Published in final edited form as:

Neuroscience. 2008 February 6; 151(3): 644–658.

ENHANCED NEURAL ACTIVATION IN BRAIN REGIONS MEDIATING SEXUAL RESPONSES FOLLOWING EXPOSURE TO A CONDITIONED STIMULUS THAT PREDICTS COPULATION

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Abstract

Stimuli associated with sexual behavior increase reproductive success if presented prior to copulation. In Japanese quail, inseminations that take place in a context that predicts the arrival of a female are more likely to result in fertilized eggs. We demonstrate here that in male Japanese quail a sexual conditioned stimulus (CS) also enhances activity in two brain regions that mediate sexual behavior, the preoptic area and the medial part of the bed nucleus of the stria terminalis. *C-fos* expression, a marker of neural activation, was higher in these areas in subjects exposed sequentially to a sexual CS and copulation than in subjects exposed to copulation or the CS alone or in subjects exposed to no sexual stimulus, either an identical, untrained CS or an empty arena. These results suggest a link between a proximate result of sexual CS presentation, male brain activation, and a known ultimate outcome, increased fertilizations.

Keywords

rhythmic cloacal sphincter movements; sexual conditioning; appetitive behavior; preoptic area; immediate early genes

Introduction

A sexual conditioned stimulus (CS) presented prior to the onset of sexual behavior enhances reproductive success. In gourami fish, a sexual conditioned red light presented to male and female pairs prior to contact between the two fish greatly increases the number of offspring produced (Hollis et al., 1997). Sexual context conditioning results in greater semen volume and more sperm release in male Japanese quail (Domjan et al., 1998). Extending these findings, Adkins-Regan and MacKillop (2003) found that inseminations that take place in a context that predicts the arrival of a conspecific are more likely to result in fertilized eggs. Similarly, a previously sexually conditioned light presented to male and female pairs of Japanese quail increases both copulatory efficacy and the percentage of fertilized eggs (Mahometa and Domjan, 2005; Çetinkaya and Domjan, 2006). Behavioral correlates of these phenomena have been well documented. In gourami, these include frontal displays and decreased aggression

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(Hollis et al., 1989, Hollis et al., 1997). Quail show conditioned approach (e.g. (Domjan et al., 1986, Holloway and Domjan, 1993, Balthazart et al., 1995), decreased latency to copulate (Gutierrez and Domjan, 1996); although see (Çetinkaya and Domjan, 2006) for an interesting case where preoccupation with the conditional stimulus delays copulation) and conditioned rhythmic cloacal sphincter movements (RCSM) (Cornil et al., 2004, Holloway et al., 2005). Little is known, however, of the neurophysiological bases of these behavior changes.

Exposing males to females is reported to activate specific brain regions. Studies of immediate early gene induction (IEG) in male Japanese quail have found that following copulation, Fos-like immunoreactivity occurs in the preoptic area/anterior hypothalamus, the medial arcopallium, and the nucleus intercollicularis (Meddle et al., 1997). Charlier and collaborators report *c-fos* and *zenk* (another IEG also commonly called *egr-1*) expression in these regions and the bed nucleus of the stria terminalis (BSTM), and an up-regulation of *c-fos* expression in the periaqueductal gray and ventral tegmental areas in sexually motivated, but not non-motivated male quail allowed to interact and eventually copulate with a quail hen (Charlier et al., 2005). The involvement of the BSTM in the male quail brain was confirmed in a subsequent study that also found copulation correlated *c-fos* induction in the sexually dimorphic medial preoptic nucleus (POM), the lateral septum (SL), and the ventro-lateral thalamus (VLT) (Taziaux et al., 2006). Several homologous regions in the mammalian brain have similarly been determined to show IEG induction correlated with exposure to sexual stimuli. For example, the odor of female urine induced Fos in the core of the nucleus accumbens of male mice with an intact vomeronasal organ (Pankevich, et al. 2006). *C-fos* immunoreactivity increased in both male and female pine voles following exposure to opposite-sex conspecifics in sexual brain regions including the rostral portions of the bed nucleus of the stria terminalis and the rostral medial preoptic area (Schwab, et al. 2004). Male rats exposed to the odor of estrous females exhibit increased Fos immunoreactivity in the accessory olfactory bulb, medial amygdala, BSTM, medial preoptic area, ventromedial hypothalamus, ventral tegmental area, and the core and shell of the nucleus accumbens (Kippin, et al. 2003; see also Veening and Coolen, 1998; and Pfaus and Heeb, 1997 for reviews).

Lesion studies with male quail indicate that copulatory behavior (Balthazart and Surlemont, 1990) and learned approach behaviors (Balthazart et al., 1998) are both affected by POM ablations, suggesting that at least in some circumstances, responses to females and to conditioned sexual stimuli are mediated by similar brain regions. If this is the case, then the enhancing behavioral effects of presenting a conditioned sexual stimulus prior to the arrival of a female may correlate with enhanced neural responses.

To explore the changes in brain activity that accompany the presentation of a sexually conditioned stimulus, we initially exposed male Japanese quail subjects to either paired or unpaired presentations of an arbitrary stimulus and copulatory opportunity with a female. Learning was assessed by evaluating the number of conditioned RCSM (Holloway et al., 2005). Ninety minutes prior to sacrifice and brain collection for *c-fos* immunocytochemistry, subjects were exposed to either two sexual stimuli, the sexual CS and a female; one sexual stimulus, either the sexual CS or a hen; or stimuli that had explicitly not been sexually conditioned.

Here we address two questions. The first is to determine what pattern of neural activation follows exposure to a CS predicting copulatory opportunity. In a previous study with male Japanese quail, exposure to a stimulus predicting visual-only access to female resulted in *c-fos* induction in different nuclei than those activated by exposure to the hen (Taziaux et al., 2007). Similar differences in *c-fos* activation following exposure to conditioned and unconditioned sexual stimuli are reported in rats (Kippin et al., 2003). The second is to explore whether presenting an increasing number of sexual stimuli results in a graded neural response.

If our hypothesis based on lesion studies of learned and unlearned sexual behavior in male quail (e.g. Balthazart and Surlemont, 1990; Balthazart et al., 1998) is correct, then subjects exposed to two stimuli should show more *c-fos* induction in sex-related brain nuclei than subjects exposed to one stimulus. This finding would be convergent with results from studies of reproductive success that indicate presenting a sexual CS, before the arrival of an opposite-sex conspecific, increases fertilizations.

Materials and Methods

Subjects and hormone treatments

Twenty-seven adult male Japanese quail served as subjects in this experiment. They were acquired from Degros Farms (Rechrival, Belgium) at approximately 1.5 month of age. Throughout experimental procedures subjects were housed individually, provided with food and water *ad libitum*, and kept on a long, 16 h L: 8 h D, photoperiod simulating the breeding season day-length. Stimulus females ($n = 8$) were housed similarly in large brooder cages.

All experimental procedures were in agreement with Belgian laws on the “Protection and Welfare of Animals” and on the “Protection of Experimental Animals,” and with 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 Liège and care was always taken to minimize the numbers of subjects and animal pain or discomfort.

Approximately two weeks after their arrival, subjects were implanted subcutaneously in the neck region with one testosterone-filled Silastic™ capsule (Silclear™ Tubing, Degania Silicone Ltd., Degania Bet, 15130, Israel; 1.57 mm i.d.; 2.41 mm o.d.; length = 20 mm), in order to avoid inter-individual plasma testosterone level variability. These implants establish in male quail testosterone plasma concentrations that are in the range of values observed in sexually active males (Balthazart et al., 1983, Balthazart et al., 1987).

Apparatus—Conditioning trials took place in a small glass aquarium that served as the test arena (40 long $\times$ 20 wide $\times$ 25 high cm). The ceiling was made of opaque mat-board. The remaining walls and floor were made of glass. One end wall was open. During conditioning trials a piece of opaque board was attached to the exterior of the glass wall facing the experimenter to prevent the subject from being distracted by the presence of the experimenter. The stimuli were presented in an apparatus that was adjacent to the open wall. A two compartment movable trolley was attached to the far side of a plywood board that served as the missing wall of the glass test arena. A 14 wide $\times$ 15.5 high cm opening in the board provided visual access to the trolley. Each compartment of the trolley was 11.5 cm deep and had the same width and height as the doorway so that only one compartment could be lined up with the doorway at a time. A vertically sliding opaque panel covered this opening and could be raised with a rope at the beginning of conditioning trials. The first compartment of the trolley contained a white, terry-cloth covered foam conditional stimulus (CS) object (9 cm long $\times$ 6 cm wide $\times$ 3 cm high with a 8 cm high $\times$ 2 cm diameter pillar at one long end). A large gauge wire mesh prevented the subject from entering this first chamber. The second compartment, open to test arena, contained a quail hen or was left empty. An overhead lamp provided illumination for both compartments. A mirror placed under the test arena at approximately a 45° angle allowed the experimenter to view the cloacal region of the subjects to assess the performance of RSCM. The experimental apparatus is represented schematically in Figure 1A.

Experimental procedures

Conditioning procedure: Behavioral procedures began one week after testosterone implantation to allow for the diffusion and action of the steroid. Prior to conditioning trials, subjects were placed into the test arena 10 times, twice per day over 5 consecutive days to habituate them to the test arena and to handling. Because of constraints of the final test trial, to be described, on half of these trials subjects were placed into the arena for 5.5 min and on half for 6.5 min. The duration in the arena was assigned each day and varied randomly between the two intervals. To further ensure that conditioning was focused on the CS object, these test-arena exposure trials continued during the conditioning phase, with all subjects being placed into the test arena 6 additional times, once per day, either in the afternoon or morning, alternating with the timing of conditioning procedures. Again, these exposures were either 5.5 or 6.5 min in duration, three of each per subject.

Immediately following the 10 initial habituation exposures, six conditioning trials were conducted for each subject, one per day for 6 days, alternating between morning and afternoon. One subject was trained at a time and the order of training was varied each day. During these 6 conditioning trials, subjects were either trained with paired presentations of an arbitrary CS (terry-cloth-covered block of foam) and sexual interaction with a female (Paired group, $n = 16$), exposed to the CS and female separated by at least 90 min (Unpaired group, $n = 6$), or placed into the test arena without exposure to the CS or female (Empty arena group, $n = 5$) and allowed to interact with a female at least 90 min later in their home cages.

For subjects in the Paired group, conditioning trials consisted of a 1 min exposure to the CS followed by 2 min of sexual interaction opportunity with a quail hen delivered from the second compartment. At the start of a conditioning trial, the vertically sliding door to the trolley compartments was closed and the CS compartment of the trolley was aligned with the opening in the plywood. The subject was placed in the test chamber for 2.5 or 3.5 min prior to stimulus presentation to reduce the likelihood that the time in the chamber would become a conditioned stimulus. After this time, the door to the trolley compartment was raised and fixed open. The subjects were exposed to the CS for 1 min and the trolley was then aligned to expose the second compartment containing a quail hen for 2 additional min. Thus, subjects were in the arena for either 5.5 or 6.5 min total on each conditioning trial. Three trials of each duration were conducted. Subjects in the Unpaired group were treated in an identical manner except exposure to the CS was followed by 2 min visual access to an empty compartment. To control for sensitization/pseudo-conditioning effects, subjects in Unpaired Group were given the opportunity to interact with a quail hen each day for 2 min in their home cage. These presentations occurred at least 90 min following conditioning trials. Subjects in the Empty Cage group were placed into the arena for either 5.5 or 6.5 min (3 times each) and were given the opportunity to interact with a female each day for 2 min in their home cage at least 90 min following exposure to the test arena. All subjects, therefore, spent the same amount of time in the arena, and the same amount of time with a female on each conditioning day. A time-line diagram of the conditioning procedures used during the 6 conditioning trials is illustrated in Figure 1B.

The number of RSCM was noted during each 2.5 or 3.5 min pre-CS and each 1 min CS period. Because RSCM occur following elimination and in response to a sexual stimulus, those RSCM that occurred after defecation were noted and excluded from analyses.

At the end of the training procedure, subjects from the Paired condition were assigned to one of three groups of six birds each that were matched based on RSCM frequency during the last trial and across the six trials. A one way ANOVA (last trial) and a mixed design ANOVA with groups as the independent factor and trials as the repeated measure were used to confirm that these groups did not differ significantly in the number of RSCM produced (sixth trial: $F_{2,13} =$

0.140, $p = 0.8702$; across trials: $F_{\text{group } 2,13} = 0.179$, $p = 0.8382$; $F_{\text{trials } 5,10} = 7.767$, $p < 0.0001$; $F_{\text{interaction } 10,65} = 0.416$, $p = 0.9339$).

Final behavioral test: Three days following the sixth conditioning trial, a test trial was administered to all subjects, one at a time. This interval was selected to maximize the probability that males would display sexual behavior in response to the presentation of the female (due to prolonged isolation in their home cage) and ensured that *c-fos* activity elicited during training would have returned to baseline for at least one or two days. As schematically represented in Fig. 1C, subjects in the Paired condition were either placed into the arena for 2.5 min, and then exposed to the CS for 1 min followed by 2 min of sexual interaction with a female (P-CS-F group, $n = 6$), placed into the arena for 3.5 min and then allowed to interact immediately with the female for 2 min (P-F group, $n = 5$), or placed into the arena for 2.5 min and exposed to the CS for 1 min after which the door to the trolley compartment was closed and subjects remained in the arena for an additional 2 min (P-CS group, $n = 5$). Subjects in the Unpaired condition were placed into the arena for 2.5 min then exposed to the CS alone for 1 min followed by 2 min exposure to the empty second trolley compartment (U-CS group, $n = 6$). Subjects in the Empty arena condition were exposed to an empty arena for 5.5 min (EA group, $n = 5$). At the end of their test, subjects were returned to their home cage. During the final behavioral test, therefore, each subject spent 5.5 min in the test arena. Further, because of the alternating 5.5 min/6.5 min training and habituation trials, the likelihood that temporal cues signaled when the various stimuli would occur was sharply reduced. The number of RSCM produced by all subjects exposed to the CS during this final test was recorded.

Fos immunocytochemistry: Ninety minutes following the end of the behavioral test, subjects were decapitated and their brain quickly dissected out of the skull. The brains were placed into acrolein (5% in phosphate buffer 0.1 M-saline 0.9%, pH 7.2) for 2.5 hours, washed twice in PBS (30 min) and cryoprotected in 30% sucrose for 24 h at 4 °C. The brains were then frozen on dry ice and stored at -80 °C until used. The forebrain of each subject (from the level of the tractus septopallio-mesencephalicus (TSM) to the caudal end of the brain) was cut in the coronal plane with a cryostat at -20°C in 4 series of 30 µm thick sections. One series of sections (120 µm between two successive sections) was stained by immunocytochemistry for the protein product of the *c-fos* gene (Fos) with a rabbit polyclonal antibody raised against an avian Fos protein sequence (Fujiwara et al., 1987) prepared and validated by D'Hondt and colleagues (D'Hondt et al., 1999). Immunocytochemical labeling was carried out by the avidin-biotin technique on free-floating sections as previously described (Taziaux et al., 2006). Briefly, sections were incubated sequentially in 0.6% hydrogen peroxide for 20 min to block endogenous peroxidases, followed by 5% normal goat serum for 30 min to decrease non specific binding. Sections were then left overnight at 4°C in the primary antibody diluted 1:2000. The antibody-antigen complex was localized using the avidin-biotin complex method performed with a Vector Elite Kit (Kit ABC Vectastain Elite PK-6100, Vector Laboratories PLC, Cambridge, UK). All reagents were in 0.01 M PBS containing 0.1% Triton X-100 and several rinses were performed between each step. Finally, the peroxidase was visualized with diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride) as chromogen. Sections were mounted on microscope slides in a gelatin-based medium and coverslipped.

Data Analysis: All sections were first qualitatively surveyed to identify brain areas expressing Fos. The numbers of Fos-immunoreactive (Fos-ir) cells were then counted in all brain areas where a qualitative examination suggested that there might be differential induction in the different experimental groups or where we had, based on previous studies investigating brain mechanisms of male sexual behavior in quail (Ball et al., 1997, Meddle et al., 1997, Tlemcani et al., 2000, Charlier et al., 2005, Taziaux et al., 2006) and male sexual learning (Taziaux et al., 2007), reasons to think that differential induction could be expected. The number Fos-ir cells present in various brain regions were quantified using computer-assisted image analysis

by the experimenter (MT) who was blind to the experimental treatment of the birds. Sections were digitized through a video camera attached to the microscope (20 × objective for all brain nuclei, except for the olfactory tubercle, OTu, where the 10 × objective was used) and Fos-ir cells were counted with a Macintosh based image analysis system using the particle counting protocol of the NIH Image (Version 1.62; Wayne Rasband, NIH, Bethesda, MD USA) program. Digital images were made binary and manual threshold was used for discriminating the labeled material from the background. Exclusion thresholds were set at 20 (low threshold; 10 for the OTu) and 200 (high threshold; 100 for the OTu) pixels to remove from the counts dark objects that did not have the size of a cell nucleus.

Fos expression was quantified in a total of 15 brain areas that are schematically illustrated in Fig. 2. In each area, the numbers of Fos-ir cells and the fractional area that they covered (ratio of the number of pixels covered by immunoreactive material to the total number of pixels in the image) were measured in one entire computer field positioned in landscape orientation, occasionally tilted (see below) to adjust to morphological landmarks (length, 0.536 mm × width, 0.360 mm = 0.193 mm²) that was placed in a standardized manner based on predefined anatomical landmarks in the sections (e.g., edge of ventricles or prominent fiber tracts). Brain structures were identified based on the atlas of the quail and chicken brain (Baylé et al., 1974, Kuenzel and Masson, 1988) but the nomenclature used in the present paper is based on the recently revised nomenclature for the avian brain (Reiner et al., 2004).

The following areas were quantified:

Medial Preoptic Area (mPOA)—Fos-ir cells were counted at 3 different rostro-caudal levels within the mPOA in sections that were approximately 240 µm apart. These levels were aligned based on the position where the anterior commissure reaches its largest extension (corresponding approximately to plate A8.2 in the chicken atlas; see (Kuenzel and Masson, 1988)). Fos-ir cells were counted in this section through the caudal mPOA and in two additional sections located 240 (intermediate mPOA) and 480 (rostral mPOA) µm more rostrally. In the caudal mPOA, two quantification fields were summed. The first quantification field was first located in the corner formed by the ventral edge of the anterior commissure and the lateral edge of the third ventricle. The computer field was then moved a half field ventrally (0.180 mm) along the third ventricle and quantification was carried out at this location. The second field was immediately adjacent and was determined by moving the quantification field ventrally the height of one field (0.360 mm) (see Fig. 2D). In the intermediate mPOA that corresponds to the section where the anterior commissure only begins to appear, two quantification fields were summed (Fig. 2C). The first quantification field was first placed in the corner formed by the lateral edge of the third ventricle and the floor of the brain. The computer field was then moved a half field dorsally (0.180 mm) along the third ventricle and quantification was carried out at this location. The second field was immediately adjacent and was determined by moving the quantification field dorsally the height of one field (0.360 mm). In rostral mPOA, a level where the lateral edge of the TSM is just below the fasciculus prosencephali lateralis (FPL), the quantification area was first delimited by the floor of the brain and the edge of the third ventricle. The computer field was then moved a half field dorsally (0.180 mm) along the third ventricle and quantification was carried out at this location (see Fig. 2B).

In each case, the entire computer field was always quantified (0.193 mm²). These fields were determined based on the location of a dense cluster of Fos positive cells at these levels. Based on previous anatomical and neurochemical studies (Foidart et al., 1995, Balthazart et al., 1996, Panzica et al., 1996), the rostral mPOA and intermediate mPOA can be assumed to be within the limits of the sexually dimorphic medial preoptic nucleus (POM) but the caudal mPOA field is overlapping with but also extend slightly ventrally to POM.

Medial part of the Bed nucleus of the Stria Terminalis (BSTM)—Fos induction was quantified in 2 different areas overlapping with the BSTM. In the rostral BSTM (rBSTM), located dorsally to the caudal edge of the anterior commissure, the quantification field (0.193 mm^2) was initially aligned with the dorsal edge of the commissure and the lateral edge of the inter-hemispherical fissure. The field was then moved laterally by one field length (0.536 mm), in a direction parallel to the dorsal edge of the anterior commissure and quantification was done at this location (see Fig. 2D). In a section located $240 \mu\text{m}$ more caudally corresponding to the caudal BSTM (cBSTM), two quantification fields (0.386 mm^2) were summed to cover the nucleus where it assumes a characteristic 'V' shape (see Fig. 2E; see definition of this nucleus by (Aste et al., 1998)). The first quantification field was initially positioned in the same manner as for the caudal mPOA (i.e. adjacent to the third ventricle and at the same dorso-ventral level); the computer field was then moved one field ventrally (0.536 mm) and quantification was carried out at this location in the entire computer field (0.193 mm^2). Then, the same computer field was moved one height (0.360 mm) dorsally along the third ventricle and one half field laterally. Quantification was performed at this level in the entire computer field (0.193 mm^2).

Lateral part of the Bed nucleus of the Stria Terminalis (BSTL)—The BSTL (formerly known as nucleus accumbens in Kuenzel and Masson, 1988) was analyzed in the same section as the caudal mPOA (see Fig. 2D). The left corner of the quantification field was positioned directly underneath the tip of the lateral ventricle and quantification was performed at this level (0.193 mm^2).

Lateral Septal nucleus (LS)—The quantification field was precisely placed in order to cover entirely the bulge of the lateral septal nucleus into the lateral ventricle (see Fig. 2D), at the level where the anterior commissure reaches its largest extension (same level as caudal mPOA).

Nucleus Ventromedialis Hypothalami (VMN)—The VMN was quantified in a section that corresponds approximately to plate A6.4 in the chicken brain atlas (Kuenzel and Masson, 1988). The quantification field was first located in the corner formed by the edge of the third ventricle and the dorsal edge of the decussatio supraoptica dorsalis (DSD) (see Fig. 2G). The field was then moved one field dorsally along the third ventricle and quantification was carried out at this location.

Periaqueductal gray (PAG; known as Substantia grisea centralis in birds)—Fos-ir cells were counted in two locations of the dorsal mesencephalon overlapping with the rostral and caudal aspects of the PAG (rPAG and cPAG). The rPAG was quantified in a section that corresponds approximately to plate A4.8 in the chicken brain atlas (Kuenzel and Masson, 1988) where the posterior commissure reaches its largest medio-lateral extension. The surface analyzed (0.193 mm^2) was delimited by the corner formed by the ventral edge of the posterior commissure (CP) and the lateral edge of the third ventricle (see Fig. 2H). The cPAG was studied in a section corresponding approximately to plate A2.0 in the chicken brain atlas (Kuenzel and Masson, 1988). The computer field was positioned between the fasciculus longitudinalis medialis on the left and the nucleus isthmo-opticus on the right (see Fig. 2I).

Ventro-Lateral nucleus Thalami (VLT)—VLT was analyzed in the same section as the cBSTM (see Fig. 2E). Quantification was performed in one field (0.193 mm^2) delimited ventrally by the dorsal edge of the optic chiasm (OC) and laterally by the ventral tip of the fasciculus prosencephali lateralis (FPL).

Nucleus taeniae of the amygdala (TnA)—Fos induction was quantified in a rostral and caudal part of the TnA (respectively rTnA and cTnA). rTnA was quantified in the section also containing the LS and caudal mPOA (see Fig. 2D). The quantification field was aligned with the ventral surface of the brain and delimited dorsally by the lateral ramification of the anterior commissure. cTnA was studied in a section corresponding approximatively to plate A7.0 in the chicken brain atlas (Kuenzel and Masson, 1988) with the quantification field positioned between the tractus occipitomesencephalicus (OM) and the floor of the brain (see Fig. 2F).

Hippocampus (Hp)—Three quantification fields were summed to quantify the Hp at a single rostro-caudal level corresponding to the rTnA, LS and cPOA (see Fig. 2D). The first field was moved ventrally as far as possible between the interhemispheric fissure and the lateral ventricle. The second and third fields were immediately adjacent and positioned one and two field heights (0.360–0.720 mm) more dorsally.

Olfactory tubercle (OTu)—The most rostral section where the TSM begins to appear between the lateral ventricles was used for the quantification of Fos-ir cells in the olfactory tubercle (see Fig. 2A). The microscope field was delimited by the floor of the brain and the medial edge of the telencephalon. Inside this field, quantification was performed in a standardized bean-shaped area (0.203 mm²) that was overlaid on the section to cover only the olfactory tubercle.

Data analysis—Data collected during conditioning trials were first analyzed by two tailed Fisher exact probability tests to examine the proportion of males from both groups that showed RCSM both at the start and the end of conditioning. Then, a repeated measure ANOVA including the paired group only was performed to show the increased RCSM frequency over trials. Data from the final conditioning trial and those from the final tests performed just before brain collection were analyzed by Mann Whitney U tests. When multiple fields were used to quantify Fos-ir cells in a brain area (3 fields in Hp; 2 in intermediate mPOA, caudal mPOA and cBSTM), these data were averaged before any further analysis. Data for each brain area were then analyzed by appropriate one-way ANOVA followed when appropriate by Fisher protected least significant difference (PLSD) post-hoc tests to compare groups two by two. All data in the text and figures are summarized by the mean $\pm$ standard error of the mean (S.E.M.).

Results

Behavioral data: Training trials

As illustrated in Figure 3, subjects in the Paired condition demonstrated a rapid acquisition of RCSM behavior elicited by the 1 min exposure to the CS across the six conditioning trials. Subjects in the Paired condition increased the mean number of RCSM produced during the CS presentation from 0.75 ± 0.512 on the first trial to 14.85 ± 3.713 on trial six (15 out of 16 birds active in the paired group during the last trial), whereas subjects in the Unpaired condition exhibited means of 0.00 ± 0.00 RCSM on both the first and last conditioning trials. As expected, a Fisher exact probability test showed that groups did not differ significantly in the number of subjects displaying RCSM during the first trial ($2p > 0.5$), whereas a significant difference between the two groups was observed at the end of the conditioning ($2p < 0.0001$). The frequency of RCSM was also higher in the Paired than in the Unpaired group at the end of conditioning while such a difference was not present at the beginning (Mann Whitney U test: end: $U = 3$, $2p < 0.001$; beginning: $U = 39$, $2p > 0.10$) Furthermore, a repeated measure ANOVA for the Paired group only showed that a significant increase in RCSM frequency occurred over trials ($F_{5, 75} = 8.302$, $p < 0.0001$).

All males that had access to the female during these trials (either immediately after CS presentation in the test arena for Paired condition or at least 90 min after CS presentation in the home cage for Unpaired and Empty arena conditions) copulated regularly.

Behavioral data: Final trial

During this final 5.5 min test, only two groups of birds from the Paired condition (P-CS-F and P-CS groups) were given the opportunity to express conditioned RCSM in reaction to the 1 min CS exposure. As could be expected based on the training trials, subjects in the Paired condition produced a large number of RCSM (18.0 ± 3.7) while subjects in the Unpaired condition (U-CS group) produced no RCSM. This difference was determined to be significant ($U = 3$, $2p < 0.002$; see Bars on the right side of Fig. 3). Moreover, no difference in conditioned RCSM frequencies was observed between the P-CS-F and the P-CS groups ($U = 10$, $2p = 0.428$).

Copulatory sexual behavior could be expressed during the final test by the P-CS-F and P-F groups only. As usually observed in quail, these birds displayed cloacal contact movements immediately after being allowed access for 2 min to the stimulus female. These two groups showed similar copulatory frequencies (numbers of cloacal contact movements: $U = 7$, $2p = 0.178$).

Quantification of the Fos-ir cells

As demonstrated in previous studies in quail (Charlier et al., 2005, Taziaux et al., 2006), immunoreactivity for the Fos protein was confined to the nuclei of activated neurons with no specific staining in the cytoplasm. The mean numbers of Fos-ir cells observed in the 15 selected brain areas of the five experimental groups are summarized in Table 1. For each brain area, these data were analyzed by one-way ANOVA, and these statistical results are also summarized in Table 1. Due to various technical problems (poor staining, section torn or lost), accurate counts could not always be obtained for each bird and nucleus. The actual number of available data points in each case is provided in Table 1.

A differential Fos expression in the five experimental groups was observed in four brain areas, the caudal mPOA, both the rostral and caudal BSTM and the BSTL. A similar differential Fos expression was observed in these same brain areas (except for the BSTL) when taking account of the number of sexual stimuli that subjects received during the final 5-min trial (2 sexual stimuli : P-CS-F group; 1 sexual stimulus : P-CS and P-F groups; no sexual stimulus : U-CS and EA groups).

Medial Preoptic Area

Comparison of the mean number of Fos-ir cells in the five experimental groups by one-way ANOVA demonstrated a significant Fos induction in the caudal mPOA only ($F_{4,21} = 4.422$, $p = 0.0095$). *Post hoc* analysis showed that the numbers of Fos-ir cells in the P-CS-F group was significantly higher relative to all other groups (all p 's < 0.05 ; Fig. 4A). The mean number of Fos-ir cells in the P-CS group (26.4 ± 5.9) was quite similar to that found in the P-F group (28.6 ± 5.6). Both were close to 50% higher than the mean found in the U-CS group (17.6 ± 3.4), however, these differences were not significant (all p 's > 0.05 ; Fig. 4A). Representative photomicrographs of sections through the caudal mPOA for the five experimental groups are shown in Figure 5 (left column, panels A through E).

A similar pattern of results was found when analysis was performed with the number of sexual stimuli received by subjects as the independent variable ($F_{2,23} = 9.604$, $p = 0.0009$). *Post hoc* tests revealed that males exposed to two sexual stimuli (the CS followed by copulation with a female [P-CS-F group]) showed enhanced Fos expression in the caudal mPOA relative

to males exposed to just one sexual stimulus (the CS alone [P-CS group] or copulation alone [P-F group]), and to males exposed to no sexual stimulus (U-CS and EA groups; Fig. 4D). Subjects exposed to just one sexual stimulus showed an intermediate level of Fos expression, and displayed a greater number of Fos-ir cells in the caudal mPOA (27.5 ± 3.8) than subject not exposed to a sexual stimulus (17.1 ± 1.9), however, this difference was not statistically significant ($p = 0.08$).

Bed nucleus of the stria terminalis, medial part (BSTM)

One-way ANOVAs also identified significant effects of the experimental conditions during the final trial on the number of Fos-ir cells observed both in the rostral ($F_{4,21} = 4.160$, $p = 0.0123$) and the caudal part of the BSTM ($F_{4,20} = 3.069$, $p = 0.0401$). Interestingly, the pattern of *c-fos* induction was slightly different in these two sub-regions of the BSTM (see Fig. 4B–C for comparisons). In the rostral BSTM, *post hoc* tests revealed that Fos expression was significantly greater in the P-CS-F group relative to all other groups (p 's < 0.05 ; Fig. 4B), which all displayed a nearly identical Fos expression (see Table 1 for mean comparisons; Fig. 5A–E for representative photomicrographs). In the caudal BSTM, the level of Fos expression in the P-CS-F group was significantly higher relative to subjects in the U-CS and EA groups only (both p 's < 0.05 ; Fig. 4C). Subjects in the P-CS and P-F groups showed intermediate *c-fos* induction (41.1 ± 8.5 and 37.9 ± 4.9 , respectively) that was over 50% more than that found in the U-CS group (25.2 ± 6.0), however, these differences were not significant (p 's > 0.05) (Fig. 5, panels F–J for representative photomicrographs).

A similar difference in the pattern of results was obtained when considering only the number of sexual stimulus as the independent variable in the statistical analysis. One-way ANOVAs with the number of sexual stimuli as factor revealed the presence of significant overall differences between conditions both in the rostral ($F_{2,23} = 8.981$, $p = 0.0013$) and the caudal BSTM ($F_{2,22} = 6.522$, $p = 0.0060$). Again, *post hoc* testing revealed that the pattern of Fos expression was slightly different between these two sub-regions of the BSTM (see Fig. 4E–F). In the rostral BSTM, subjects exposed to two sexual stimuli (P-CS-F groups) showed a level of Fos induction significantly higher relative to levels found in subjects exposed to one sexual stimulus (subjects from the P-F and P-CS groups) or no sexual stimulus (subjects from the U-CS and EA groups). Subjects exposed to just one or no sexual stimulus had nearly identical *c-fos* expression (Fig. 4E). In contrast, in the caudal BSTM, the pattern of activation was comparable to that observed in the caudal mPOA. Exposure to two sexual stimuli significantly enhanced the level of Fos expression in comparison with the levels found in subjects exposed to no sexual stimulus ($p < 0.05$). Subjects exposed to one sexual stimulus, however, showed an intermediate level of activation and did not significantly differ from either the subjects exposed to two stimuli or those exposed to no sexual stimulus (Fig. 4F).

Other brain areas

Cells expressing *c-fos* were also observed in a large number of other areas and quantification of this signal was performed in 12 separate brain regions selected based on previous studies implicating them in the control of male sexual behavior and learning. One-way ANOVAs only identified significant effects of the experimental conditions in the BSTL ($F_{4,20} = 4.191$, $p = 0.0126$). *Post hoc* analysis showed that the numbers of Fos-ir cells in the EA group was significantly lower relative to all other groups (p 's < 0.05), except the P-F group where the number of Fos-ir cells was significantly lower compared to the U-CS group ($p < 0.05$). When considering only the number of sexual stimuli as independent variable in the statistical analysis, no significant effect was observed in the BSTL. In the other eleven brain nuclei, no significant difference in Fos expression could be detected between the five experimental groups (see detail of results in table 1). These data were also analyzed after subjects were clustered in three groups

based on the number of sexual stimuli they had been exposed to during the last trial before sacrifice and these analysis also failed to detect any significant effect (data not shown).

Discussion

The results from this study demonstrate enhanced neural activation that occurs following presentation of a conditioned stimulus (CS) that predicts copulation. Further, the behavioral outcome of the present experiment confirms and extends the findings from previous studies.

A previously arbitrary stimulus that was not inducing any specific sexual behavior will if repeatedly paired with access to a hen elicit conditioned RCSM in male Japanese quail. Female quail are most receptive and lay eggs in the afternoon (Wilson and Huang, 1962, Delville et al., 1986). RCSM occur in the presence of a hen and whip a muco-protein into a foam (Seiwert and Adkins-Regan, 1998) that is delivered to the female cloaca during copulation. This foam enhances sperm competition and motility (Cheng et al., 1989a, Cheng et al., 1989b), and following afternoon copulations, increases the probability of fertilization, perhaps because the foam reduces the number of sperm swept out by a passing egg (Adkins-Regan, 1999). Conditioned RCSM may contribute to the enhanced fertility associated with a sexual CS presentation because they allow for foam production to begin before the arrival of a hen (Holloway et al., 2005). Here, acquisition proceeded more rapidly, and resulted in more conditioned RCSM than reported in other conditioning procedures (e.g. (Cornil et al., 2004, Holloway et al., 2005). In those studies, access to the hen following CS exposure was limited to visual exposure. The copulatory access in this study likely contributed to the steeper learning curve. In a direct comparison of visual-only versus full-copulatory access to a hen, it was found that both types of female exposure support classically conditioned sexual approach behavior (Holloway and Domjan, 1993). Copulation, however, resulted in faster learning and more time spent engaging in the conditioned approach response. Although not a direct comparison, the results from the current experiment suggest that the same is true for conditioned RCSM. It should be noted that in the present experiment copulation with the female included visual exposure and tactile interactions with female. Therefore, the putative reward of copulation is likely a stimulus complex that supports greater numbers of RCSM. Similarly, the CS may also be a stimulus complex. Although the non-reinforced exposures to the test chamber probably reduced any accidental conditioning of handling and test cage sounds and lighting, we cannot rule out minor conditioning of these stimuli. Further, as training progressed, the proprioceptive feedback from the performance of conditioned RCSM may have served as a CS, although the contribution of these cues is also likely to be minor, as sexual classical conditioning of male quail occurs during tests of omission contingency in which the reward is withheld if the conditioned response is made (Crawford & Domjan, 1993).

Immunohistochemical staining of Fos, the protein product of *c-fos* expression, is a widely used technique to determine brain regions involved in sexual responses (see Pfau and Heeb, 1997 and Veening and Coolen, 1998 for reviews). Important for the present study, a previous investigation of *c-fos* expression in male quail following interactions with a female revealed that consummatory and appetitive behaviors are mediated by different brain regions (see Beach, 1956 for a discussion of appetitive and consummatory behavior, and Mills et al., 1997 for detailed descriptions of male quail responses). Male quail allowed to copulate with a female show increased Fos-ir cells in both rostral and caudal sections of the POM, the BSTM, the SL, and the VLT (Taziaux et al., 2006). Males allowed only visual access to a hen make appetitive RCSM responses but only show elevated *c-fos* expression in the rostral region of the POM and the VLT. In a subsequent study, it was demonstrated that exposure to a CS predicting only visual access to a hen differentially activated two brain regions, the nucleus taeniae of the amygdala (TnA) and the hippocampus (Hp) (Taziaux et al., 2007). In that study, prior to brain collection and *c-fos* immunohistochemistry, males were exposed to either the

sexual CS or the same arbitrary stimulus that had not been conditioned. Here, we exposed males to both a CS that predicts copulatory opportunity and sex with a female (2 stimuli), either the sexual CS or copulation (1 stimulus), or to a stimulus that was not associated with sex (no stimulus). As we anticipated, presenting a sexual CS prior to copulation enhances neural activity in regions known to mediate sexual responses. This effect mirrors the behavioral data demonstrating that presenting a sexual CS prior to sex increases reproductive success in quail (Adkins-Regan and MacKillop, 2003, Mahometa and Domjan, 2005, Çetinkaya and Domjan, 2006). Further, the regions affected by a CS associated with consummatory copulation, the caudal preoptic area (POA; a region including the POM), and the rostral and caudal BSTM, are different than those areas reported to be affected by exposure to a CS associated with exposure to a female that permits only appetitive responses (compare (Taziaux et al., 2006) and (Taziaux et al., 2007)).

Preoptic Area

We found that exposure to a CS predicting copulation prior to sex with a female caused enhanced *c-fos* expression in the caudal POA over that seen in subjects exposed to just one sexual stimulus, the CS or copulation alone, and over subjects exposed to no sexual stimulus. Indeed subjects exposed to two sexual stimuli had over two-thirds more Fos-ir cells in the caudal POA than subjects exposed to a single sexual stimulus, and over 2.5 times more than subjects not exposed to a sexual stimulus. Subjects exposed to a single sexual stimulus also showed some enhancement of *c-fos* expression, having slightly more than 60% more Fos-ir cells in this region than subjects not exposed to a sexual stimulus, however, this difference did not reach a significant level ($p = .08$).

The enhanced activity in this region is of particular reproductive importance. The POA has been clearly established as a critical region for the control of sexual behavior in male Japanese quail (see (Panzica et al., 1996) for a review). Lesions here disrupt both appetitive and consummatory aspects of male sexual behavior (Balthazart et al., 1998). Testosterone implants into the POM sub-region of castrated male quail are sufficient to activate learned appetitive approach responses, and consummatory copulation (Riters et al., 1998). Additionally, as previously mentioned, copulation activates the rostral to caudal extent of the POM as revealed by increased Fos-ir cells, and visual exposure to a female activates the rostral POM (Taziaux et al., 2006).

The presentation of a sexual CS prior to copulation then increased neural activity in a region known to mediate both appetitive and consummatory reproductive behaviors. Interestingly however, the activation of *c-fos* expression took place in the caudal part of the POM, not in its rostral part that was previously found to be associated with RSCM production (Taziaux et al., 2006). Importantly, the POA regulates RSCM (Balthazart et al., 1998), found here to be sharply activated in subjects exposed to a sexual CS. While the relationship between enhanced activity in the POA and increased RSCM is correlational in these findings, there is reason to believe a causal relationship exists. Disruptions of normal POA functioning, either by lesions (Balthazart et al., 1998) or castration (Riters et al., 1998) reduce or eliminate RSCM. In castrated males, implants of exogenous testosterone into the POM sub-region also tend to reinstate RSCM (Riters et al., 1998). We suspect, therefore, that the enhanced activity in the POA contributes to the performance of the conditioned RSCM. Because, as mentioned, RSCM produce a fertilization facilitating foam (Cheng et al., 1989a, Cheng et al., 1989b, Seiwert and Adkins-Regan, 1998), it is likely that the enhanced activity in the POA, when copulation is preceded by a sexual CS, substantially contributes to the increased reproductive success reported following sexual CS presentation (Adkins-Regan and MacKillop, 2003, Mahometa and Domjan, 2005, Çetinkaya and Domjan, 2006).

Bed Nucleus of the Stria Terminalis

Activation of the BSTM as revealed by IEG, both *c-fos* and *zenk*, occurs following copulation (Ball et al., 1997, Meddle et al., 1997, Charlier et al., 2005, Taziaux et al., 2006). Here we demonstrated that there is enhanced *c-fos* expression in both the rostral and caudal BSTM if copulation is preceded by a sexual CS. Interestingly, the pattern of activation was different in the two regions. In the rostral BSTM it appears that a CS predicting copulation presented before copulation was necessary to activate the region above levels found in subjects exposed to either copulation or the sexual CS alone, the untrained CS or the empty arena. Subjects exposed to two sexual stimuli had almost twice the Fos-ir cells in the rostral BSTM as subjects exposed to just one or no sexual stimulus. Subjects exposed to just one or no sexual stimulus had nearly identical *c-fos* expression.

In the caudal BSTM, exposure to a sexual CS before copulation also enhanced *c-fos* expression to almost twice the levels found in subjects exposed to no sexual stimulus. Subjects exposed to one sexual stimulus, however, showed an intermediate level of activation and did not significantly differ from either the subjects exposed to two stimuli or those exposed to no sexual stimulus.

It has been suggested that the expectation of a female may induce IEG in the BSTM (Charlier et al., 2005). In that study, castrated male quail given exogenous testosterone and exposed to an arena where they had previously encountered a female had more *c-fos* expression than castrated males without testosterone implants exposed to a hen. Castrated, testosterone-implanted males exposed only to their home cages did not show this difference. The present findings suggest that the caudal BSTM may be sensitive to the expectation of a female as subjects exposed to the sexual CS alone had over 40% more Fos-ir cells in this area than subjects exposed to the empty arena or an untrained CS, however, this difference did not reach significance. The significantly enhanced activity found in subjects presented with both the sexual CS and copulation, though, likely reflects this expectation.

The results from the rostral BSTM are particularly intriguing. Activation above that following a neutral-stimulus, baseline exposure appears to require sequential presentation of a sexual CS and copulation. It may be that a CS associated with copulation primes this region for subsequent activation by sex with a female, but more detailed studies of the BSTM need to be conducted to fully support this conclusion.

Other Brain Regions

Among all other brain regions studied, a significant difference between the five experimental groups was only found in the BSTL. Previous studies showed that *c-fos* expression is induced in this brain region after performance of both appetitive and/or consummatory components of male sexual behavior in quail (Tlemçani et al., 2000) and rodents (Pfaus and Heeb, 1997). Moreover, this structure seems to be critical for the acquisition and expression of Pavlovian stimulus-reward relationships (Day and Carelli, 2007). However, Fos induction observed here in BSTL did not follow the pattern of expression displayed by the other regions (see detail in Table 1), suggesting that the differences between groups were not directly related to the sexual stimuli but to other aspects of the experimental manipulations.

We did not find additional significant differences in *c-fos* expression in any of the other regions we examined. Although these areas were targeted for investigation based on IEG expression following sexual behavior in other studies with quail, there are substantial differences among the protocols that likely account for the lack of further findings here. For example, in this experiment subjects were exposed to sexual classical conditioning procedures, and their controls, prior to the final trial. Previous work in quail suggests that repeated presentations to

a female cause a decrease in expression of the IEG *zenk* in the preoptic area (Can et al., 2006). During the present study, the lack of induction of *c-fos* in many brain areas that were previously shown to be activated during the expression of male copulatory behavior (Meddle et al., 1997, Charlier et al., 2005, Taziaux et al., 2006) may similarly relate to the repeated testing that birds were exposed to before the final test preceding brain collection. Perhaps most significantly, the exposure to the female was limited to 2.5 min in this study whereas in previous studies male subjects were exposed to females for 90–120 min (Meddle et al., 1997), 1 hour (Charlier et al., 2005), or 5 min (Taziaux et al., 2006). The absence of a differential *c-fos* expression between the five experimental groups in the other regions in this study most likely represents different sensitivities to sexual stimuli and may indicate that induction in different nuclei depends on different physiological events (see Taziaux et al., 2006).

Sexual Conditioning in Mammals

Sexual classical conditioning in rats has consequences similar to those reported for Japanese quail. The latency to copulate is significantly decreased in male rats exposed to a sexual CS (Zamble et al., 1985). Further, sexual conditioning reduces the number of intromissions necessary before ejaculation (Silberberg & Adler, 1974). An odor previously paired with sexual opportunity (i.e., with ejaculation) results in a preference to ejaculate with a female scented with that odor (Kippin & Pfaus, 2001a) and will even elicit copulation with unreceptive females (Kippin & Pfaus, 2001b). Sexual classical conditioning has also been identified as a factor in female rat mate preference. A scent placed on a male in a paced mating situation will later elicit a conditioned partner preference in females if they are given the choice between a scented and unscented male (Coria-Avila et al., 2005). In a subsequent study, this conditioned partner preference in females was demonstrated to also occur with naturally occurring stimuli in strains of male rats (Coria-Avila et al., 2006).

Interestingly, the neural activation in male rats that results from exposure to a sexually conditioned odor differs from the activation that follows exposure to estrous odors. As previously mentioned in male rats, exposure to estrous odors results in increased Fos-ir in the accessory olfactory bulb, medial amygdala, BSTM, medial preoptic area, ventromedial hypothalamus, ventral tegmental area, and the core and shell of the nucleus accumbens. Exposure to an odor paired with copulation results in increased Fos-ir in the piriform cortex, basolateral amygdala, the anterior aspect of the lateral hypothalamus, and the core of the nucleus accumbens (Kippin et al. 2003).

In the present study, there is considerable overlap in areas of *c-fos* expression following exposure to a quail hen and to a conditioned stimulus predicting copulation. The differences between the patterns of neural activation in male quail and rats to conditioned and unconditioned stimuli may reflect species differences or may be due to differences in experimental procedures. Further tests are required to explore these possibilities.

The Significance of Fos expression in the POA and BSTM

The findings of our study raise an interesting related question. Here we addressed the number and nature of the stimuli to which subjects were exposed. The exposure protocol presented subjects with stimuli identical to those encountered during training. Necessarily, therefore, the duration of subjects' encounters with the sexual stimulus(i) differed across groups. It is unlikely that time of exposure accounted for our results, as subjects exposed to copulation-only had 2 min of stimulus exposure and subjects exposed to the sexual CS had only 1 min of stimulus exposure and these two conditions did not differ significantly, and expressed nearly identical numbers of Fos-ir cells, in all three regions where significant differences were found. We cannot, however, rule out that a threshold exists for *c-fos* expression in these and other regions

and only further experiments manipulating the duration of stimulus exposure will identify whether such a threshold exists and whether it differs across brain regions.

Even if the duration of stimulus exposure is found to influence *c-fos* expression, our conclusion remains unchallenged: Exposing male Japanese quail to a stimulus predicting copulatory opportunity prior to sex enhances brain activity in regions known to mediate male sexual behavior.

Of interest, exposure to an increasing amount of sexual stimulation results in patterns of *c-fos* expression that are proportional to the number of sexual stimuli presented. This was found across the three nuclei. As expected, the Fos induction in groups P-CS and P-F was nearly identical. The observed numerical increase of Fos-ir cells in these two groups was, however, not significant as compared to the U-CS group. We believe that the time of exposure to the stimulus and, more importantly, the amount of behavior that could be expressed during this testing duration can explain the differences in brain activation between this study and previous studies in our laboratory (numerical increase of Fos-ir cells in POA and BSTM following copulation that is or is not statistically significant). Indeed, as previously demonstrated by work in rodents on brain circuitry implicated in male sexual behavior and male sexual satiety, Fos expression (numbers of positive neurons and their localization) varies considerably as a function of the types of behavioral responses that have been produced (anogenital investigation, mounting, intromission or ejaculation) (Veening and Coolen, 1998; Parfitt and Newman, 1998; Phillips-Farfan and Fernandez-Guasti, 2007). The very short behavioural tests performed in the present experiment presumably did not allow birds to produce enough behavior to fully activate Fos expression in relevant brain areas.

Significantly, the changes we report occur rapidly, after only six conditioning trials. Further, the enhanced activity in the POA and BSTM, regions that have positional and functional mammalian homologues (Panzica et al., 1996; Pfau and Heeb, 1997; Aste et al., 1998; Veening and Coolen, 1998), takes place after relatively brief exposures to sexual stimuli (1 to 3 min). This result is interesting because it allows specifying with more precision the stimuli responsible for the activation of IEG and avoids uncertainties concerning the nature of these stimuli. Lastly if, as we believe is likely the case, there is a causal relationship between the enhanced brain activity and RCSM which increase reproductive success (a causal relationship that should be specifically tested), then the ultimate result of the neural activation in the male brain has a very important functional outcome since it should result in fertilizing more eggs.

Acknowledgements

This work was supported by grants from the National Institutes of Health (R01 MH50388) and the Belgian FRFC (2.4562.05) to JB and by a Vassar College Faculty Research grant and a fellowship for visiting professors from the Belgian FNRS to KSH.

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Figure 1.

Schematic presentation of the experimental procedures. **(A)** Drawing of the test apparatus. A small glass aquarium contained the experimental male quail. During conditioning trials, a piece of opaque board was attached to the front glass wall facing the experimenter. A mirror placed at 45° under the aquarium allowed the experimenter to view the cloacal area of the subject. A two-compartment movable trolley was attached to the far side of a plywood board that completely covered the open right wall of the test arena. An opening in the board provided visual access to the trolley. A vertically sliding opaque panel covered this opening and could be raised with a rope at the beginning of conditioning trials. The first compartment of the trolley contained the CS. The second compartment contained a female (Paired condition) or was left

empty (Unpaired condition). **(B)** Time-line diagram of the conditioning procedures used during the 6 conditioning trials. Time in the empty arena was alternated between 2.5 and 3.5 min for subjects in the Paired and Unpaired conditions, and between 5.5 and 6.5 min for subjects in the Empty arena condition to reduce the likelihood of temporal conditioning signaling stimuli presentations. **(C)** Time-line diagram of the behavioral procedures on the final trial. Subjects from the Paired condition were assigned to one of three groups: the CS followed by copulation (P-CS-F group), the CS alone (P-CS group), or copulation alone (P-F group). Subjects from the Unpaired condition were exposed to the CS that had not, for them, acquired sexual association during the conditioning trials (U-CS), and subjects exposed only to the empty arena were placed into the arena only (EA group).

Figure 2.

Schematic drawings of coronal sections through the quail brain presented in a rostral to caudal order from Panels A through H illustrating the areas where Fos expression was quantified (dark areas). Quantification took place in a box of standardized dimensions (0.536×0.360 mm) but for a few nuclei (POA in 2B and 2C, Hp in 2C, BSTM in 2D), multiple adjacent boxes of the same size were used to cover the field of interest. Aq, aqueduct of Sylvius; BSTM, bed nucleus of the stria terminalis, medial part; CA, commissura anterior; Cb, cerebellum; CO, chiasma opticum; CP, commissura posterior; CPa, commissura palii; DSD, decussatio supraoptica dorsalis; E, entopallium; FA, tractus fronto-archistriaticus; FLM, fasciculus longitudinalis medialis; FPL, fasciculus prosencephali lateralis; GLv, nucleus geniculatus lateralis, pars

ventralis; Hp, hippocampus; IO, nucleus isthmo-opticus; LaM, mesopallial lamina; M, mesopallium; mPOA, medial preoptic area; N, nidopallium; OM, tractus occipitomesencephalicus; OTu, olfactory tubercle; PAG, periaqueductal grey; ROT, nucleus rotundus; SM, medial septal nucleus; SL, lateral septal nucleus; TeO, tectum opticum; TnA, nucleus taeniae of the amygdala; TSM, tractus septopallio-mesencephalicus; VL, ventriculus lateralis; VLT, ventro-lateral nucleus thalami; VMN, nucleus ventromedialis hypothalami; VT, ventriculus tecti mesencephali.

Figure 3.

Mean ($\pm$) number of RCSM produced by subjects in the Paired and Unpaired condition during the 1 min CS presentation. The lines graphs summarize RCSM produced during the conditioning phase (six trials) during which subjects in the Paired condition were exposed to the CS for 1 min immediately followed by sexual interaction with a female. Subjects in the Unpaired group were treated in an identical manner except exposure to the CS was followed by 2 min visual access to an empty compartment. They were given the opportunity to interact with a female in their home cage at least 90 min following conditioning trials. RCSM produced during the final trial are summarized by the bar graphs.

Figure 4.

Distribution of Fos-ir cells in the caudal mPOA (A, D) and both the rostral (B, E) and caudal BSTM (C, F) in the five experimental groups (A–C) and with the number of sexual stimuli received by the subjects during the final 5-min trial considered as independent variable (D–F). P-CS-F: subjects in the Paired condition were either exposed to the CS for 1 min followed by 2 min of sexual interaction with a female; P-F: subjects in the Paired condition were allowed to interact immediately with the female for 2 min; P-CS: subjects in the Paired condition were exposed to the CS for 1 min not followed by the female; U-CS: subjects in the Unpaired condition were exposed to the CS alone; EA: subjects in the Empty arena condition were exposed to an empty arena. Symbols above the bars summarize the results of the *post hoc* Fisher PLSD tests as follows : * $p < 0.05$ vs. P-CS-F. In panels D–F, the 5 experimental groups were organized in 3 categories based on the number of sexual stimuli they were exposed to during the final test: 2 stimuli (group P-CS-F), one sexual stimulus (groups P-F and P-CS) or no sexual stimulus (groups U-CS and EA).. Symbols above the bars summarize the results of the *post hoc* Fisher PLSD tests as follows : * $p < 0.05$ vs. no sexual stimulus; # $p < 0.05$ vs. 1 sexual stimulus.

Figure 5.

Photomicrographs illustrating areas of the quail brain where a differential induction of Fos expression was observed in the five experimental groups (**A,F** : P-CS-F group; **B,G** : P-F group; **C,H** : P-CS group; **D,I** : U-CS group and **E,J** : EA group). Panels **A** through **E** illustrate Fos expression in: (**a**) the caudal mPOA and the rostral BSTM at low magnification (5× objective); (**b**) the rostral BSTM at high magnification (40× objective); (**c**) the caudal mPOA at medium magnification (20× objective). Magnification bars: 500 μm in **a**, 50 μm in **b** and 100 μm in **c**. Panels **F** to **J** illustrate Fos expression in the caudal BSTM at low magnification (5× objective). The inset shows a higher magnification (40× objective) of the caudal BSTM. Magnification bars: 500 μm and 50 μm in the inset. The boxes in **A** and **F** illustrate the areas

re-photographed at higher magnification. CA, commissura anterior; CPa, commissura pallii; FPL, fasciculus prosencephali lateralis.

Table 1

Mean ($\pm$ SEM) number of Fos-ir cells and summary of the statistical analysis by one-way ANOVAs in the thirteen brain nuclei in the five experimental conditions

Nucleus	P-CS-F	P-CS	P-F	U-CS	EA	One-way ANOVAs		
						F	(df)	p
Rostral mPOA	31.8 $\pm$ 3.8 (n=5)	16.6 $\pm$ 5.3 (n=5)	29.2 $\pm$ 9.1 (n=5)	20.7 $\pm$ 2.0 (n=3)	20.2 $\pm$ 4.5 (n=5)	1.245	(4,18)	0.3277
Intermediate mPOA	31.8 $\pm$ 5.3 (n=4)	24.9 $\pm$ 5.9 (n=4)	31.7 $\pm$ 7.5 (n=4)	14.2 $\pm$ 3.1 (n=3)	20.8 $\pm$ 6.3 (n=5)	1.334	(4,15)	0.3027
Caudal mPOA	46.2 $\pm$ 8.5 (n=6)	26.4 $\pm$ 5.9 (n=5)	28.6 $\pm$ 5.6 (n=5)	17.6 $\pm$ 3.4 (n=5)	16.5 $\pm$ 2.2 (n=5)	4.422	(4,21)	0.0095
Rostral BSTM	56.7 $\pm$ 7.0 (n=6)	30.0 $\pm$ 6.1 (n=5)	27.6 $\pm$ 4.4 (n=5)	31.0 $\pm$ 6.2 (n=5)	33.2 $\pm$ 6.0 (n=5)	4.160	(4,21)	0.0123
Caudal BSTM	106.8 $\pm$ 15.7 (n=6)	82.2 $\pm$ 17.1 (n=4)	75.8 $\pm$ 9.8 (n=5)	50.4 $\pm$ 12.0 (n=5)	57.8 $\pm$ 9.8 (n=5)	3.069	(4,20)	0.0401
BSTL	32.7 $\pm$ 5.6 (n=6)	31.0 $\pm$ 13.7 (n=5)	23.2 $\pm$ 9.8 (n=5) [∞]	38.2 $\pm$ 8.3 (n=4)	17.0 $\pm$ 3.8 (n=5)	4.191	(4,20)	0.0126
Rostral TnA	14.5 $\pm$ 4.0 (n=6)	15.6 $\pm$ 3.7 (n=5)	20.7 $\pm$ 2.8 (n=4)	13.7 $\pm$ 1.4 (n=4)	13.2 $\pm$ 2.6 (n=5)	0.725	(4,19)	0.5857
Caudal TnA	21.0 $\pm$ 5.2 (n=6)	23.0 $\pm$ 5.3 (n=5)	32.0 $\pm$ 5.9 (n=4)	21.2 $\pm$ 5.2 (n=4)	27.8 $\pm$ 6.3 (n=5)	0.666	(4,19)	0.6237
Rostral PAG	27.2 $\pm$ 4.3 (n=6)	10.6 $\pm$ 1.6 (n=5)	18.0 $\pm$ 1.8 (n=5)	20.8 $\pm$ 7.5 (n=5)	24.2 $\pm$ 5.4 (n=5)	1.867	(4,21)	0.1539
Caudal PAG	15.3 $\pm$ 1.9 (n=3)	6.4 $\pm$ 2.5 (n=5)	15.1 $\pm$ 3.4 (n=5)	8.7 $\pm$ 4.6 (n=4)	15.7 $\pm$ 4.6 (n=5)	1.409	(4,17)	0.2733
LS	61.0 $\pm$ 12.6 (n=5)	60.0 $\pm$ 8.4 (n=5)	42.8 $\pm$ 8.3 (n=5)	75.5 $\pm$ 7.4 (n=4)	60.2 $\pm$ 21.3 (n=5)	0.736	(4,19)	0.5789
Hp	30.0 $\pm$ 2.9 (n=6)	30.9 $\pm$ 3.2 (n=5)	28.7 $\pm$ 3.5 (n=4)	22.9 $\pm$ 3.2 (n=5)	30.2 $\pm$ 11.9 (n=3)	0.565	(4,18)	0.6913
OTu	55.7 $\pm$ 30.9 (n=6)	31.4 $\pm$ 23.5 (n=5)	51.4 $\pm$ 19.3 (n=5)	47.8 $\pm$ 4.6 (n=4)	15.7 $\pm$ 4.6 (n=5)	1.409	(4,17)	0.2733
VMN	28.0 $\pm$ 4.8 (n=6)	22.8 $\pm$ 7.0 (n=5)	23.0 $\pm$ 4.7 (n=5)	16.0 $\pm$ 15 (n=5)	52.7 $\pm$ 32.2 (n=4)	0.751	(4,20)	0.569
VLt	56.0 $\pm$ 8.1 (n=5)	60.4 $\pm$ 6.4 (n=5)	76.2 $\pm$ 11.5 (n=5)	84.7 $\pm$ 10.7 (n=3)	83.2 $\pm$ 15.6 (n=5)	1.359	(4,18)	0.2870

Results shown in the table indicate for the main factor in the analysis (experimental group) the F ratio (F), corresponding degrees of freedom (df) and associated probability (p).

Symbols next to the data indicate results of post-hoc Fisher PLSD test as follows:

* p<0.05 vs. P-CS-F

p<0.05 vs. EA

∞ p<0.05 vs. U-CS