were analyzed by non-parametric Kruskal Wallis ANOVAs. These analyses indicated the frequencies of NG, MA or M were significantly different between the three groups ($H = 13.588$, $p = 0.0011$). The difference was obviously caused by the presence of behaviors in all birds of the Fem+VOR+T group and their absence in the others (comparison with the control Fem group: $U = 0$, $p = 0.0039$). The difference between groups of CCM frequencies observed during the last test did not reach statistical significance ($H = 5.923$, $p = 0.0517$, comparison of Fem+VOR+T with Fem group: $U = 6$, $p = 0.0662$) but if pre-tests are considered, then the treatment with VOR+T significantly increased this behavior also ($U = 0$, $p = 0.0039$).

Fos-ir cells—A general two-way ANOVA of the numbers of Fos-ir cells counted in the POM (sum of 3 sections) indicated the presence of overall differences related to the

endocrine conditions of the females ($F_{2,35} = 4.56$, $p = 0.017$) and to the type of test they had been subjected to before brain collection ($F_{2,35} = 19.40$, $p < 0.001$; see Fig. 2B). The interaction between these two factors was not significant even if a strong trend was detected ($F_{4,35} = 2.38$, $p = 0.069$). The average data in Figure 2B indicated indeed that the 3 groups of females had reacted quite differently to the behavior tests and therefore, data from each of these three groups were analyzed by separate one-way ANOVA with the behavior test conditions as factor. No effect of test conditions was detected in the control group (Fem; $F_{2,14} = 1.82$, $p = 0.198$) but in contrast very significant differences were detected both in the Fem+T ($F_{2,12} = 7.03$, $p = 0.009$) and in the Fem+VOR+T groups ($F_{2,9} = 15.93$, $p < 0.001$).

Post-hoc Fisher PLSD tests indicated that, as suggested by the average values, these overall effects had different origins. In Fem+T subjects, as observed in experiment 1, Fos-ir cells were more numerous in the ASB than in the other two subgroups whereas, in Fem+VOR+T birds, both the birds tested after ASB and after CSB had more Fos-ir cells in POM than the control subjects (See Fig. 2B for detail of statistical results). Females that displayed CCM did not express Fos in more cells of the POM than females that did not express this behavior during the last tests before brain collection.

The same pattern of results was detected specifically in POM sections collected at the level of the anterior commissure (CA; Endocrine condition: $F_{2,35} = 3.67$, $p = 0.036$; Behavior test: $F_{2,35} = 7.30$, $p = 0.002$; Interaction: $F_{4,35} = 2.25$, $p = 0.083$). In the more rostral sections (CA-2 and CA-4) treatment effects were no longer significant (CA-2: $F_{2,35} = 1.11$, $p = 0.341$; CA-4: $F_{2,35} = 2.03$, $p = 0.146$) and numbers of Fos-ir cells were affected only by the type of behavioral test (CA-2: $F_{2,35} = 12.30$, $p < 0.001$; CA-4: $F_{2,35} = 9.86$, $p < 0.001$). No interaction was found (CA-2: Interaction: $F_{4,35} = 1.09$, $p = 0.377$; CA-4: $F_{4,35} = 1.25$, $p = 0.309$).

No significant effect of the endocrine condition of the subjects, type of behavior test or interaction was detected in the six other nuclei investigated ($p > 0.132$ in all cases) except for the test condition in VLT and the endocrine condition in BSTM and VLT where statistical trends were detected [$0.10 < p < 0.05$]. However, these statistical tendencies do not correspond to obvious means' differences (see Table 2 for detail).

DISCUSSION

Previous research indicated that female Japanese quail are able to display high rates of RSCM, a measure of ASB that naturally occurs in males, provided they are treated with exogenous T (Adkins-Regan & Leung, 2006; Cornil *et al.*, 2011). In contrast, this endocrine treatment still fails to activate consummatory (copulatory) sexual behavior (i.e., CSB) in females unless their demasculinization by estradiol during embryonic life was blocked by exposure to an antiestrogen (Adkins, 1976) or an aromatase inhibitor (Balthazart *et al.*, 1992; Cornil *et al.*, 2011). These results were fully replicated here.

We now demonstrate that in the female POM, a nucleus underlying expression of both ASB and CSB in male quail (Panzica *et al.*, 1996; Balthazart *et al.*, 1998), Fos expression is similarly affected by the adult or embryonic endocrine treatments. Exposure to another female providing an opportunity to express ASB or CSB did not increase Fos expression in the POM of control untreated females. After treatment with exogenous T, expression of RSCM was activated and in parallel, a significant Fos induction was detected in the POM. However, no copulatory behavior and no Fos induction was observed when an opportunity to freely interact with another female was offered. Finally, if the same T treatment was administered to females whose demasculinization had been blocked by *in ovo* injection of the aromatase inhibitor Vorozole, they expressed both ASB and CSB and in both cases a significant Fos induction was detected in the POM.

These data therefore confirm that the sex differences affecting sexual behavior and the related brain activity in quail do not relate directly to the genetic sex of the subjects but to specific organizational (for CSB) or activational (for ASB) effects of sex steroids. These findings also provide additional support for the notion that the neural substrates supporting expression of ASB and CSB are not identical and differentiate sexually in an independent manner. The neural circuit mediating male-typical ASB is probably present in adult females, as it is obviously in adult males; it only needs to be activated by exogenous T to be revealed. Alternatively, it cannot be excluded that parts of this circuit, in particular local connections (e.g., within POM), are not normally present in adult females but are capable of growing within a two week-period under the influence of exogenous testosterone. We demonstrated indeed that in male quail testosterone induces significant changes in POM anatomical organization (Charlier *et al.*, 2008) and similar effects with definite behavioral consequences could take place in females. In contrast, the circuit controlling CSB is either absent or completely non-functional in genetic females unless estrogen's action is blocked during ontogeny.

The induction of the immediate early gene Fos provides an excellent marker of brain areas that are activated during and after sexual interactions (Bialy & Kaczmarek, 1996). The exact nature of brain activity that results in Fos expression remains however difficult to specify. In theory, Fos expression could reflect the perception of stimuli originating from the sexual partner, the performance of motor acts related to copulation or reflect some of the endogenous processes often grouped under the general notion of motivation that relate to the endocrine or neurochemical control of behavior and to the perception of the partner.

In mammals specifically, nerve cut experiments demonstrated that Fos expression observed in the preoptic area following male copulatory behavior largely reflects the detection of olfactory information originating from the female and somatosensory feedback from the penis (Baum & Everitt, 1992). In quail, previous work in males indicated that, surprisingly, Fos induction in the POM is inhibited in males who had copulated with a female after their nostrils had been plugged, suggesting that olfactory information from the sexual partner is significantly involved in Fos induction (Taziaux *et al.*, 2008b). An earlier experiment had, however, detected a correlation between the number of preoptic Fos-ir cells and the frequency of cloacal contact movements performed by the male (Meddle *et al.*, 1997) suggesting that sexual performance is also implicated in Fos induction, as observed in the preoptic area and lumbar spino-thalamic neurons of male rats where Fos induction is specifically induced by ejaculation (Truitt & Coolen, 2002).

The present analysis of Fos induction in different female groups in which dissociations in the expression of ASB and CSB were experimentally induced provides new critical information on the nature of the neural activity that is responsible for increased Fos expression in the preoptic area. Control females (Fem groups in both experiments) when presented to another female were exposed to the same environmental and socio-sexual stimuli as females in the other groups but did not show any increase in preoptic Fos expression. An increase in Fos expression was only observed if females were treated with T before being exposed to the stimulus female. This could suggest that Fos activation relates to the performance of RSCM (after T treatment) or copulatory behavior (after Vorozole and T treatment). However, a previous study showed that preoptic Fos expression is decreased in olfactory deprived males even if their copulatory activity is unaffected (Taziaux *et al.*, 2008b). This suggests therefore that expression of copulatory behavior *per se* is not the critical factor in Fos induction. This is also supported by the present study showing that birds in the ASB group showed the same level of Fos expression as CSB birds. It is of course possible that Fos induction concerned somewhat different cell populations in the ASB and CSB groups as suggested by previous studies (Taziaux *et al.*, 2006) but this

anatomical specificity was not detected in the present experiment possibly due to the more limited sample size.

Therefore it appears likely that the simple perception of the female is a critical aspect of Fos induction similar to what has been observed for the stimulus which regulates the decrease in aromatase activity that has been observed following sexual interactions in male quail (de Bournonville *et al.*, 2012). However, a subject will only pay attention to the female stimuli if he/she is in the proper motivational state that is induced by the presence of T. Males and females could thus interpret similar stimuli in different ways depending on their endocrine condition and this difference would contribute to the differences in Fos expression detected between groups.

The findings described in this paper are relevant to some controversial issues that have been recently debated in the field of behavioral neuroendocrinology. One issue concerns the organization of motivated behaviors such as sexual behavior. We have argued (Ball & Balthazart, 2008), in response to some strong criticisms (Sachs, 2007), that the appetitive/consummatory distinction, does provide one with a useful way to subdivide behavior when trying to understand the underlying neuroendocrine mechanisms even though it is not always clear where to implement precisely a separation between these two aspects of male-typical behavior (Ball & Balthazart, 2008). In the current study we found that both appetitive and consummatory aspects of male-typical sexual behavior (including the associated expression of Fos) can be sex reversed by steroid hormone treatment. However, the Fos expression associated with male-typical appetitive responses could be reversed by adult hormone treatment while the pattern of Fos expression associated with consummatory behavior could only be reversed by steroid hormone manipulation of the embryo. The finding that the hormonal regulation of the sex difference observed for both aspects of the behavior is so different is consistent with the notion that the appetitive/consummatory distinction based on behavioral analyses does map onto important variation in the neuroendocrine mechanisms mediating behavioral activation.

A second controversy relates to the neural basis of sex differences in behavior. Sexual differentiation of brain and behavior utilized concepts derived from the study of the sexual differentiation of the genitals and related secondary sexual characteristics (Feder, 1981). The basic concept is that males and females are born with a bipotential substrate to develop either in a male-typical or female-typical fashion (Feder, 1981). This may be because males and females both possess two structures (such as the Wolffian ducts and the Mullerian ducts) and one structure needs to atrophy and the other develop in order to have a male-typical or female-typical morphological phenotype or there is one structure, (e.g., the genital tubercle) that has the potential to develop either into a male-typical or female-typical genitals. In either case a bipotential system differentiates into a system that is specific to one sex. Recently Dulac and colleagues (Dulac & Kimchi, 2007; Kimchi *et al.*, 2007) have proposed that the mouse brain is actually bipotential to exhibit the full complement of male-typical and female-typical behaviors but the required sensory input needs to be present in order to reveal this potential. Many aspects of these studies have been criticized (Martel & Baum, 2009) but it is true that there is extensive evidence that male and female mice are bipotential in many respects as regards the activation of male-typical sexual behavior (see references in (Martel & Baum, 2009)). Our findings illustrate how complex these sorts of questions can be. Fos expression is a property of the sex-typical sexual behavior circuit and expression related to appetitive aspects of male-typical behavior seems to be biopotential while expression related to consummatory aspects is not. Thus a very careful behavioral analysis is necessary in order to parse out properties of the neural substrate controlling sex-typical behavior and one single rule may not explain all examples of sex differences in behavior even in the same species concerning the same behavioral system!

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Figure 1.

Frequency (means + SEM) of the Rhythmic Cloacal Sphincter Movements (RCSM) and of consummatory sexual behaviors (CSB) observed during experiments 1 (Panel A) and 2 (panel B). The figure represents RCSM frequencies displayed before (no female) and after (with female) a stimulus female was presented to the experimental females of this experiment. In panel A (Experiment 1), females were either untreated (Fem) or had received implants filled with testosterone (Fem+T). In panel B, top part (Experiment 2), females were either untreated (Fem) or had received implants filled with testosterone (Fem+T) or had received testosterone implants but had also been injected on embryonic day 9 with the aromatase inhibitor Vorozole™ (Fem+VOR+T). Panel B, bottom part (Experiment 2) shows the frequencies of consummatory sexual behavior (CSB) displayed during the last test before brain collection. NG= neck grab, MA= mount attempt, M= mount and CCM= cloacal

contact movements. *= $p < 0.05$ compared to the control Fem group in the same test condition; # = $p < 0.05$ compared with the no female condition for the same experimental group.

Figure 2.

Numbers of Fos-ir cells (Mean + SEM) in the POM of female quail that were visually exposed to another female so that they could express appetitive sexual behavior (ASB) in the form of Rhythmic Cloacal Sphincter Movements (RCSM) or could freely interact with a female and express consummatory sexual behavior (CSB) even if they did not necessarily do so, or were just handled and returned to their home cage as control (C). In panel **A**, females were either untreated (Fem) or had received implants filled with testosterone (Fem+T). In panel **B**, females were either untreated (Fem) or had received implants filled with testosterone (Fem+T) or had received two testosterone implants but had also been injected on embryonic day 9 with the aromatase inhibitor Vorozole™ (Fem+VOR+T). Number of data points is indicated in each bar. * (**)= $p < 0.05$ (0.01) compared to the control (Fem) group in the same test condition; \$= $p < 0.05$ compared to the ASB group in the same endocrine condition.

Table 1

Numbers of Fos-ir cells (Mean $\pm$ SEM) in six brain nuclei of female quail that were visually exposed to another female so that they could express appetitive sexual behavior (ASB) in the form of Rhythmic Cloacal Sphincter Movements (RCSM) or could freely interact with a female and express consummatory sexual behavior (CSB) even if they did not necessarily do so, or were just handled and returned to their home cage as control (C). Females were either untreated (Fem) or had received implants filled with testosterone (Fem+T). Data were analyzed by Two-Way ANOVA with the type of behavior test and endocrine condition of the females or their interaction as factors and results (F values and associated probabilities [p]) are provided in the last 3 columns.

	Fem		Fem+T		Behavior test		Endocrine condition		Interaction	
Nucleus	C	ASB	CSB	C	ASB	CSB	F (p)	F (p)	F (p)	F (p)
BSTM	30.6 $\pm$ 3.1	41.2 $\pm$ 6.2	37.4 $\pm$ 9.7	50.3 $\pm$ 7.8	50.0 $\pm$ 6.7	32.5 $\pm$ 6.0	1.23 (0.303)	2.11 (0.154)	1.63 (0.208)	
SL	54.3 $\pm$ 8.3	56.1 $\pm$ 8.4	60.4 $\pm$ 7.5	48.1 $\pm$ 6.9	79.9 $\pm$ 14.5	70.9 $\pm$ 5.3	1.81 (0.179)	1.33 (0.257)	1.16 (0.324)	
TnA	37.8 $\pm$ 4.0	41.4 $\pm$ 5.2	48.4 $\pm$ 1.8	29.8 $\pm$ 5.9	38.1 $\pm$ 5.5	47.3 $\pm$ 7.1	3.20 (0.054)	0.78 (0.381)	0.20 (0.813)	
VLT	27.0 $\pm$ 3.1	34.6 $\pm$ 7.0	33.2 $\pm$ 3.0	27.0 $\pm$ 4.08	42.2 $\pm$ 8.8	34.3 $\pm$ 5.4	2.02 (0.144)	0.39 (0.536)	0.26 (0.772)	
VMN	20.0 $\pm$ 3.0	25.6 $\pm$ 3.9	35.4 $\pm$ 5.8	27.2 $\pm$ 10.3	47.4 $\pm$ 6.4	19.2 $\pm$ 3.0	2.30 (0.114)	0.73 (0.398)	4.59 (0.016)	
PAG	27.7 $\pm$ 3.3	19.5 $\pm$ 2.0	27.9 $\pm$ 6.6	23.5 $\pm$ 5.3	30.7 $\pm$ 5.2	26.2 $\pm$ 7.6	0.07 (0.935)	0.15 (0.698)	1.03 (0.367)	

Table 2

Numbers of Fos-ir cells (Mean + SEM) in six brain nuclei of female quail that were visually exposed to another female so that they could express appetitive sexual behavior (ASB) in the form of Rhythmic Cloacal Sphincter Movements (RCSM) or could freely interact with a female and express consummatory sexual behavior (CSB) even if they did not necessarily do so, or were just handled and returned to their home cage as control (C). Females were either untreated (Fem) or had received implants filled with testosterone (Fem+T) or had received testosterone implants but had in addition been injected on embryonic day 9 with the aromatase inhibitor Vorozole™ (Fem+VOR+T). Data were analyzed by Two-Way ANOVA with the type of behavior test and endocrine condition of the females or their interaction as factors and results (F values and associated probabilities [p]) are provided in the last 3 columns.

Nucleus	Fem		Fem+T		Fem+ VOR+T		Behavior test	Endocrine condition		Inter-action
	C	ASB	CSB	C	ASB	CSB		F (p)	F (p)	
BSTM	13.1 ± 3.1	22.5 ± 1.4	20.1 ± 4.9	21.4 ± 6.9	25.5 ± 4.9	34.8 ± 11.2	24.5 ± 5.3	43.1 ± 11.2	23.1 ± 5.6	1.16 (0.345)
SL	31.8 ± 13.0	21.8 ± 4.3	27.4 ± 7.2	24.4 ± 5.7	27.2 ± 7.0	35.0 ± 6.9	32.9 ± 6.6	45.0 ± 6.1	34.2 ± 3.0	0.73 (0.575)
TnA	15.5 ± 3.4	17.3 ± 4.9	9.7 ± 2.3	15.4 ± 3.5	21.4 ± 4.3	14.2 ± 3.1	17.8 ± 5.6	20.5 ± 7.3	18.7 ± 3.7	0.21 (0.928)
VLT	2.5 ± 0.8	2.9 ± 0.3	6.0 ± 1.8	3.6 ± 0.4	5.7 ± 1.2	7.0 ± 1.7	2.2 ± 1.6	3.2 ± 1.9	2.8 ± 1.1	0.54 (0.704)
VMN	10.7 ± 2.2	14.8 ± 3.1	11.4 ± 1.6	14.9 ± 4.6	20.6 ± 5.7	28.9 ± 10.2	18.5 ± 9.0	15.9 ± 7.9	12.1 ± 4.3	0.85 (0.501)
PAG	8.1 ± 3.0	7.6 ± 2.2	9.6 ± 4.4	9.5 ± 1.8	7.1 ± 0.6	9.5 ± 2.8	7.2 ± 1.8	16.4 ± 6.7	5.0 ± 1.0	1.50 (0.224)