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Fos Expression in Monoaminergic Cell Groups in Response to Sociosexual Interactions in Male and Female Japanese Quail

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Abstract

Monoaminergic neurotransmitters regulate different components of sexual behaviors, but how the different monoaminergic cell groups selectively regulate these behaviors is not well understood. We examined the potential contribution of these different cell groups in the control of different aspects of sexual behaviors in male and female quail. We used double-label immunohistochemistry, labeling the protein product of the immediate early gene, Fos, along with tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH), markers for catecholaminergic or indolaminergic cells, respectively. Rhythmic Cloacal Sphincter Movements (RCSM) were recorded as a measure of male appetitive sexual behavior. Consummatory sexual behaviors were evaluated based on the species-typical copulation sequence. Enhanced Fos expression in the medial preoptic nucleus and bed nucleus of the stria terminalis was observed in association with both physical and visual contact to the opposite sex for males, but not for females. Fos induction associated with physical contact was observed in the ventral tegmental area and anterior periaqueductal gray in both sexes. In males only, the number of Fos-immunoreactive (ir) cells increased in the visual contact condition in these two dopaminergic cell groups, however no significant effect was observed for double-labeled TH-Fos-ir cells. In addition, consummatory but not appetitive sexual behavior increased Fos expression in TPH-ir cells in the raphe pallidus of males. This increase following physical but not visual contact agrees with the notion that activation of the serotonergic system is implicated in the development of sexual satiation but not activated by simply viewing a female, in contrast to the dopaminergic system.

Keywords

preoptic area; bed nucleus of stria terminalis; monoaminergic cell groups; sexual behavior; immediate early genes

Social behavior generally defined includes any interactions among conspecifics. Species can vary greatly in the degree to which they spend time with one another so the extent and intensity of social behavior can vary greatly (Robinson et al., 2008). Fundamental aspects of social relationships start in the context of reproduction (i.e., sexual and parental behaviors; Insel & Fernald, 2004). For example, in sexually reproducing species, especially those with internal fertilization, a male and a female, must meet up for gamete transfer to occur so even if a species is largely solitary, engaging in sexual reproduction requires some sort of social interaction (Ball & Balthazart, 2002; Adkins-Regan, 2005). Depending on the specific

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context in which sexual behavior occurs males and females may be in different motivational states and may approach these sexual interactions in different ways (Ball & Balthazart, 2002; Adkins-Regan, 2005). In order to understand the neural regulation of social behaviors, males as well as females should be investigated and their respective behavioral contexts should be specified (Insel & Fernald, 2004).

Japanese quail (*Coturnix japonica*) readily exhibit robust sexual behaviors under captive conditions. In standard testing arenas males exhibit both appetitive aspects of male-typical sexual behavior (searching and pursuing a female) as well as consummatory behaviors (i.e., the copulatory sequence per se; Balthazart & Ball, 1998). Under these same conditions females do not reliably display behaviors that can be considered as appetitive behaviors but do engage readily in consummatory behaviors (i.e., female-typical copulatory behaviors). There is substantial evidence indicating that dopaminergic, noradrenergic and serotonergic cell groups are involved in the regulation of social interactions associated with sexual reproduction in a variety of vertebrate species (Balthazart & Ball, 1998; Pfau, 2009). In particular, numerous studies have addressed the excitatory role of DA and inhibitory role of serotonin (5-HT) especially in the regulation of male sexual behavior (Hull, Muschamp, & Sato, 2004). For example, *in vivo* microdialysis in the medial preoptic area (mPOA) demonstrated an increase in extracellular DA activity during precopulatory exposure to female conspecifics in male quail (Kleitz-Nelson, Dominguez, & Ball, 2010) and rats (Hull, Du, Lorrain, & Matuszewich, 1995). Furthermore, D₁ dopamine receptor agonists facilitated copulatory behaviors and prolonged the time spent with the estrus female in male rats (Beck, Bialy, & Kostowski, 2002). In male quail, appetitive and consummatory behaviors decreased when animals were treated with D₁ receptor antagonists, but increased when animals were treated with D₁ receptor agonists (Balthazart, Castagna & Ball, 1997). This line of research indicated the importance of DA both in initiation of sexual behavior and copulatory performance, and emphasized the role of dopamine on motivational aspects of the sexual behavior.

In contrast, serotonin is known to have an inhibitory effect on male and female sexual behavior. Studies in various vertebrate species indicate that endogenous serotonin release contributes to the onset of sexual satiety (Hull et al., 2004). For example, administration of 5-HT to mPOA impaired male sexual behavior in rats (Fernández-Guasti, Escalante, Ahlenius, Hillegaart, & Larsson, 1992). Additionally, *in vivo* microdialysis demonstrated that 5-HT levels increased in the lateral hypothalamic area after ejaculation in rats but were stable during the presence of female and copulation (Lorrain, Matuszewich, Friedman, & Hull, 1997), which is in agreement with the notion that activation of the serotonergic system is implicated in the development of sexual satiation. Pharmacological studies indicate that noradrenaline (NA) plays a similar role to 5-HT in male sexual behavior. A NA receptor antagonist, yohimbine, reversed the sexual inhibition due to sexual exhaustion (Rodríguez-Manzo & Fernández-Guasti, 1994). Also, injection of noradrenergic neurotoxin (DSP4) facilitated sexual behavior in male quail, indicating NA's inhibitory role in sexual behavior (Balthazart, Libioulle & Sante, 1988).

Immediate early gene studies (IEG) have been very valuable in identifying brain areas that are involved in the various components of sexual behavior in quail (e.g., Charlier, Ball, & Balthazart, 2005; Meddle et al., 1997; 1999; Taziaux et al., 2006). These studies demonstrated an increase in IEG expression in a number of nuclei in response to different components of sexual behavior and therefore established critical evidence for the parts of the neural circuitry controlling the different aspects of sexual behavior. In addition, studies that are investigating effects of testosterone on female appetitive and consummatory behavior indicated that different neuroendocrine mechanisms are involved in different aspects of

sexual behavior as evident from differentiated IEG expression in preoptic medial nucleus (POM) of female quail (Balthazart, Corbisier de Meaultsart, Ball, & Cornil, 2013).

Even though, these pharmacological and IEG studies provided valuable evidence concerning the roles of different monoaminergic neurotransmitters in modulation of sexual behavior, the specific role of different monoaminergic cell groups is still not well understood. In particular these studies did not investigate the roles of: 1) indolaminergic cell groups in different aspects of sexual behavior, 2) catecholaminergic cell groups in relation to appetitive sexual behavior in males 3) either of these cell groups in naturally occurring female appetitive or consummatory behaviors. Therefore in the present study, we attempted to dissociate the roles of these monoaminergic cell groups in different aspects of sexual behavior in male and female quail.

One particular way of investigating the effects of specific monoaminergic cell groups on sexual behavior is combining expression of immediate early genes (IEGs), like Fos, with tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) immunohistochemistry. TH is the rate-limiting enzyme for catecholamine biosynthesis and TPH plays the same role in the synthesis of indolamine. Hence, we mapped here the induction of the c-fos protein via immunohistochemistry in two different sets of sections that were the double-labeled for TH or TPH in brains of quail of both sexes collected after they expressed both aspects of sexual behavior.

Experimental Procedures

Method

Subjects—A total of 48 experimentally naïve adult (10 weeks old) male (24) and female (24) Japanese quail (*Coturnix japonica*) were obtained from a local breeder. All subjects were maintained on a standard 16L/8D cycle at approximately 22°C and had food and water available *ad libitum*. Both male and female quail were housed in individual cages throughout the experiment.

Apparatus—Behavioral testing took place in an aquarium that consisted of two compartments separated by opaque and transparent sliding panels. A camcorder was placed under the aquarium to allow recordings of the cloacal area to allow assessment of rhythmic cloacal sphincter movements (RCSMs), a measure of appetitive sexual behavior (see below) and another one placed in front of aquarium to provide measures of the consummatory sexual behavior, namely the frequency of neck-grabs (NG), mount attempts (MA), mounts (M) and full cloacal contact movements (CCM).

Experimental Procedures—Quail were randomly assigned to three groups in consistent pairs of one male and one female: visual contact (VC), physical contact (PC), and Control groups (n=8 per group) that remained the same throughout the experiment. At the start, all subjects experienced five consecutive days of a 15 minute pretest in which males and females interacted freely and gained sexual experience. Before the first pretest, baseline RCSM frequencies of all males were collected during 5 minutes without the presentation of female. In addition, animals were individually placed daily in a holding cage (HC) for 15 minutes for nine successive days to habituate them to the environment and handling. A five day break was given between pretests and the final testing while subjects continued to be placed daily in the HC (see figure 2).

The final testing took place on the 10th day. Like during the pretests, one male and one female quail were placed in the two different sides of the experimental chamber. In the VC group, first, RCSM frequencies of males were measured for 5 minutes, and then the opaque

panel was removed for 15 min while the Plexiglas panel remained in place allowing only visual access to the bird of the opposite sex. Once again, the RSCM frequencies of all male subjects were measured for 5 minutes starting with the onset of visual access to the female because previous work showed that RSCM frequencies rapidly decrease in these conditions (De Bournonville et al., 2013). For females, RSCM measurements were not collected, because these contractions are not produced in the presence of male quail as a correlate of appetitive behavior under natural conditions (Adkins-Regan & Leung, 2006).

For the PC group both panels were removed and the male and female quail freely interacted for 15 minutes. In this group subjects thus experienced bodily contact with the partner of the opposite sex associated with somatosensory stimulation as well as visual, auditory and possibly olfactory interactions. During the first 5 minutes of this period, frequencies and latencies of the first occurrences of NG, MA, M and CCM were recorded. These behavioral frequencies also markedly decline after the first few minutes of interaction (De Bournonville et al., 2013). Animals in the control group were placed in the holding cage they had been habituated to for 15 minutes. Animals from all three groups returned to their home cages after the 15 minute manipulation and remained in the home cages for the next 75 minutes until their brains were collected.

Fixation and Immunohistochemistry—Ninety minutes following onset of the behavioral tests, subjects were decapitated and their brain dissected out of the skull. The brains were placed into acrolein (5% in phosphate buffer 0.1 M saline) for 3 hours, washed four times in PBS (15 min) and cryoprotected in 30% sucrose for 24 h at 4 °C. The brains were then frozen on dry ice and stored at –70 °C until used. All brains were cut at 35 µm in the coronal plane using a cryostat at –20°C and sections were collected in four series.

Fos expression was then visualized by immunohistochemical procedures with the Avidin Biotin Complex (ABC) technique. Three rinses with 0.01 M PBS containing 0.1% Triton X-100 were performed between each step. First, sections were incubated for 60 min in 0.3% hydrogen peroxide and in 20% normal goat serum to remove endogenous peroxidase and decrease non-specific binding. This step was followed by Avidin-biotin blocking (Vector SP-2001) for 15 min, to block possible biotin binding sites in the tissue. Then sections were incubated in the primary Fos antibody (1:10,000) for 48 hours. Afterwards, sections were incubated for 60 min in goat anti-rabbit serum. The antibody-antigen complex was localized using the avidin-biotin complex method performed with a Vector Elite Kit (ABC Vectastain Elite PK-6100, Vector Laboratories PLC) and finally, the peroxidase enzymatic activity was visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride) intensified with Nickel ammonium sulfate and chloride.

A similar technique was used for immunohistochemical labeling of TH and TPH in two different series of sections that were already labeled for Fos with the following exceptions. Sections were incubated in anti-TH antibody (1:10,000, Immunostar AB22941) or anti-TPH antibody (1:10,000, Millipore AB938) for 48 hours. Afterwards, sections were incubated for 60 min in biotinylated horse anti-mouse IgL for TH and biotinylated donkey anti-sheep IgL for TPH. The peroxidase enzymatic activity was visualized with DAB alone. Reactions were terminated by several rinses in PBS and sections were mounted and coverslipped.

Quantification of immunohistochemical results—Cells immunoreactive for Fos and cells double-labeled by Fos and either TH or TPH were quantified in several brain nuclei selected based on previous work either identifying Fos induction by sexual behavior or presence of catecholaminergic or indolaminergic cells groups or both (Ball, Tlemcani & Balthazart, 1997; Charlier, Ball & Balthazart, 2005; Meddle et al., 1997; Meneghelli et al., 2009; Tlemcani et al., 2000; Taziaux et al., 2006). Quantification of all Fos-ir, Fos-ir+TH-ir

& Fos-ir+TPH-ir cells was done by an experimentally blind observer under a light microscope by direct observation (see figure 4 & 6). To validate these counts a different experimentally blind observer collected data for POM, BSTM, ventral tegmental area (VTA, A10 dopaminergic cells), periaqueductal gray (PAG, A11 dopaminergic cells) and raphe pallidus (Rp) and a high correlation between the observers was present ($r(186)=0.923, p<.001$).

Preoptic Medial Nucleus (POM)—Fos-ir nuclei were quantified in a rectangular field of 0.58 mm^2 aligned with the ventral edge of the anterior commissure and the lateral edge of the third ventricle at a rostro-caudal level including the largest extension of the anterior commissure that corresponds to plate 14 (Interaural 3.76 mm) of the chicken atlas of Puelles, Martinez-de-la-Torre, Paxinos, Watson, & Martinez, (2007) (see figure 3 A & B).

Bed Nucleus of Stria Terminalis (BSTM)—Fos-ir cells were quantified in the BSTM at a slightly more caudal level corresponding to plate 15 (Interaural 3.28 mm) where the anterior commissure has just disappeared and the BSTM has adopted a recognizable V-shape. Quantification was performed in two rectangular 0.42 mm^2 fields, one adjacent to the third ventricle and one moved one full field more dorsally and half a field laterally to capture most of the “V” shape of the BSTM (Figure 3 C-F).

Catecholaminergic areas—Catecholaminergic areas (A10 dopaminergic cells of ventral tegmental area, VTA; locus coeruleus, LoC; A9 dopaminergic cells of substantia nigra SN; subceruleus ventrale, SCv; A11 dopaminergic cells of anterior (a) and posterior (p) periaqueductal gray, PAG) were localized based on the chicken atlas of Puelles et al., (2007) and (Charlier, Ball & Balthazart, 2005). The borders of these nuclei were identified by the high density TH labeling and/or surrounding anatomical markers (Figure 4 B-D). Single FOS-ir, and double FOS-ir/TH-ir cells were counted under a light microscope (40X objective) by direct observation (see figure 4 A).

Serotonergic raphe pallidus nucleus—The Raphe Pallidus Nucleus (RP) is located ventral to the medial longitudinal fasciculus in plate 49 (Interaural -4.88mm) of the chicken atlas of Puelles et al., (2007) and can be defined by the high density of TPH positive cells (see Figure 4 F). Single Fos-ir and double Fos-ir/PTH-ir cells were counted under a light microscope (40X objective) by direct observation in this nucleus (see Figure 4 E).

Results

Behavioral Data

In the VC male group, the RSCM frequencies were collected in three different 5 minute time periods. The first one was on the first testing day to assess the baseline RSCMs, prior to any sexual experience. The second one was on the last day of testing prior to visual access to female and the third one was on the onset of visual presentation of female. A repeated measures oneway ANOVA indicated a significant difference ($F(2, 14) = 69.16, p<.05$, corrected for nonsphericity with Greenhouse-Geisser) between these three conditions. The contrast analysis between baseline RSCM vs. pre-visual RSCM and pre-visual RSCM vs visual RSCM exceeded the critical F value depicted by Scheffe's S test ($F_s=(2)(2,14)=7.4$) which indicated a significant difference between them (see figure 5 A).

In the PC male group, the mean latency of sexual approach was 156.6 sec. on the first day of testing whereas it was 2.75 sec on the 10th day just before brain collection. Paired sample t test demonstrated a significant decline in latency of sexual approach ($t(7)= 3.390, p < .05$; Figure 5 B). A significant increase of the frequency of neck-grabs ($t(7)= 3.389, p < .05$),

mount attempts ($t(7)=2.434, p<.05$), mounts ($t(7)=2.538, p<.05$) and cloacal contact movements ($t(7)=4.432, p<.05$) was also observed between these two tests performed on day 1 and 10 (see Figure 5 C).

These behaviors are not displayed by females during their physical interaction with males and the corresponding data are thus not available.

Fos expression in POM & BSTM

An analysis of variance (ANOVA) comparing the mean number of Fos-ir cells in POM among different experimental conditions showed a significant difference between male groups ($F(2,21)=3.972, p<.05$) but not in females ($F(2,20)=1.042, p>.05$). Post hoc analysis by the Fisher's least significant difference (LSD) test indicated an increased number of Fos-ir nuclei in POM in the PC ($p<.05$) and VC ($p<.05$) groups when compared to control condition in male quail (Figure 6 A-B).

Similarly, ANOVAs indicated a significant difference among groups ($F(2,18)=4.023, p<.05$) in the mean number of Fos-ir in the BSTM of male quail. Post hoc analysis (LSD) demonstrated a significant increase in Fos immunoreactivity in PC ($p<.05$) and VC ($p<.05$) groups when compared to control. No significant difference was found between female groups, ($F(2,21)=.735, p>.05$) (Figure 6 C-D).

Fos expression in VTA

Figure 7 presents the mean number of Fos-ir and TH+Fos-ir double labeled cells within VTA. Significant group differences were found in male quail for Fos-ir cell numbers ($F(2,19)=4.124, p<.05$) but not for TH+Fos double labeled cells ($F(2,19)=2.037, p>.05$). Post hoc analysis by the LSD test showed a significant difference between the PC and Control ($p<.05$) and between VC and Control ($p<.05$) groups (see figure 7 A-B).

In female quail, ANOVA also indicated a significant difference in number of Fos-ir cells between the three experimental groups ($F(2,22)=16.337, p<.05$). Post hoc analysis (LSD) indicated a significant difference between PC and Control ($p<.05$) and between PC and VC ($p<.05$) groups. No significant difference between groups was observed for the double labeled cells in the female VTA ($F(2,20)=0.797, p>.05$; see figure 7 C-D).

Two of the analyses reported above that concern male and female Fos-ir in VTA did not meet the homoscedasticity assumption. Therefore, to avoid drawing erroneous conclusions related to a possible violation of the ANOVAs conditions, we additionally ran non parametric Kruskal-Wallis tests for all these data. The statistical outcomes were the same as reported above for parametrical analyses, which confirms the conclusions reported in this paper.

Fos expression in PAG

In the anterior PAG, a significant group difference was present for Fos-ir cells of male quail ($F(2,19)=5.365, p<.05$). The post hoc analysis indicated a significant difference between the PC ($p<.05$) and VC ($p<.05$) and the control condition. No significant group difference was present for the double labeled cells within the anterior PAG ($F(2,19)=2.296, p>.05$) (see figure 8 A & C).

In females a significant difference among groups was also present in the anterior PAG, for single ($F(2,17)=6.130, p<.05$) and also for double labeled cells ($F(2,17)=5.114, p<.05$). Post hoc analysis (LSD) demonstrated that female quail in the PC group had elevated

number of Fos-ir ($p < .05$) and of TH+Fos-ir ($p < .05$) cells compared to the control and VC groups respectively (see figure 8 B & D).

In the posterior PAG a single significant group difference was found: numbers of Fos-ir cells differed between groups in male quail ($F(2, 18) = 6.212, p < .05$) but not in female quail ($F(2, 18) = 1.205, p > .05$). In males, post hoc analysis (LSD) yielded a significance difference between PC and control ($p < .05$) and between VC and control ($p < .05$) groups for single Fos-ir cells. No significant difference was present in numbers of double labeled cells in male ($F(2, 17) = 1.420, p > .05$) nor female quail ($F(2, 21) = 2.267, p > .05$) (see figure 8 E-H).

IEG expression in SN

ANOVAs comparing the mean number of Fos-ir cells in SN among different experimental conditions showed no significant difference for males ($F(2, 16) = 1.743, p > .05$) nor females ($F(2, 19) = 1.351, p > .05$). In addition, no significant difference was found for TH-Fos double labeled cells in males ($F(2, 16) = 0.440, p > .05$) and females ($F(2, 19) = 1.619, p > .05$).

Fos expression in noradrenergic cell groups LoC and SCv

No significant group difference in the number of Fos-ir cells, in males ($F(2, 18) = 2.287, p > .05$) and females, ($F(2, 21) = .876, p > .05$), or in double labeled cells in males, ($F(2, 18) = 2.220, p > .05$) and females, ($F(2, 21) = 1.964, p > .05$) were observed.

Similarly, in SCv, ANOVA also did not indicate any significant group differences in single Fos-ir cells for males ($F(2, 18) = 1.238, p > .05$) and females ($F(2, 19) = 1.407, p > .05$) or in double labeled cells for males ($F(2, 18) = 1.088, p > .05$) and females ($F(2, 19) = 0.730, p > .05$).

Fos expression in serotonergic Raphe Pallidus Nucleus (Rp)

The ANOVAs comparing the number of single labeled Fos-ir cells in the three groups of males indicated a significant difference ($F(2, 19) = 5.323, p < .05$; figure 9 A). In addition post hoc LSD tests indicated a significant difference between PC - VC ($p < .05$) and PC - Control ($p < .05$). The corresponding analysis found no significant difference in the number of Fos-ir cells between the three groups of females ($F(2, 19) = .182, p > .05$; figure 9 B).

The ANOVA indicated a significant group difference in the numbers of TPH+Fos immunopositive cells within the raphe pallidus nucleus of male quail ($F(2, 19) = 9.11, p < .05$). Post hoc analysis by LSD test yielded a significant difference between the PC and VC ($p < .05$) and the PC and Control ($p < .05$) groups (see figure 9 C). No significant group difference in the numbers of double-labeled cells was found in female quail ($F(2, 19) = 0.66, p > .05$; figure 9 D).

Discussion

The present study revealed an enhanced Fos immunoreactivity in a variety of brain nuclei subsequent to expression of appetitive sexual behaviors in male quail and of consummatory sexual behaviors in male and female quail. In POM and BSTM, this increase was present after expression of both aspects of sexual behavior but only in males. Fos induction associated with consummatory behavior was also observed in different dopaminergic nuclei — the VTA and the anterior PAG — in both sexes but a similar increase in Fos-ir cells number was observed only in males in the appetitive (visual contact) condition. In the posterior PAG, the increase of Fos expression was associated with both aspects of sexual

behavior and was observed exclusively in males. In noradrenergic cell groups, the LC and SCv, we observed no significant difference after expression of both components of sexual behavior. In addition, we observed that consummatory, but not appetitive, sexual behavior induced Fos expression in the serotonergic nucleus of raphe pallidus in male but not in female quail. Overall, the present study demonstrates that different monoaminergic cell groups are specifically activated, in a sex specific manner, in relation to different aspects of sexual behavior.

Behavioral results

In concordance with previous studies on appetitive sexual behavior (Seiwert & Adkins-Regan, 1998), a clear increase of Rhythmic Cloacal Sphincter Movements (RCSMs) frequency was present in male quail visually exposed to a female. In addition, just placing the male in the experimental chamber where copulation had occurred during previous tests significantly increased the RCSM frequency without any visual access to a female although to a lower level, suggesting the presence of a form of conditioning such that the anticipation of a female and of the possibility to express sexual behavior became sufficient to activate this behavior. A similar form of conditioning of RCSMs has been previously reported in experiments that associated the repeated presentation of a neutral stimulus with the view of a female (Cornil, Holloway, Taziaux & Balthazart 2004; Holloway, Balthazart & Cornil, 2005).

It should be noted that this behavioral conditioning, if it potentially influenced the frequency of RCSM on the final test day, cannot be invoked to explain the group differences in Fos induction observed after the final behavioral test. All birds in the three different groups had indeed been exposed to the same pretests and had spent similar amounts of time in the holding cage. This identical past experience thus cannot be the source of any differential pattern of Fos induction.

A pronounced increase of RCSM frequency was also observed when we compared the pre-visual with the visual condition in male quail which is indicative of an enhancement of the appetitive behaviors in these males. Under physiological endocrine conditions, i.e., in the absence of treatment with exogenous testosterone, female quail do not show male-typical RCSMs when provided with visual access to a male quail (Adkins-Regan & Leung, 2006; Balthazart et al., 2013). Their female-typical appetitive responses are also very discrete and irregularly observed when birds are tested in a confined enclosure so that they are difficult if not impossible to quantify. Therefore, although no behavioral measurements were collected for female in quail in the appetitive condition, one of the aims of the present study was to investigate the neural correlates of the visual exposure to a male in order to obtain in both sexes estimates of the neural responses to the same conditions i.e. visual or physical exposure to a sexual partner. This investigation allowed us to evaluate possible sex differences in this aspect of sexual behavior.

IEG results

Findings of the present study strengthen the view that POM has a critical role in male sexual behavior. Converging evidence from different lines of research including other IEG studies (Charlier, Ball & Balthazart, 2005; Meddle et al., 1997; Taziaux et al., 2006), effects of electrolytic lesions (Balthazart & Surlmont, 1990a), stereotaxic injections of aromatase inhibitors (Balthazart, & Surlmont, 1990b) and testosterone implants (Balthazart Evrard, & Surlmont, 1990) all support this conclusion.

The current study did not find any difference in the expression of Fos-ir material in POM between different groups of females. Previous IEG studies have also failed to find a

significant effect of sexual interactions on Fos expression in the POM of female quail exposed to their endogenous gonadal secretions (Balthazart et al., 2013; Meddle et al., 1999). Anatomical and hodological studies also imply that POM is more critical for regulation of sexual behavior in males. For instance, the volume of the POM is sexually dimorphic and smaller in females than in males (Viglietti-Panzica et al., 1986). Moreover, female quail have fewer aromatase-positive neurons projecting to the PAG than males (Carere et al., 2007).

We also found an increased number of Fos-ir cells in the BSTM of male, but not female, quail subsequent to both appetitive and consummatory sexual behavior conditions. Electrolytical lesions to POM and BSTM were previously shown to differentially influence male appetitive and consummatory behaviors: lesions to BSTM had no effect on another measure of the male appetitive sexual behavior, the learned social proximity response but they did have an inhibitory effect on consummatory sexual behaviors (Balthazart et al. 1998). However, findings of the present study indicate that both appetitive and consummatory sexual behaviors increase the number of Fos-ir nuclei in POM and BSTM for males suggesting that both structures are implicated in both component of sexual behavior.

IEG Induction in Dopaminergic cell groups

Male quail that expressed the consummatory sexual response showed increased Fos expression in the VTA, as reported in a previous study (Charlier, Ball & Balthazart, 2005). The present study also revealed a consummatory behavior-induced Fos expression in the VTA of females. However, we observed an increase in Fos-ir following appetitive sexual behavior in males but not in females.

We failed to identify a significant increase in TH-Fos double labeled cells in VTA and PAG in association with sexual behavior. Similar to our findings, initial analysis of the Charlier et al., (2005) study, also failed to demonstrate changes of TH-Fos-ir cell numbers in the VTA and PAG. However further analysis in the aforementioned study, which combined different experimental groups engaged in sexual behavior or controls, demonstrated a significant difference in TH-Fos-ir expression. Unlike in the Charlier et al. (2005) study, the design of the present study was not suitable for combining groups to increase the sample size. Thus our sample size was possibly insufficient to provide the statistical power needed to detect the changes in Fos expression specifically in TH-positive cells in VTA and PAG.

Previous work employing IEG expression has also identified changes in gene expression specific to midbrain dopaminergic cells in association with sexual behavior. For instance in zebra finches courting and mounting behaviors were associated with an increase in Fos expression in dopaminergic cells of VTA and PAG (Bharati & Goodson, 2006). The same study, also demonstrated a correlation between directed songs, as an indicator of appetitive sexual behavior, and TH-Fos-ir cell numbers in the PAG. This activation of midbrain dopaminergic neurons in association with sociosexual interactions has also been reported in mammals (i.e. Balfour, Yu & Coolen, 2004). Other studies also noted a positive correlation between TH density and directed songs and courtship behaviors in VTA (Alger, Juang, & Ritters, 2011). Overall, these studies strongly suggest that dopaminergic neurons within VTA and PAG are associated with sexual behaviors and the effect observed here on the total number of Fos-ir cells in VTA and PAG is also in concordance with these findings."

It could in addition or alternatively be considered that the neuronal activation observed in the VTA and PAG of sexually active males concerns mostly the non-dopaminergic neurons (e.g. GABAergic cells; see Tolu et al., 2013) and that these neurons indirectly activate dopaminergic cells, either after a longer latency or through another metabolic route that does not involve Fos expression.

Hodological studies of POM and VTA have demonstrated that there are ascending dopaminergic projections originating from VTA and incertohypothalamic DA cells to POM (Balthazart & Absil, 1997). Also, in vivo microdialysis studies demonstrated a release of dopamine in the male mPOA during performance of appetitive and consummatory sexual behavior (Kleitz-Nelson et al., 2010) and during copulation in rodents (Hull et al., 1995). Studies in rodents also demonstrated that incertohypothalamic DA projections to mPOA are associated with sexual behavior (Bitran et al., 1987).

It is important to note that VTA also plays a critical role in the mesolimbic reward system via its projections to nucleus accumbens, and in mammals a number of studies showed that this circuitry is involved in sexual behavior (For a review: Frohmader, Pitchers, Balfour, & Coolen, 2010; Pfaus, 2009). It is important to note that VTA also plays a critical role in the mesolimbic reward system via its projections to nucleus accumbens, and in mammals a number of studies showed that this circuitry is involved in sexual behavior (For a review: Frohmader, Pitchers, Balfour, & Coolen, 2010; Pfaus, 2009). There are a number of studies indicating a similar role of VTA in the mesolimbic reward system in birds. For example, a positive correlation has been noted between directed songs and courtship behaviors and the density of TH-ir cells in the VTA and nucleus accumbens in zebra finches (Alger, Juang, & Ritters, 2011). Also, an increase was observed in the number of IEG-ir cells in the VTA and nucleus accumbens of female white-throated sparrows in response to hearing conspecific song (Earp & Maney, 2012). Thus, the observed increase of the Fos-ir in VTA in association with sexual behavior could be related to activation of the mesolimbic reward system.

Induction of Fos in PAG was different in males and females and specialized in different subdivisions of the nucleus. There was no significant Fos induction in the posterior PAG of females whereas increased Fos-ir was present after expression of PC in the anterior PAG of females. In males, elevated Fos expression was present after performance of VC and PC throughout the rostro-caudal extent of PAG. Previous studies demonstrated sexual behavior-induced Fos immunoreactivity within PAG in males (Charlier, Ball & Balthazart, 2005) and our study now adds consummatory behavior-induced Fos-ir in females. Moreover, when only double-labeled cells are considered (TH-ir and Fos-ir), an increased expression of Fos was only observed in the female consummatory behavior group in anterior PAG. A previous study in males found no effect of copulatory behavior on the number of TH+Fos-ir cells in this structure (Charlier, Ball & Balthazart, 2005). Studies in ferrets also indicated a similar sex difference in Fos immunoreactivity in PAG TH-ir neurons (Wersinger & Baum, 1997). In mammals, a columnar organization of PAG has been proposed based both on morphological and functional criteria (Bandler & Shipley, 1994) and there is evidence for a similar organizational pattern in avian species (Kingsbury, Kelly, Schrock, & Goodson, 2011). In quail, anterior and posterior parts of the PAG correspond to different functional zones proposed by Kingsbury et al., 2011, thus the anatomically discrete patterns of IEG expression in PAG of female quail identified in this study may well be a reflection of this topographical organization.

IEG Induction in Noradrenergic cell groups

In the noradrenergic cell groups, LC and SCv, we found no significant difference in Fos induction after expression of both components of sexual behavior. A number of studies have indicated that the noradrenergic system plays an inhibitory role in male sexual behavior (Balthazart, Libiouille, & Sante, 1988; Rodríguez-Manzo & Fernández-Guasti, 1994). However, the present study did not demonstrate a significant increase in Fos-ir in either LoC or SCv suggesting that either these structures were not activated during the performance of the behavior in our testing conditions or more probably that this activation does not result in an induction of the IEG under study.

Serotonergic nucleus of raphe pallidus

The inhibitory role of serotonin in sexual behavior is well established in rodents (Fernandez-Guasti et al., 1992; Lorrain et al., 1997). For instance, an increase in 5-HT concentrations has been documented in the lateral hypothalamic area after ejaculation (Lorrain et al., 1997). The present study found elevated Fos-ir expression in the raphe pallidus only following consummatory sexual behavior in male quail. In agreement with previous findings, the present results are thus consistent with the notion that serotonergic inputs rooted in the raphe pallidus may be responsible for the modulation of the satiation of male sexual behavior.

Implications for Our Understanding of the Functional Organization of the Sexual Behavior Circuit

What have the studies reported in this manuscript told us that is new about our understanding of the neural control of sex-typical sexual behavior? The POM is a key integrative site in the male sexual behavior circuit in quail (Ball & Balthazart, 2004; 2010; Wild & Balthazart, 2013) as is the case in other vertebrate species (Hull, 2011). Although there are many gaps in our knowledge concerning its hodology, this brain area seems to receive the relevant sensory inputs and then projects via the PAG to the brain areas needed to implement the motor output required to engage in both appetitive and consummatory components of male-typical sexual behaviors (Ball & Balthazart, 2004; 2010; Wild & Balthazart, 2013). Another key component of the regulation of male sexual behavior includes the many projections to the basic sexual behavior circuit such as those by the monoamines and by various neuropeptide systems. Based on behavioral studies by Beach (1956), Everitt (1990) suggested that appetitive and consummatory aspects of male-typical sexual behavior could be differentially controlled by various aspects of the integrated hypothalamic-limbic circuit that controls sexual performance and motivation. Work completed in quail suggested that there were differences in the control of these different aspects of male sexual behavior within the POM itself (Balthazart et al., 1998) with the more rostral POM being important in the regulation of appetitive behaviors while the more caudal part was important in consummatory aspects of the behavior. In this study we have confirmed the significance of the POM and the closely connected BNST in mediating both aspects of male-typical sexual behavior and we have identified new insights into the monoaminergic modulation of the circuit.

In the current study we have observed that cell groups previously implicated in the control of consummatory aspects of male sexual behavior (Charlier, Ball & Balthazart, 2005) are involved in appetitive sexual behavior as well. So for example, the VTA exhibits gene expression when males are engaged in appetitive as well as consummatory aspects of male-typical sexual behavior as was shown previously (Charlier, Ball & Balthazart, 2005). Similar observations have been made concerning the rostral and caudal PAG. Interestingly, cell groups such as the SN and the noradrenergic cells groups such as the LoC and the SCV that did not exhibit significant IEG expression in association with consummatory behaviors in the previous study (Charlier, Ball & Balthazart, 2005) also did not for male-typical appetitive sexual behaviors in the current study. These findings suggest that modulatory catecholamine systems seem to be generally involved in the regulation of male-typical sexual behavior, in other words if a particular cell group expresses Fos in association with engaging in one component of male-typical sexual behavior it will also express IEGs in association with the other.

A novel feature of the current study involves the investigation of possible modulatory action by the serotonergic system. Cells in the Raphe pallidus did not exhibit Fos expression in association with appetitive behaviors but did in association with consummatory behaviors.

Thus the indoleamine serotonin unlike the catecholamine systems exhibits a more selective pattern of IEG expression. The number of TPH cells expressing Fos was only elevated when males engaged in copulatory behavior per se. Interestingly, in all our studies there did not tend to be a significant effect if one focused on double labeled cells, i.e. cells labeled with TH that also expressed Fos. The only exception was in females engaging in consummatory sexual behavior where Fos expression increased in TH cells in the posterior PAG. In the Raphe pallidus, the Fos expressing cells correlated with engaging in consummatory but not appetitive behaviors in males also tended to be double-labeled with TPH.

Our studies of gene expression and female sexual behaviors are harder to put in a broader context. One previous study did investigate Fos expression in female quail (Meddle et al. 1999), but much less is known about the circuit mediating the control of the female genitals in mammals or birds (Marson & Murphy, 2006; Ball & Balthazart, 2009). The somewhat preliminary evidence that is available suggests that there is much in common between male and female quail in the circuit that controls genital responses associated with sexual behavior (Wild & Balthazart, 2013). In our study we collected female quail that were each interacting with a male just prior to copulation and in females after they had engaged in sex-typical sexual behavior. Under the standard laboratory testing conditions female quail often do not exhibit clear indications of appetitive sexual behaviors so in this testing situation we describe them as having visual access to males but do not claim that are actively engaging in appetitive sexual behavior. We found no increase in Fos expression in the visual contact group for female quail; the possible variation in the appetitive state of each female may have contributed to this outcome. For females in the physical contact group an increase in Fos-ir was present in VTA and Anterior PAG but not in POM or BNST after interacting with the male and engaging in copulatory behavior. Thus VTA and PAG seem to exhibit signs of cellular activation in a sexual context in both males and females. VTA is one of the regions involved in the mesolimbic reward circuitry, and had been associated with sexual behavior in mammals. The lack of effects in other nuclei in females requires further studies of the female sexual behavior circuit using different testing conditions for females to interpret them properly.

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Figure 1.
A schematic representation of testing apparatus.

Figure 2.

A schematic representation of experimental time line indicating days of pretesting for gaining sexual experience, holding cage (HC) placements for environmental habituation and experimental manipulations.

Figure 3.

Photomicrographs illustrating the brain regions where Fos-immunoreactive (ir) cells were quantified. Panels A-B illustrate the Fos-ir cells at the rostro-caudal level of the medial preoptic nucleus (POM) located just ventral to the anterior commissure (AC) and lateral to the third ventricle (3v) for males in the physical contact (A) and control (B) groups. Sections C-F illustrate Fos-ir cells in the dorsolateral (C-D) and ventral (E-F) bed nucleus of stria terminalis (dlBSTM and vBSTM) respectively for males in the physical contact (C and E) and control (D and F) groups.

Figure 4.

Photomicrographs illustrating the Fos-ir+TH-ir (A-D) and Fos-ir+TPH-ir (E-F) double labeled cells within the regions of interest. Panel A illustrates a Fos-ir positive (blue arrow), TH-ir positive (red arrow) and doubled labeled cell (black arrow) in the substantia nigra (A9). Panels B-D illustrates Fos and TH positive cells in the ventral tegmental area (A10) (B), anterior periaqueductal gray (A11) (C), and posterior periaqueductal gray (A11) (D), blacks arrows demarcate the borders of these nuclei. Panel E illustrates a Fos-ir positive (blue arrow), TPH-ir positive (red arrow) and doubled labeled cell (black arrow) in the raphe pallidus nucleus. Panel F illustrates the Fos-ir and TPH-ir positive cells in raphe pallidus nucleus, blacks arrows demarcate the borders of the nucleus.

Figure 5. (A) Mean (± 1 SE) frequency of RSCMs within 5 min quantification periods for baseline, prior to visual access and during visual access. (B) Mean (± 1 SE) latency of first sexual approach within 5 min quantification periods. (C) Mean (± 1 SE) Frequency of neck-grabs (NG), mount attempts (MA), mounts (M), and cloacal contact movements (CCM).

Figure 6. Mean (± 1 SE) number of Fos-ir cells in the POM (A-B) and BSTM (C-D) of male (left) and female (right) quail. Asterisks indicate significant differences by post hoc LSD analysis.

Figure 7. Mean (± 1 SE) number of cells in the VTA (A10) of males (left) and females (right) that were labeled for Fos only (A and B respectively) or were double labeled for TH and Fos (C and D respectively). Asterisks indicate significant differences identified by post hoc LSD analysis.

Figure 8. Mean ($\pm$ 1 SE) number of Fos-ir (A-B, E-F) or TH+Fos-ir (C-D, G-G) cells in males (left) and females (right) in posterior and anterior PAG (A11). Asterisks indicate significant differences as identified by post hoc LSD analysis.

Figure 9. Mean (± 1 SE) number of Fos-ir (A-B) and of TPH+Fos-ir (C-D) cells in the raphe pallidus nucleus of males (left) and females (right). Asterisks indicate significant differences by post hoc LSD analysis.