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# Role of aromatase in distinct brain nuclei of the social behavior network in the expression of sexual behavior in male Japanese quail

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## ABSTRACT

In male Japanese quail, brain aromatase is crucial for the hormonal activation of sexual behavior, but the sites producing neuro-estrogens critical for these behaviors have not been completely identified. This study examined the function of aromatase expressed in several nuclei of the social behavior network on a measure of sexual motivation known as the frequency of rhythmic cloacal sphincter movements (RCSM) and on copulatory behavior. Sexually experienced castrated males chronically treated with testosterone were stereotaxically implanted with the aromatase inhibitor vorozole (VOR), or cholesterol as control, and tested for sexual behavior. In experiment 1, males were implanted in the medial preoptic nucleus (POM) with VOR, a manipulation known to reduce the expression of copulatory behavior. This experiment served as positive control, but also showed that VOR implanted in the dorsomedial or lateral portions of the POM similarly inhibits male copulatory behavior compared to control implants. In experiments 2 to 4, males received stereotaxic implants of VOR in the periaqueductal gray (PAG), the nucleus taeniae of the amygdala (TnA) and the ventromedial nucleus of the hypothalamus (VMN), respectively. Sexual behavior was affected only in individuals where VOR was implanted in the PAG: these males displayed significantly lower frequencies of cloacal contact movements, the last step of the copulatory sequence. Inhibition of aromatase in the TnA and VMN did not alter copulatory ability. Overall, RCSM frequency remained unaffected by VOR regardless of implantation site. Together, these results suggest that neuro-estrogens produced in the POM contribute the most to the control of male copulatory behavior, while aromatase expressed in the PAG might also participate to premotor aspects of male copulatory behavior.

Keywords: Aromatase, Neuro-estrogens, Sexual behavior, Medial preotic nucleus, Periaqueductal gray, Nucleus taeniae of the amygdala, Ventromedial nucleus of the hypothalamus

## 1. INTRODUCTION

Social behavior, including sexual behavior, plays a crucial role in the enhancement of individual reproductive success and the growth of populations. In vertebrates, neural circuits underlying social behavior involve a conserved set of interconnected brain nuclei known as the social behavior network. This neural network includes a set of diencephalic (medial preoptic area, POA; ventromedial nucleus of the hypothalamus, VMN), telencephalic/limbic (medial bed nucleus of the stria terminalis, BST; medial amygdala, MeA; lateral septum) and mesencephalic (periaqueductal gray) nuclei that are differentially activated to mediate the regulation of different components of social behavior[1-5]. In parallel, studies have suggested dissociations between components of neural circuits underlying the expression of appetitive (i.e., sexual motivation) and consummatory components (i.e., copulatory behavior) of male sexual behavior in vertebrates (reviewed in [6,7]).

In many vertebrates, male sexual behavior is activated, at least in part, by estrogens synthesized in the brain, also known as neuro-estrogens[8,9]. Aromatase acts as a key enzyme in the conversion of testosterone into estradiol. It is expressed in most brain nuclei of the social behavior network of vertebrates[10-15], including humans[16-18], along with estrogen receptors[19-22]. The POA has been identified as a key region in the control of male copulatory behavior[23,24]. Within this region, neuro-estrogens play a critical role in the expression of copulatory behavior in many vertebrate species. Stereotaxic implantation of an aromatase inhibitor in the POA results in a reduced expression of copulatory behavior in male rodents and birds[25-27], whereas estradiol implants in the POA restore copulatory abilities in gonadally intact males implanted with a subcutaneous osmotic pump loaded with an aromatase inhibitor[28]. However, the function of the aromatase expressed in the other brain nuclei of the social behavior network remains elusive.

Sparse and sometimes indirect evidence suggests that neuro-estrogens produced in several brain nuclei of the social behavior network also play a role in the expression of male sexual behavior. Site-specific estrogen receptor alpha (ERα) knockdown results in a reduction of sexual behavior when it targets the POA or the VMN, but not the MeA, of male mice[29]. Optogenetic manipulations of ERα neurons in the ventrolateral subdivision of the VMN alter close investigation of a female stimulus and mounting behavior of male mice, although this effect largely depends on the photostimulation intensity and the number of activated cells[30]. Inhibition or ablation of aromatase-expressing neurons within the posterodorsal MeA does not alter mating behavior in male mice[31], whereas male rats chronically infused with an aromatase inhibitor into the MeA through an osmotic pump take longer to mount and ejaculate[32]. Another study employing pharmacological inhibition and activation of ERα-expressing cells in the posterior amygdala that project to the medial preoptic nucleus also showed a disruption in the expression of social investigation and sexual behavior of male mice[33]. To our knowledge, the behavioral function of aromatase expressed in the PAG has never been explored.

The Japanese quail (*Coturnix japonica*) is an excellent experimental model to study brain aromatase and examine its role in discrete brain regions on the expression of male sexual behavior[34]. Males of this species exhibit a dense

expression of aromatase and estrogen receptors in most nuclei of the social behavior network[10,21,35-37]. Brain aromatase plays a crucial role in the control of appetitive and consummatory components of sexual behavior and its blockade by systemic injections or intracerebroventricular infusion of aromatase inhibitors decreases the expression of sexual behavior[38-40]. The full range of aromatase-positive brain sites implicated in the regulation of various aspects of sexual behavior have not been identified.

Inhibition of aromatase in the medial preoptic nucleus (POM) prevents the expression of copulatory behavior[26,27], but its blockade in the whole brain only weakly affects other social behaviors[38]. Chronic stereotaxic implants of an aromatase inhibitor into the BST also delay the appearance of copulatory behavior, but similar implants into VMN do not seem to impair this behavior[26,27]. Note however that in this latter experiment, all implants were targeted to the junction between POM and BST and implants in VMN were consequently in small number (3 subjects treated with an aromatase inhibitor and 4 controls). The power of this study was therefore limited.

The frequency of rhythmic cloacal sphincter movements (RCSM), a measure of sexual motivation, produced in response to the visual presentation of a female was not significantly affected by aromatase blockade in POM, BST or VMN[27]. Yet, two studies had shown that RCSM are decreased by chronic systemic or intracerebroventricular treatments with an aromatase inhibitor[38,39]. However, in these experiments, the behavioral inhibition was only fully revealed after multiple tests with a female. It was thus impossible to determine whether this inhibition resulted from a direct effect of the treatment on sexual motivation or whether it indirectly resulted from a decreased value of the female as a sexual stimulus due to the inability of males to copulate.

In summary, the effects on sexual behavior of a site-specific inhibition of aromatase in the VMN, the nucleus taeniae (TnA; homologous of the mammalian medial amygdala[41]) and PAG have never been precisely investigated in male quail. The main goal of the present experiments was thus to address this question by implanting stereotaxic bilateral cannulas filled with the aromatase inhibitor vorozole (VOR) in these nuclei. Implants were additionally placed in the POM as a positive control.

## 2. METHODS

### 2.1. Subjects

Five experiments were conducted to analyze the effects of a site-specific administration of the aromatase inhibitor VOR on the sexual behavior in male Japanese quail (*Coturnix japonica*). Each experiment targeted a different aromatase-expressing nucleus of the social behavior network including the medial preoptic nucleus (POM; Experiment 1), the periaqueductal gray (PAG; Exp. 2), the nucleus taeniae of the amygdala (TnA; Exp. 3) and the ventromedial nucleus of the hypothalamus (VMN; two separate experiments Exp.4.1 and Exp.4.2, see details in the general procedures section).

Each experiment was conducted separately on distinct batches of quail hatched at our own colony (Central Animal Facility from the University of Liège, agreement number: LA1610002). All males were castrated between the age of 2 and

3 weeks and implanted with one 20 mm long Silastic™ capsule (Silclear® tubing, 1.57 mm i.d; 2.41 mm o.d.; Degania silicone, Ref: 20301502431) filled with crystalline testosterone (Sigma, B6500) at the age of 5 weeks. This size of testosterone implant maintains circulating concentrations that are in the physiological range usually observed in gonadally intact sexually mature male quail[42,43]. Experimental subjects remained in same-sex group of males until the age of 6 (Exp. 1, 2 and 4.1) or 8 weeks (Exp. 3 and 4.2) before their transfer to individual cages. All birds were maintained under a photoperiod simulating long summer days (16h light and 8h dark) and provided with food and water *ad libitum*.

### 2.2. General procedures

At the age of 8-9 weeks, males were given a series of copulatory pre-tests to provide them with extensive experience of the testing procedures, ensure that all subjects were sexually active and establish their baseline sexual performance. Depending on the experiment, subjects required 10 (Exp. 1), 7 (Exp. 2), 8 (Exp. 3 and 4.2) or 3 (Exp. 4.1) pre-tests for male copulatory behavior on different days to reach a plateau in copulatory frequency. These tests were performed within a period of one to three weeks depending of the number of tests completed by week and the time that was needed for birds to reach the copulatory criterion. In any case, there were never multiple tests on a same day. They then received on a different day one test of sexual motivation measuring rhythmic cloacal sphincter movements (RCSM; see quantification of sexual behaviors section) in response to the view of a female. Groups of experimental males were then matched based the behaviors displayed during the pre-test phase as well as on the mean body mass used as a measure of health and the mean cloacal gland area as a measure of circulating androgen concentrations[44]. Some subjects died during the subsequent stereotaxic surgery or during the experiment sometimes resulting in minor differences between groups during the pre-test phase.

At the age of 19 (Exp. 1), 11 (Exp. 2), 10 (Exp. 3 and 4.2) or 9 (Exp. 4.1) weeks, adult male quail were implanted with a bilateral cannula in the targeted brain nucleus (see detailed procedure in the stereotaxic implantation section) filled with either the aromatase inhibitor, vorozole (VOR or R76713, Janssen Pharmaceutica, Beerse, Belgium) or its control cholesterol (CHOL; Sigma-Aldrich, C8667; Exp. 1, 3 and 4.2). Alternatively, cannulas were left empty as controls (Exp. 2 and 4.1). Cannulas were filled by tapping 30 times the cannula tips into crystalline VOR or CHOL filling the cannula with approximatively 1 mm length powder. The inhibitor packed in the cannula diffuses slowly into the brain areas immediately adjacent to the cannula tip and this technique was shown to successfully inhibit male quail copulatory behavior for several weeks in previous studies[26,27].

Behavior testing was then resumed soon after the stereotaxic surgeries. Specifically, starting five (Exp. 1, 3 and 4.2) or seven (Exp. 2 and 4.1) days after the implantation of the cannula, depending on the time the animals needed to recover from surgery, all birds were repeatedly tested for sexual behaviors. For all experiments, a first test of sexual motivation (RCSM) was performed on day 5 (Exp. 1, 3 and 4.2) or day 7 (Exp. 2 and 4.1) and followed by series of copulatory tests (Exp. 1, days 8, 12 and 16 after surgery; Exp. 2, days 9, 11, 13, 29 and 36 after surgery; Exp. 3 and 4.2, days 7, 14, 21 and 28 after surgery; Exp. 4.1, days 9 and 14

after surgery). A final test of RCSM was conducted on day 19 (Exp. 1), 38 (Exp. 2) or 32 (Exp. 3 and 4.2).

Body mass and cloacal gland size were periodically measured during these experiments. No significant changes in these measures related to the treatment were detected and these data will therefore not be reported in detail here. All experimental procedures are in agreement with the Belgian laws on the "Protection of Experimental Animals" and were approved by the Ethics Committee for the Use of Animals at the University of Liège (Protocol #1442).

### 2.3. Stereotaxic implantation

All birds were anesthetized with 5% isoflurane (in 0.8 L $O_2$/min, Isovet, Verdifarm, using a custom-made anesthesia mask; Kopf Instruments) subsequently decreased to 2% and placed in a stereotaxic apparatus (Kopf Instruments). The head of the quail was positioned in the stereotaxic frame with the ear bar and the beak holder using an angular approach of approximately 45°. The skull was exposed by a medial skin incision and scraped with a scalpel to identify the sagittal midline between the two parietal bones. The center of the interaural axis was used as the zero-reference point for the antero-posterior axis (x). The superior sagittal sinus in the dorso-medial part of the brain presumably located under the sagittal midline of the skull was used as a reference point to determine coordinates in the vertical (y) and lateral axis (z). Craniectomy was performed to introduce the cannula as it allows the visualization of the sinus and facilitates the entry of the cannula tips by doing a small incision of the meninges at the entry point in the brain. To target the POM, PAG and VMN located in a medial position of the brain, a unique square was removed from the skull to visualize the sinus. To target the TnA, two symmetrical squares were removed on each side of the skull at the corresponding lateral stereotaxic coordinates of this nucleus. In this case, the sagittal midline rather than the sinus was used as a reference point to avoid performing a too large craniectomy. Holes were drilled in the bone surrounding the square to secure the cannula to the skull with dental cement. When a home-made cannula was used (see next paragraphs), the part of the cannula protruding out of the skull was removed with a wire cutter and clogged with dental cement. Commercial cannulas were closed with a dust-cap to prevent the contamination of the inside of the cannula. Finally, skin was sutured.

Stereotaxic coordinates were based on the quail and chicken brain stereotaxic atlases[45,46] and adjusted by trial and error. Final coordinates of the bilateral cannula were 1.2 mm anterior to the zero-reference point, 6 mm deep relative to the surface of the sinus, and 0.4 or 0.75 mm lateral from the sinus for the POM (Exp. 1), 4.4 mm posterior to the zero-reference point using a rostral angular approach of 20° to avoid the cerebellum, 4.6 mm deep relative to the surface of the sinus, and 0.8 mm lateral from the sinus for the PAG (Exp. 2), 0.25 mm posterior to the zero-reference point, 5.6 mm deep relative to the surface of the skull, and 4 mm lateral from the sagittal midline for the TnA (Exp. 3), and 0.2 mm anterior or 0.25 mm posterior to the zero-reference point, 7.75 mm or 7.5 mm deep relative to the surface of the sinus, and 0.75 or 0.6 mm lateral from the sinus for the VMN (coordinates for Exp. 4.1 and 4.2 respectively). Knowing that rostral rather than caudal lesions of the TnA result in reduced expression of sexual behavior in male quail[47,48], implants were placed in the most rostral part of the nucleus but also targeted a larger aromatase cell group rostral

to the boundaries of TnA. In any case, none of the VOR implants affected sexual behaviors.

Birds were implanted with commercial bilateral cannulas in the POM (Exp. 1; two types of cannulas with different center to center (c-c) distances were used in this experiment (see detailed objectives in next paragraphs): C235G-0.8 and C232G-1.5, 7.2 mm below pedestal, 26- and 22- gauge respectively, PlasticsOne®), in the PAG (Exp. 2; C232G-1.6, 7.8 mm below pedestal, 22 gauge, PlasticsOne®), and in the VMN (Exp. 4.1; C232G-1.5, 8.8 mm below pedestal, 22 gauge, PlasticsOne®). Bilateral cannulas with large or smaller c-c distances were home made to target the TnA (Exp. 3) and VMN (Exp. 4.2) respectively. Briefly, two 24-gauge needles (BD Microlance™, 304100) were used. The plastic adaptor used to connect to a syringe was removed by melting with a Bunsen burner followed by scraping with a razor blade to completely clean the blunted tip of needle. The lumen of the needle was cleaned to receive the drug. Both needles were then fixed symmetrically in the electrode holder with a straight clamp (Model 1771, Kopf Instruments), the beveled tip being inserted in the holder of the stereotaxic apparatus such that the blunted tip could be inserted in the brain.

A previous study[27] and post-mortem histological analyses performed after preliminary experiments had shown that, when using a c-c distance of 1.5 mm, the cannula tips were located laterally to the POM, questioning whether the drug could efficiently diffuse to the targeted nucleus. Therefore, in experiment 1 we used two types of bilateral cannulas with c-c distances of 1.5 or 0.8 mm. Three groups of males were used in this experiment: a control group implanted with a bilateral cannula filled with cholesterol with a c-c distance of 0.8 mm (N=9) and two groups implanted with a bilateral cannula filled with VOR with a c-c distance of 0.8 mm (N=7) or 1.5 mm (N=6). The selection of the shorter c-c distance was based on surgical limitations: 0.8 mm is the shortest distance allowing implantation without touching the dorsal sinus. This experiment targeting the POM was used as a positive control as previous results showed a reduced expression of copulatory behavior induced by aromatase inhibitors implanted in this region[26,27]. Contrary to these previous studies, this experiment used sexually experienced males carefully matched based on body mass, cloacal gland area and sexual performance. Since it is easier to maintain with testosterone an established sexual behavior than to activate its appearance and correspondingly more difficult to inhibit established behavior than to block its appearance [49,50], this experiment constituted an additional test of the role of preoptic aromatase in the control of sexual behavior.

For experiments targeting the PAG, TnA and VMN (Exp. 2, 3, 4.1 and 4.2), the c-c distance was based on the location of each brain nucleus in the brain atlases and corrected by our preliminary investigations. Only two groups were compared in these experiments: a CTL group (Exp. 2, N=10; Exp. 3, N=8; Exp. 4.1, N=10; Exp. 4.2, N=8) and a VOR group (Exp. 2, N=14; Exp. 3, N=7; Exp. 4.1, N=8; Exp. 4.2, N=8). Two experiments were performed to target the VMN. Indeed, post-mortem histological results from a first experiment (Exp. 4.1) indicated that cannula tips were located in the rostro-lateral part of the VMN for most subjects and were possibly too deep to target the dorsal and more medial portions of the aromatase-positive cell populations in this nucleus. A second experiment (Exp. 4.2) thus attempted to target

a larger proportion of the aromatase neurons of the VMN by adjusting stereotaxic coordinates.

### 2.4. Quantification of sexual behaviors

Male sexual behavior is classically divided into an appetitive phase, indicative of the motivation to mate, and a consummatory phase, the actual sexual performance[51,52]. Stimulus females were gonadally intact and gained sexual experience as they interacted with the experimental males during (pre)tests. Behavioral scoring and analyses were carried out by the investigator who was blind to the treatment of animals.

#### 2.4.1. Appetitive sexual behavior - Rhythmic cloacal sphincter movements (RCSM)

The frequency of RCSM produced in response to the view of a female is commonly used as a measure of sexual motivation in quail[34]. RCSM were quantified by placing the experimental male quail in a glass aquarium (50 [length] x 30 [width] x 40 [height] cm). The experimental device was covered on top with wire mesh and divided in two compartments. The largest of the two compartments (33.4 cm long) was separated by a transparent glass partition and an adjacent vertically sliding cardboard from a smaller compartment (16.7 cm long) to prevent the birds located on each side from seeing each other. The experimental bird was placed in the larger compartment while the view of the gonadally intact female stimulus located in the smaller compartment was obstructed by the cardboard. Few seconds after the introduction of the experimental male, the cardboard was lifted, allowing visual access to the female for 150 seconds although physical interaction was still prevented by the glass partition. The experimental device was positioned on an elevated transparent platform with a mirror placed underneath at a 45° angle to provide an unobstructed view of the cloacal area of the experimental bird[52,53]. The number of RCSM was quantified in real time for the 150 seconds while the experimental male was exposed to the view of the female.

#### 2.4.2. Consummatory sexual behavior

The frequency and latency of neck grabs (NG), mount attempts (MA), mounts (M) and cloacal contact movements (CCM; detailed description in [54,55]) were scored in real time during 5 minutes periods immediately following the introduction of the experimental male in a small arena (60 [length] x 40 [width] x 50 [height] cm) containing a gonadally intact sexually mature female. Birds who did not display a given behavior were assigned a latency of 5 minutes for statistical purposes. As NG and M frequencies and latencies were nearly identical to MA and CCM frequencies and latencies respectively, only data for MA and CCM will be presented.

### 2.5. Histological verification of the stereotaxic implant

#### 2.5.1. Immunohistochemistry

At the end of each experiment, birds were euthanized either by transcardiac perfusion with 4% paraformaldehyde (Sigma-Aldrich, 441244) in 0.1 M phosphate

buffer (PB, pH=7.2) following anesthesia with pentobarbital-natrium (Euthasol vet. 400mg/ml; 200ml per quail; Exp. 1, 3 and 4.2) or by rapid decapitation (Exp. 2 and 4.1). Following perfusion, brains were removed from the skull, post-fixed in PFA 4% overnight and then rinsed three times in phosphate-buffered saline (PBS 0.01M, pH 7.4) for 30 min. Brains from birds that were decapitated were fixed in 5% acrolein (Sigma-Aldrich, 110221) in PBS for 2.5 hours followed by three 30 min rinses in PBS. Brains were then immersed in 30% sucrose until they sank (approximatively two nights), frozen on dry ice and stored at -80°C until further use. Brains were cryosectioned in four series of 30 µm thick coronal slices and stored in antifreeze at -20°C until further use.

One series of brain sections was immunostained for aromatase by procedures previously described and validated [10,56]. Briefly, sections were first rinsed three times in 0.01 M tris-buffered saline (TBS, pH 7.6) for 5 min each (same procedure applied for all following rinses). Sections were then incubated in 0.1% of sodium borohydride (Sigma-Aldrich, 452882) in TBS for 15 min to retrieve antigen from the acrolein fixed tissue and followed by rinses (this step is not necessary for PAF fixed tissue). Peroxidase activity was blocked with 0.6% of hydrogen peroxide ($H_2O_2$; Carl Roth, 8070.1) in TBS at room temperature (RT) for 20 minutes. After rinses, sections were left for 1 hour in 5% of normal goat serum (NGS; Vector Laboratories, S-1000) and TBS containing 0.1% Triton X-100 (TBST) to reduce non-specific binding of proteins and antibodies to reaction surfaces and non-specific binding sites. Sections were then incubated with an anti-aromatase primary antibody (1/3000, Harada QR 02/05) in TBST for two nights at 4°C. Sections were rinsed and incubated for 2 hours at RT with a goat anti-rabbit biotinylated secondary antibody (1/400, Dako, E0432) in TBST. Sections were washed in TBS and incubated for one hour and a half in the vectastain® Elite ABC-HRP Kit, Peroxidase (Vector Laboratories, PK-6100) with TBST at RT, then rinsed in TBS. The peroxidase was finally visualized with 0.04% of 3,3'-diaminobenzidine tetrachlorhydrate (Carl Roth, CN75.1) used as chromogen along with 0.012% of $H_2O_2$ in TBS at RT. Sections were finally rinsed, mounted on slides, dried overnight, left ten minutes in xylene and coverslipped using Eukitt (Sigma-Aldrich, 03989).

#### 2.5.2. Image analysis

Brain sections were examined with an Olympus BH-2 microscope at the magnification x4 for the POM and VMN and x2 for the PAG and TnA. Photographs from figure 1 were acquired with a Scion Corporation MTV-3 camera. Stereotaxic implants were considered as being in the targeted brain nucleus when the tip of the lesion made by the implant was located at the dorsal edge of, at the lateral boundary or within the population of aromatase immunoreactive cells of the targeted nucleus. All nuclei of interest in the present study (POM, PAG, TnA, VMN) contain large populations of neurons that express aromatase (see Fig. 1; [10,37]).

Insert figure 1 about here

The two tips of the cannulas had to target the population of neurons immunoreactive for aromatase to be considered as having targeted the nucleus (Figure 1). It is indeed well established than an inhibitor must act bilaterally to exert a significant effect on

behavior. Implant tip locations were then plotted in specific figures for each experiment. Post-mortem analyses revealed that some birds with a cannula filled with VOR or CTL had their implants outside the targeted brain nucleus. Specifically, one implant filled with CHOL and one filled with VOR were out of the POM (Exp. 1) and TnA (Exp. 3), one implant filled with VOR was out of the PAG (Exp. 2), one empty implant and two implants filled with VOR were out of the VMN targeted in the first experiment (Exp. 4.1), whereas all implants were at the intended location for the second experiment targeting VMN (Exp. 4.2). Data from these few birds were thus moved to the control group (see above for sample size including individuals whose cannula was outside the POM in CTL). Note that all conclusions presented here are not affected when these birds are included in the control groups or excluded. More generally, it was shown here and in previous experiments on the POM that vorozole implants do not affect sexual behavior when located outside the target nucleus[26,27]. This strongly suggests that the inhibitor only diffuses short distances away from the cannula tip in behaviorally effective concentrations.

### 2.6. Statistical analyses

All behaviors were analyzed by two-way repeated measures ANOVAs followed by Sidak's post-hoc tests when significant effects were revealed by using GraphPad Prism 8.0 for Windows 10. All ANOVAs were performed without assuming sphericity. The deviation from sphericity was accounted for by the correction of Greenhouse and Geisser, which results in degrees of freedom sometimes becoming fractional (see the Prism Graph User guide at https://www.graphpad.com/guides/prism/6/statistics/stat_sphericity_and_compound_symmet.htm). Effects were considered significant for $p < 0.05$ and are presented in the text and figures by their mean ± standard error of mean.

## 3. RESULTS

### 3.1. Appetitive sexual behavior

As illustrated in figure 2, the chronic administration of the aromatase inhibitor implanted bilaterally in the POM, PAG, TnA or VMN did not change the frequency of RCSM over time.

Accordingly, separate two-way repeated measures ANOVAs for each brain nucleus, with treatment as an independent factor and the tests as a repeated measure, revealed no effect of treatment (POM: $F_{2,19}=0.2436$, $p=0.7862$; PAG: $F_{1,22}=0.2178$, $p=0.6453$; TnA: $F_{1,13}=0.0477$, $p=0.8305$; VMN (Exp.4.1): $F_{1,16}=0.0319$, $p=0.8505$; VMN (Exp.4.2): $F_{1,14}=0.0199$, $p=0.890$) and no interaction between treatment and test (POM: $F_{4,38}=0.6698$, $p=0.6170$; PAG: $F_{2,43}=0.7629$, $p=0.4725$; TnA: $F_{2,26}=1.503$, $p=0.2411$; VMN (Exp.4.1): $F_{1,16}=0.7524$, $p=0.3985$; VMN (Exp.4.2): $F_{2,28}=0.8855$, $p=0.4238$). However, an effect of repeated testing was found for birds implanted in the POM ($F_{1.985,37.72}=7.235$, $p=0.0022$), the PAG ($F_{1.988,42.74}=3.772$, $p=0.0312$) and the TnA ($F_{1.50,19.51}=4.425$, $p=0.0350$), but not in those implanted in the VMN (Exp.4.1; $F_{1,16}=0.0590$, $p=0.8112$; Exp.4.2; $F_{1.529,21.41}=0.8775$, $p=0.4037$). The Sidak's post hoc tests revealed that this main effect of tests reflects a reduction of the RCSM frequency between the test performed before surgery (PRE) and/or the test

performed the first week following surgery (POST 1) compared to the last test performed several weeks after surgeries (POST 2; see figure 2 for details about the individual comparisons).

Insert figure 2 about here

### 3.2. Consummatory sexual behavior

#### 3.2.1. Experiment 1: Medial preoptic nucleus (POM)

The first goal of this experiment was to build on previous findings showing a reduced expression of copulatory behavior induced by VOR implanted in the POM[26,27] as a positive control to extend the experimental procedure to different aromatase expressing brain nuclei of the social behavior network. By utilizing sexually experienced males matched based on sexual performance, body mass and cloacal gland area, the second goal was to confirm the ability of VOR to inhibit the established behavior instead of blocking its appearance, which had never been done before. Finally, the third goal was to determine the best location of the bilateral guide cannula tips relative to the brain nucleus by using two types of cannulas with a larger and shorter distance between the tips (1.5mm and 0.8mm, respectively; see details in the methods).

As confirmed by the two-way repeated measures ANOVA, the behaviors displayed during the pre-test phase by the three groups of males did not differ (groups: $F_{2,19}<2.305$, $p>0.1269$; interaction: $F_{18,171}<1.440$, $p>0.1185$), but their sexual performance improved over time as shown by an effect of test for all measures ($F>3.016$, $p<0.0453$) except MA latency ($F_{2.606,49.51}=1.304$, $p=0.2835$; Figure 3, PRE).

Sexual performance was drastically reduced in both groups implanted with VOR, whereas a relatively stable level of copulatory behavior was observed in the CTL males (Figure 3, POST). Accordingly, ANOVAs of data collected during the 3 post-tests identified an effect of treatment, but no effect of test and no interaction between treatment and test for the MA (treatment: $F_{2,19}=8.838$, $p=0.0019$; test: $F_{1.940,36.87}=1.298$, $p=0.2847$; interaction: $F_{4,38}=0.7398$, $p=0.5708$) and CCM frequencies (treatment: $F_{2,19}=5.872$, $p=0.0103$; test: $F_{1.595,30.30}=0.2919$, $p=0.6992$; interaction: $F_{4,38}=0.1534$, $p=0.9603$). However, no effect of treatment, test or interaction were found for the MA (treatment: $F_{2,19}=1.650$, $p=0.2184$; test: $F_{1.906,36.22}=2.958$, $p=0.0669$; interaction: $F_{4,38}=1.746$, $p=0.1601$) and CCM latencies (treatment: $F_{2,19}=3.316$, $p=0.0582$; test: $F_{1.937,36.81}=0.5874$, $p=0.5557$; interaction: $F_{4,38}=0.3476$, $p=0.8440$). The Sidak's post hoc tests indicated that CTL males displayed higher frequencies of MA and CCM compared to VOR treated males regardless of the distance between the two cannulas (for details about post hoc comparisons, see figure 3). Importantly, no difference was found between the two groups of VOR treated males (MA: $p=0.7796$; CCM: $p=0.3588$). Implant tip locations for this nucleus were shown in figure 4.

These results confirm that preoptic aromatase plays a key role in the expression of copulatory behavior. Its inhibition largely blocks the expression of the behavior even if it was fully established during the pre-experimental period. In addition, the absence of difference between bilateral cannulas with different c-c

distances suggests that the inhibitor is able to diffuse sufficiently through the nucleus to result in a significant effect on behavior.

Insert figure 3 and 4 about here

### 3.2.2. Experiment 2: Periaqueductal gray (PAG)

The two-way repeated measures ANOVA on behaviors displayed during the pre-test phase, confirmed that the frequencies and latencies of behaviors displayed by the two groups of males did not differ before treatment (groups: $F_{1,21}<0.9159$, $p>0.3494$; interaction: $F_{6,126}<1.629$, $p>0.1444$), but sexual behavior expression improved over time (Figure 5, PRE) as expected and supported by an effect of test for the CCM frequency ($F_{3.149,\ 66.13}=4.623$, $p=0.0047$) and latency ($F_{4.859,\ 102.0}=3.656$, $p=0.0048$).As illustrated in figure 5, the aromatase inhibitor implanted in the PAG led to a slight reduction of the CCM frequency, while other aspects of sexual behavior were not affected. Accordingly, the ANOVA of data from the post-test phase revealed an effect of treatment ($F_{1,21}=7.675$, $p=0.0115$) and test ($F_{3.282,68.10}=4.159$, $p=0.0074$) for the CCM frequency, but not for other behavioral measures (treatment: $F_{1,21}<1.187$, $p>0.2882$; test: $F<1.833$, $p>0.1544$). Regardless of the measure, no interaction was found between treatment and test ($F_{4,83}<2.025$, $p>0.0984$). Implant tip locations for this nucleus were illustrated in figure 6.

These results thus indicate that the aromatase expressed in the PAG participates to the control of the CCM frequency.

Insert figure 5 and 6 about here

### 3.2.3. Experiment 3: Nucleus taeniae of the amygdala (TnA)

The copulatory behavior of the two groups of birds did not differ prior to surgery as confirmed by the two-way repeated measures ANOVA run on the data of the pre-test phase which revealed no group difference or interaction between group and test (groups: $F_{1,13}<0.5298$, $p>0.4796$; interaction: $F_{7,91}<0.9906$, $p>0.4432$) except for an interaction affecting the MA frequency ($F_{7,91}=2.385$, $p=0.0276$). The Sidak's post hoc tests revealed however no specific differences ($p>0.05$; Fig. 7, PRE). This interaction may be explained by the differential expression of MA frequency in the two groups over the five first tests that disappeared in the last three tests resulting in two groups with similar frequencies of MA prior to surgery. As expected, the behavioral performance also improved with repeated testing as supported by a main effect of the test for the CCM frequency and latency ($F>3.226$, $p<0.0143$). Sidak's post doc tests performed on the CCM frequency and latency revealed however no significant differences between tests ($p>0.05$).

As illustrated in figure 7 (POST), no change could be detected in behavior frequency and latency following treatment with VOR in TnA. Accordingly, an ANOVA of data from the post-test phase did not reveal any effect of the treatment, test, or interaction between these two factors for any measure (treatment: $F_{1,13}<1.258$, $p>0.2824$; test: $F<2.197$, $p>0.1135$; interaction: $F_{3,39}<2.145$, $p>0.1101$) with the exception of an effect of test on CCM latency ($F_{2.364,30.73}=4.243$, $p=0.0187$), possibly

explained by an increased latency between T3 and T4 (p=0.0907). Implant tip locations for this nucleus were shown in figure 8.

Together, these results suggest that the aromatization taking place in TnA or in the aromatase-expressing neurons that are rostral to the nucleus does not play a key role in the expression of copulatory behavior in Japanese quail.

Insert figure 7 and 8 about here

### 3.2.4. Ventromedial nucleus of the hypothalamus (VMN)

Two experiments were conducted to target the aromatase expressing neurons of the ventromedial nucleus of the hypothalamus (VMN). In the first experiment, the behavioral tests were stopped after two tests as most animals displayed levels of sexual behaviors similar to those observed in the pre-test phase. Post-mortem analysis revealed that the cannula was located for most subjects in the rostro-lateral part of the VMN. It was also possibly too ventral for the treatment to reach the dorsal edge of the nucleus even if part of the aromatase expressing neurons were successfully targeted. The second experiment aimed at replicating this finding following adjustments of the stereotaxic coordinates to target the dorsal portion of the nucleus.

#### 3.2.4.1 Experiment 4.1: Rostral part of the VMN

As illustrated in figure 9, there was no difference between groups or tests and no interaction between these factors prior to surgery but also after the cannulas were implanted in the VMN (PRE: treatment: $F_{1,16}$<1.953, p>0.1813; test: F<3.916, p>0.0621; interaction: $F_{2,32}$<2.013, p>0.1502; POST: treatment: $F_{1,16}$<4.226, p>0.0565; test: $F_{1,16}$<1.531, p>0.2339; interaction: $F_{1,16}$<3.922, p>0.0651). Implant tip locations for the rostro-lateral portion of this nucleus were shown in figure 10.

Insert figure 9 and 10 about here

#### 3.2.4.2 Experiment 4.2: Medial part of the VMN

The two groups of males did not differ prior to surgery as confirmed by the two-way repeated measures ANOVA of the pre-test phase data that revealed no group difference and no interaction between groups and test (groups: $F_{1,14}$<1.120, p>0.3078; interaction: $F_{7,98}$<1.238, p>0.2894; Figure 11, PRE). As expected, the behavioral performance improved with the repetition of the tests as supported by an effect of test on CCM frequency ($F_{2.872,40.20}$=3.399, p=0.0284).

After surgeries, no difference in MA and CCM frequencies and latencies was observed between the CTL and VOR groups (treatment: $F_{1,14}$<3.665, p>0.0762; time: F<0.9917, p=0.3926; interaction: $F_{3,42}$<0.7216, p>0.5447). Implant tip locations for the medial portion of this nucleus were shown in figure 12.

Both experiments 4.1 and 4.2 clearly indicate that the aromatization in the VMN parts targeted in this study does not play a key role in the expression of copulatory behavior in Japanese quail.

Insert figure 11 and 12 about here

It must be noted, however, that there was in experiment 4.2. a sharp decline in the CCM frequencies of both groups between the last pretest and the first test post implantation. A specific two-way repeated measures ANOVA of this subset of data indicates as expected a significant effect of time on CCM frequency ($F_{1,14}$<10.34, p=0.0062), but again no effect of treatment ($F_{1,14}$=0.4879, p=0.4963) and no interaction ($F_{1,14}$=0.8109, p=0.3831). This decreased frequency of CCM for both groups following stereotaxic surgery suggests an effect of lesions induced by the surgery in the VMN at this location, independent of aromatase inhibition. Note also that this decrease of CCM frequency only occurred when the medial part of VMN but not its rostral part was targeted (Experiment 4.2, but not 4.1). This decrease also specifically concerned the CCM frequency and not other behavioral measures. This decrease might thus represent a localized lesion effect confined to this behavioral measure. This effect identified by an ex-post facto analysis should be confirmed in future studies.

## 4. DISCUSSION

The main goal of this study was to determine the site-specific role on male sexual behavior of the aromatase expressed in discrete brain nuclei of the social behavior network. To achieve this goal, male quail were implanted with bilateral cannulas filled with the aromatase inhibitor VOR in the POM, PAG, TnA and VMN and the effects of these treatments on both appetitive (i.e., sexual motivation) and consummatory (i.e., copulation) components of sexual behavior were quantified. As previously demonstrated, copulatory behavior was drastically reduced when VOR targeted the POM[26,27]. VOR implants in the PAG also decreased the frequency of cloacal contact movements, the last step of copulatory behavior. However, VOR did not produce any effect on copulatory behavior when implanted in the TnA and VMN. Surprisingly, RCSM frequencies were not affected by the treatment in any of the targeted regions. These data indicate that the aromatization within the POM is most critical for the expression of male copulatory behavior in Japanese quail, while in the PAG the enzyme may play a minor role in the premotor control of male copulatory behavior.

### 4.1. Aromatase expressing brain nuclei and RCSM

The present study did not provide any evidence supporting a role of the aromatase expressed in the POM, PAG, TnA and VMN in the control of RCSM, a measure of sexual motivation in male Japanese quail. This finding is somewhat surprising as peripheral injections[39,40] or intracerebroventricular infusions[38,40] of the same aromatase inhibitor were previously shown to result in a reduction of sexual motivation. It was however unclear whether this reduction was triggered by the direct effect of the aromatase inhibitor on the behavior itself or by the indirect effect of the treatment resulting from the inability of the males to copulate with the females. The former hypothesis tends however to be supported by the fact that an acute intracerebroventricular injection of VOR inhibits within 30 min the expression of RCSM[40] (see [38] for additional discussion). In the present study, RCSM frequency was

not influenced by VOR neither before nor after the series of copulatory tests, even if copulatory behavior was successfully reduced in males implanted with the drug in the POM. By inference, it could be assumed that, as the inability to copulate with a female did not necessarily result in a later reduction of sexual motivation, there is no indirect effect of the treatment on male sexual motivation as measured by RCSM.

It has been proposed that distinct sub-populations of aromatase expressing cells in the POM differentially control the appetitive and consummatory aspects of sexual behavior[6,57]. Specifically, lesions affecting the caudal POM inhibited copulatory behavior, while those in rostral POM inhibited the learned social proximity response, another measure of sexual motivation[58]. Similarly, expression of copulatory behavior induced FOS expression in the caudal POM, while RCSM expression induced FOS in the rostral POM[59]. Finally, additional comparisons between male quail implanted with VOR or controls indicated a significant difference for the RCSM frequency when the medial and caudal portions of the POM was targeted (see detailed results in [27]). The sample size in the groups of individuals implanted in the more rostral versus more caudal parts of the POM was however too low to statistically determine whether inhibiting aromatase in one of these two sub-populations results in a higher inhibition of RCSM frequency. In the present study, VOR was implanted in both the medial and caudal part of the POM. It is possible that the VOR did not sufficiently diffuse in the more rostral portion on the POM to produce an effect on RCSM frequency. Alternatively, and in contrast to the study of de Bournonville and colleagues[27], the present study only used sexually experienced males carefully matched based on their sexual performance and it might be more difficult to inhibit RCSM by blocking aromatase when the behavior has been fully established. This would however contradict the results by Seredynski and colleagues[40] who showed that acute intracerebroventricular administration of VOR acutely reduced RCSM in sexually experienced males. Further studies should thus be conducted to compare the effect of a stereotaxic inhibition of aromatase in the rostral and caudal POM on the frequency of RCSM.

It should also be emphasized that RCSM are only one aspect of appetitive sexual behavior reflecting sexual motivation that has many dimensions (e.g., search for females, attraction by their calls or view, approach...). Sexual motivation can thus be quantified by multiple behavioral measures including the learned social proximity response[60] or the male approach to a female stimulus[61]. Conversely, consummatory sexual behavior is a stereotyped behavioral sequence in quail that is easier to measure. There may thus be a differential sensitivity to the effects of aromatase inhibition depending on the dependent measure selected. Alternatively, it is possible that other measures of sexual motivation would have been affected by the implants of VOR. Finally, RCSM frequency might be controlled by a sub-population of cells that was not inhibited in one of the targeted brain nuclei, by an additional brain nucleus expressing aromatase not considered in this study or by the combined action of two or more aromatase expressing brain nuclei. The bed nucleus of the stria terminalis (BST) is the only major nucleus expressing aromatase that was not investigated in the present study. A previous study had however shown that the stereotaxic inhibition of aromatase located in the BST does not alter RCSM frequency in male quail[27]. It is thus very unlikely that another candidate is involved in the control of RCSM.

### 4.2. Aromatase expressing brain nuclei and copulatory behavior

#### 4.2.1. POM

Consistent with previous studies[26,27], aromatase expressed in the POM was shown here to play an essential role in the copulatory behavior of male quail. Compared to previous studies where preoptic aromatase was blocked prior to the development of sexual experience[26,27], this experiment demonstrates that blocking preoptic aromatase in sexually experienced males also results in a marked reduction of copulatory behavior. Another interesting aspect of this study lies in the fact that the inhibition of aromatase was observed in two groups of animals implanted with either a long or short distance between the tips of the two bilateral cannulas (center to center (c-c) distances of 1.5 mm and 0.8 mm) which respectively targeted the lateral or the dorso-medial portions of the nucleus. Despite these differences in the c-c distances, the two groups of males showed a similar behavioral inhibition, thus confirming that VOR successfully diffused to at least a few hundred microns from the cannula tip and inhibited aromatase activity in the POM. In conclusion, the experiment targeting the POM not only confirmed the effects of chronic bilateral inhibition of the aromatase on established copulatory behavior, but also suggested that overall, VOR implanted in the dorso-lateral part of a nucleus is sufficient to inhibit aromatase activity within the targeted nucleus.

#### 4.2.2. PAG

In line with studies suggesting that aromatase expressing neurons located in the POM may control the premotor aspects of male copulatory behavior through their projection to the PAG[56,62], the present experiment revealed a significant reduction in CCM frequency following aromatase inhibition in the PAG. This effect was however modest but probably underestimated as it was difficult to precisely quantify the subtle disruption in the frequency of cloacal contact movements (CCM) observed in this experiment. CCM are commonly scored when the male grabs the female neck feather with his beak, both legs are placed at the top of female's back and more importantly when a movement of the male tail is performed to try to make contact between his cloaca and the cloaca of the female, before the animal falls backwards. While this tail movement is usually produced quickly (<1 sec) and with intensity and precision in control animals, it seems that the VOR implanted birds which displayed CCM did it with less intensity and precision, suggesting a disruption at the premotor level. This subtle disruption of the copulatory pattern was however not sufficiently large to make this behavior not recognizable.

This deficit also raises the question of whether the close apposition between the male and female cloaca was successful and more importantly, whether sperm exchange occurred. If this was not the case, then the minor disruption of CCM expression observed after implantation of VOR in the PAG might have an important biological impact since insemination success would be decreased. It is well established that male Japanese quail exhibit a large androgen-dependent cloacal gland that produces a meringue-like white foam[53] that is transferred to the female cloaca during copulations and is known to enhance male fertilization success[63]. The presence of this foam in female cloaca should be quantified in future experiments and considered as an indicator of insemination success to fully assess the possible

biological role of PAG aromatization in reproduction. Further studies should also be conducted on sexually naive males to determine whether aromatase blockade in the PAG would have more pronounced effects on the expression of CCM in birds with no prior experience with the experimental setup. An effect of experience was indeed identified in rats in which it was demonstrated that, in sexually naïve males, copulatory behavior was prevented by lesion in the medial amygdala[64], but this was not the case in sexually experienced subjects[65].

It must also be recognized that the PAG in mammals is organized in separate columns with a dorso-ventral organization that each have specific functions[66]. Specifically, the dorsal and lateral columns seem to be involved in the control of female sexual behavior. Injections of a GABA antagonist in these columns indeed caused a significant inhibition of lordosis behavior and these columns express estrogen receptors[67]. The anatomical organization of the PAG in birds is very different, but one study provided detailed evidence indicating also the presence of discrete fields with specific neurochemical characteristics and a differential activation, as measured by increased expression of immediate early genes, following expression of a variety of behaviors[68]. In birds, these different fields are however not organized along a dorso-ventral but rather a medio-lateral axis and they potentially extend laterally to the nucleus intercollicularis. It remains therefore possible that our treatment with an aromatase inhibitor failed to affect the fields that are relevant to the control of male appetitive or consummatory sexual behavior. It is in this respect worth mentioning that a recent experiment in canaries demonstrated that the injection of a GABA antagonist in the PAG increased the latency to resume singing behavior which can be considered as a form of appetitive sexual behavior[69]. To our knowledge, the present study is the first to examine the role of aromatase in the PAG in the context of sexual behavior *per se,* but additional work would therefore deserve to be undertaken.

#### 4.2.3. VMN

The present study identified no effect of the aromatase inhibitor implanted in the two sub-populations of the VMN including the rostro-lateral and the medial portions of the VMN. This is in line with previous preliminary data from our group[27]. In other species, studies targeting the VMN to examine the role of sex steroids hormones in the regulation of sexual behavior have produced contradictory results. Some studies found that implanting testosterone in this nucleus does not affect the behavior of castrated male rabbits or house mice[70,71], while stereotaxic implants of testosterone located in the VMN restored sexual motivation, but not copulation in castrated male rats[72]. Androgen receptor blockade in the anterodorsal portions of the VMN impairs the restoration of sexual motivation and copulation in castrated male rats treated with systemic testosterone[73]. In contrast, in male mice, estrogen receptor alpha (ERα) knockdown in the VMN reduces sexual behavior[29]. Optogenetic activation targeting ERα neurons in the ventrolateral subdivision of the VMN also increases close investigation towards a female stimulus and mounting behaviors, but this effect largely depends on the photostimulation intensity and the number of activated cells[30]. In mice, estrogens in the VMN might thus play a key role in male sexual behavior. Together, these somewhat contradictory studies indicate that if VMN plays a role in the activation of male sexual behavior, this effect seems to depend on the sub-

population of the targeted nucleus and the identity of the hormones mediating this effect may vary depending on the species. Based on these mammalian studies, it seems possible that effects of aromatase inhibition in VMN might be highly variable depending on the specific sub-population of the VMN that is affected. Additional work targeting different sub-regions might thus be needed to confirm or refine the lack of effect observed here.

#### 4.2.4. TnA

In mammals, sex steroid hormones in the medial amygdala (MeA), the mammalian homolog of the avian nucleus taenia of the amygdala (TnA)[41] are known to play a role in the control of male sexual behavior although this conclusion may vary depending on the species considered. In castrated hamsters, implants filled with either testosterone[74-77] or estradiol (E2)[78], but not the non-aromatizable androgen DHT[78] located in the MeA restore the appetitive and consummatory components of male sexual behavior. Similarly, in castrated rats, estradiol benzoate (EB) implants in the corticomedial amygdala increase the number of mounts and decrease the mount latency[79]. In addition, a DHT implant in the MeA of castrated rats treated with EB increases mounting and intromission rates and the incidence of ejaculation[80]. These studies suggest that aromatization in the amygdala plays a role in sexual behavior of male hamsters and a synergy between estrogens (estradiol) and androgens (DHT) may be necessary to fully activate sexual behavior in male rats. In line with this suggestion, stereotaxic implants of the androgen receptor antagonist hydroxyflutamide in the MeA are not effective enough to fully prevent the testosterone induced sexual behavior, as shown by the partially reduced but not significantly inhibited percentage of mounts, intromissions and ejaculations displayed by castrated males treated with testosterone[81]. In contrast, manipulations of the estrogen receptors in the MeA clearly affect male sexual behavior in most cases. The anti-estrogen tamoxifen implanted in the MeA reduces the frequencies of intromission and ejaculation in castrated hamsters treated with testosterone[77].

The role of local aromatization was also investigated more specifically. Indeed, in gonadally intact male rats systemically treated with the aromatase inhibitor fadrozole, implanting estradiol in the MeA maintained a sexual activity while copulatory behavior was decreased in untreated subjects[82]. Conversely, fadrozole implanted in the MeA of gonadally intact males increased the latencies of mounts and ejaculations and the post-ejaculatory interval[32]. Furthermore, bilateral implants of estradiol coupled to bovine serum albumin (BSA, a large protein that does not cross the plasma membrane, thereby restricting the action of estradiol to cell-surface signaling) in the MeA slightly increased the frequencies of intromissions and ejaculations, and decreased the post-ejaculatory interval in castrated males treated with DHT[32]. Together, these studies indicate that in the MeA, neuro-estrogens play a role in the activation of male sexual behavior in rats and hamsters. In mice, however, the contribution of estrogens produced in the amygdala to sexual behavior seems less clear. Indeed, one study showed that ERα knockdown in the MeA does not alter sexual behavior[29] and no effect was either observed after inhibition or ablation of aromatase-expressing neurons within the posterodorsal MeA[31]. But one study showed that chemogenetic inhibition and activation of ERα-expressing cells in the posterior amygdala that project to the medial preoptic nucleus disrupt the expression

of social investigation and sexual behavior[33]. Species differences may thus exist in the role played by the amygdala in the control of male sexual behavior or the relative contribution of estrogens and androgens to this control. There might also exist sub-populations playing different role in behavioral control. Finally, it is important to realize that manipulating the neuronal (electrical) activity of neurons expressing aromatase or estrogen receptors is not the same as blocking the synthesis or action of a given hormone. An absence of effect following optogenetic or chemogenetic manipulation is thus not necessarily in contradiction with the idea that estrogens are involved in a given behavior.

In male quail, the present study identified no effect of the aromatase inhibitor implanted in the anterior part of the nucleus taeniae (TnA). Yet, a previous study found that lesions of the anterior part of TnA reduce sexual motivation measured by the latency to approach and to stay close to a female stimulus, while copulatory behavior is partially disrupted [48]. Surprisingly, in another study targeting the posterior part of the TnA, copulatory behavior including mount attempt was facilitated by the lesion, whereas other measures of sexual motivation, including the frequency of RCSM or the learned social proximity response, remained unaffected[47]. Distinct sub-populations in this region might thus differentially affect male sexual behavior. Future studies will thus have to investigate the effect or aromatase in the posterior portion of TnA along with the role of androgens.

## 5. Limitations

The discrepancies within and between the studies previously described and our results might result from differences between species. Differences of methodology might also account for the variability observed between results. For example, manipulations of neuronal populations are more likely to produce behavioral effects than the targeting of a specific enzymatic protein. In addition, the discrepancies might be explained by the existence of distinct subpopulations of aromatase neurons implicated in distinct aspects of social behavior. This is the case in mice for example where two distinct subpopulations of estrogen receptor alpha-expressing cells were discovered in the posterior amygdala. One projects to the POA and controls sexual behavior, while the other projects to the VMN and controls aggressive behavior[33]. It is noteworthy that the PAG, TnA and VMN are large brain nuclei extending over a long distance in the rostro-caudal axis and it is very likely that they are fragmented in distinct functional cell sub-populations. It is possible that the VOR implanted in the present experiments did not diffuse to the entirety of these nuclei. The inhibitor would then have failed to affect a specific sub-population of cells involved in sexual behavior. Alternatively, copulatory behavior in male quail may only be modulated by neuro-estrogens arising from the POM and, to a lower extent from the BST[27] and PAG, but not from the VMN and TnA (present study). In these regions, sexual behavior could also rely on other mechanisms than those involving aromatization including testosterone action on androgen receptors either directly or following its conversion into its more potent androgenic metabolites, 5α-dihydrotestosterone, the synergy between androgen and estrogen action (see detailed information in [83]) or progesterone action on its receptor[84].

Finally, aromatase from the social behavior network is likely implicated in the control of other functions besides sexual behavior. For example, studies using viral strategies to site-specifically manipulate aromatase in male mice show an alteration of aggression when targeting the posterior amygdala and the bed nucleus of the stria terminalis (BST). Indeed, ablation of aromatase-expressing neurons in the posterodorsal medial amygdala reduces the number of attacks which is paralleled by an increased latency of attack[31]. Similarly, the inhibition or ablation of aromatase-expressing cells in the BST reduces the number and the percentage of males showing attacks[85]. As in mice, it is possible that this network of aromatase expressing brain nuclei plays a role in the wide range of social behavior displayed by quail in the wild[86] including sexual behavior, aggression and social motivation. Inhibition of brain aromatase by administration of VOR in the third ventricle only affected in a clear manner sexual behavior in a previous experiment where behaviors were tested in laboratory conditions[38]. However, the reliability of the tests for aggression and social motivation was probably limited (see discussion in [38]). The identification of robust measures of social behaviors in Japanese quail, combined with experiments that would utilize other animal models where such measures exist, should provide a final answer to the question of the behavioral role of aromatase in nuclei of the social behavior network.

## 6. Conclusion

The present study confirms that the POM constitutes a major center of aromatization involved in the control of copulatory behavior in male Japanese quail. The projection of aromatase neurons from the POM to other brain nuclei such as the PAG possibly ensures the optimal expression of the copulatory sequence. The role of aromatization within the TnA and VMN remains unresolved like the identity of the aromatase expressing brain region(s) involved in the control of RCSM, a measure of male sexual motivation.

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## FIGURE CAPTIONS

**Figure 1.** Representative images of the tips of the bilateral cannula implanted in the medial preoptic nucleus (POM; A and B), the ventromedial nucleus of the hypothalamus (VMN; C), the periaqueductal gray (PAG; D) and the nucleus taeniae (TnA; E). Implants in the POM included two distinct types of bilateral cannulas having a 0.8mm center-to-center (c-c) distance (A) and a 1.5mm c-c distance (B) between the tips, respectively. Arrows indicate the location of the cannula tips. Images were taken at 4x magnification for A, B and C and at 2x magnification for D and E. Abbreviations: DSD, dorsal supraoptic decussation; NIII, third nerve; PC, posterior commissure; 3V, third ventricle.

**Figure 2.** Site-specific inhibition of aromatization does not affect the frequency of rhythmic cloacal sphincter movement (RCSM) produced by male quail in response to the view of a female. RCSM frequency was quantified before (pre-test phase, PRE), approximately one week (POST 1) and several weeks after the cannula was implanted (POST 2; see methods for detailed timing). Three groups of birds were used to target the medial preoptic nucleus (POM; A): control quail implanted with a bilateral cannula filled with cholesterol as control (CTL) and two groups of quail treated with the aromatase inhibitor vorozole (VOR) administered through a bilateral cannula characterized by either a 0.8 mm (VOR 0.8) or a 1.5 mm (VOR 1.5) center-to-center (c-c) distance between the tips. Experiments targeting the periaqueductal gray (PAG; B), the nucleus taeniae of the amygdala (TnA; C) and the ventromedial nucleus of the hypothalamus (VMN; two experiments Exp.4.1 in fig. D and Exp.4.2 in fig. E) included control (CTL) and vorozole (VOR) groups of male quail. Data from

each brain nucleus (A to E) were analyzed by separate two-way repeated measures ANOVAs (boxed text) with the successive tests (TEST) as a repeated measure and the treatments (TRT) as an independent factor followed by Sidak's post-hoc tests when the effect of repeated testing was significant. *p<0.05, **p<0.01. INT= interaction; ns= not significant.

**Figure 3.** Effect of the inhibition of aromatization in the medial preoptic nucleus (POM) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 10 pretests (pre-test phase, PRE) and the 3 tests performed after cannula implantation (post-test phase, POST, gray square), but statistical results are only presented for the POST phase (for the PRE phase, see the main text). Three groups of male quail received cannulas filled either with cholesterol or with the aromatase inhibitor vorozole (VOR) with a center-to-center distance between the tips of 0.8 mm (VOR 0.8) or 1.5mm (VOR 1.5). The statistical boxes present the results of the analyses on POST data. The data were analyzed by two-way repeated measures ANOVA followed by Sidak post-hoc tests: $^{(*)}$p<0.10, *p<0.05, **p<0.01, ***p<0.001. TRT= treatment; INT= interaction; ns= not significant.

**Figure 4.** Neuroanatomical localization of the bilateral implants in the medial preoptic nucleus (POM). Panel A shows the individuals implanted with vorozole, whereas panel B shows the control individuals. Circles represent cannula tips that were in or outside the population of aromatase immunoreactive neurons. Bilateral cannulas with a 1.5 mm center-to-center (c-c) distance (green circles) and a 0.8 mm c-c distance (blue circles) between the tips were used in this experiment. Red circles represent implants that were outside of any aromatase immunoreactive population. A straight line connects both sides of a given bilateral cannula. Brain nuclei expressing aromatase are represented in color shapes. Pink: POM, green: BST and orange: TnA Abbreviations: AC, anterior commissure; BST, bed nucleus of the stria terminalis; TnA, nucleus taeniae of the amygdala; TSM, septopalliomesencephalic tract. (See web version of this article for the color code used in the image).

**Figure 5.** Effect of the inhibition of aromatization in the periaqueductal gray (PAG) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 7 pretests (pre-test phase, PRE) and the 5 tests performed after cannula implantation (post-test phase, POST; gray square), but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA: $^{(*)}$p<0.10, *p<0.05, **p<0.01, ***p<0.001. Sidak's post-hoc tests for the test effect on CCM frequencies were not significant. TRT= treatment; INT= interaction; ns= not significant.

**Figure 6.** Neuroanatomical localization of the bilateral implants in the periaqueductal gray (PAG). Panel A shows the individuals implanted with vorozole, whereas panel B shows the individuals with the implants left empty. Blue and red circles represent

cannula tips that were in and outside the population of aromatase immunoreactive neurons, respectively. A straight line connects both sides of a given bilateral cannula. PAG is represented in red. Abbreviations: ME, median eminence; NIII, third nerve; PC, posterior commissure. (See web version of this article for the color code used in the image).

**Figure 7.** Effect of the inhibition of aromatization in the nucleus taeniae of the amygdala (TnA) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 8 pretests (pre-test phase, PRE) and the 4 tests performed after cannula implantation (post-test phase, POST) but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA followed by Sidak post-hoc tests. TRT= treatment; INT= interaction; ns= not significant.

**Figure 8.** Neuroanatomical localization of the bilateral implants in the nucleus taeniae of the amygdala (TnA). Panel A shows the individuals implanted with vorozole, whereas panel B shows the control individuals. Blue and red circles represent cannula tips that were in and outside the population of aromatase immunoreactive neurons, respectively. A straight line connects both sides of a given bilateral cannula. Brain nuclei expressing aromatase are represented in color shapes. Pink: POM, green: BST, orange: TnA and blue, VMN. Abbreviations: AC, anterior commissure; BST, bed nucleus of the stria terminalis; DSD, dorsal supraoptic decussation; OT, optic tectum; POM, medial preoptic nucleus; TSM, septopalliomesencephalic tract; VMN, ventromedial nucleus of the hypothalamus. (See web version of this article for the color code used in the image).

**Figure 9.** Effect of the inhibition of aromatization in the ventromedial nucleus of the hypothalamus (VMN; Exp. 4.1) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 3 pretests (pre-test phase, PRE) and the 2 tests performed after cannula implantation (post-test phase, POST), but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA: TRT= treatment; INT= interaction; ns= not significant.

**Figure 10.** Neuroanatomical localization of the bilateral implants in the ventromedial nucleus of the hypothalamus (VMN) for Exp. 4.1. Panel A shows the individuals implanted with vorozole, whereas panel B shows the individuals with the implants left empty. Blue and red circles represent cannula tips that were in and outside the population of aromatase immunoreactive neurons, respectively. A straight line connects both sides of a given bilateral cannula. Brain nucleus expressing aromatase are represented in orange for the TnA and in blue, for the VMN. Abbreviations: DSD, dorsal supraoptic decussation; OT, optic tectum; TnA, nucleus taeniae of the amygdala. (See web version of this article for the color code used in the image).

**Figure 11.** Effect of the inhibition of aromatization in the ventromedial nucleus of the hypothalamus (Exp. 4.2) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 8 pretests (pre-test phase, PRE) and the 4 tests performed after cannula implantation (post-test phase, POST), but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA: TRT= treatment; INT= interaction; ns= not significant.

**Figure 12.** Neuroanatomical localization of the bilateral implants in the ventromedial nucleus of the hypothalamus (VMN) for Exp. 4.2. Panel A shows the individuals implanted with vorozole whereas panel B shows the control individuals. All cannula tips were in the population of aromatase immunoreactive neurons for this experiment and were represented by two dark blue circles connected on both sides by a straight line. Brain nucleus expressing aromatase are represented in orange for the TnA and in blue, for the VMN. Abbreviations: DSD, dorsal supraoptic decussation; OT, optic tectum; TnA, nucleus taeniae of the amygdala. (See web version of this article for the color code used in the image).

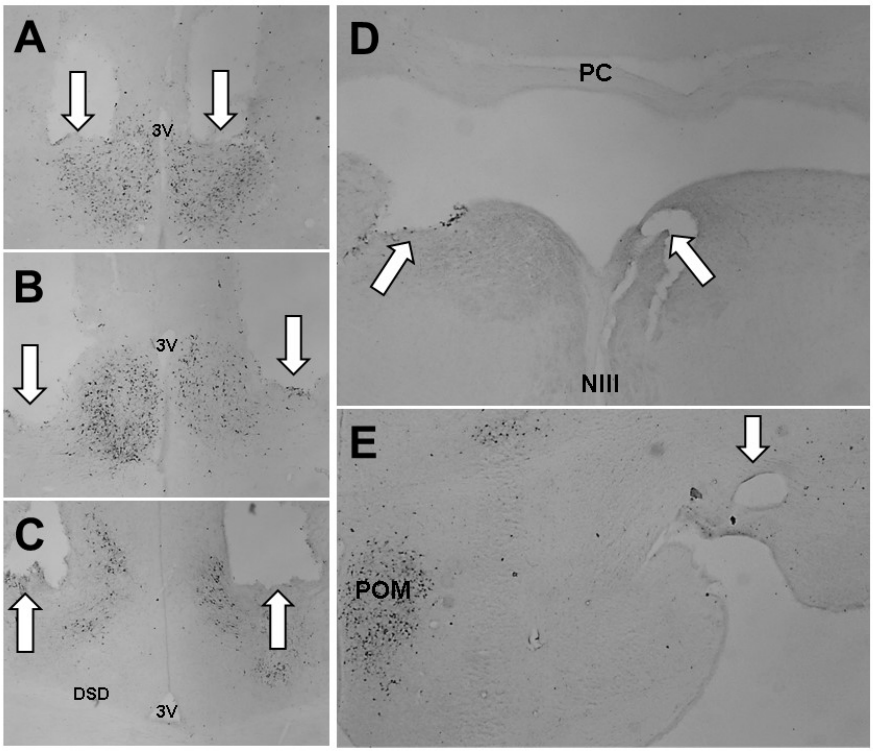


Figure 1. Representative images of the tips of the bilateral cannula implanted in the medial preoptic nucleus (POM; A and B), the ventromedial nucleus of the hypothalamus (VMN; C), the periaqueductal gray (PAG; D) and the nucleus taeniae (TnA; E). Implants in the POM included two distinct types of bilateral cannulas having a 0.8mm center-to-center (c-c) distance (A) and a 1.5mm c-c distance (B) between the tips, respectively. Arrows indicate the location of the cannula tips. Images were taken at 4x magnification for A, B and C and at 2x magnification for D and E. Abbreviations: DSD, dorsal supraoptic decussation; NIII, third nerve; PC, posterior commissure; 3V, third ventricle.

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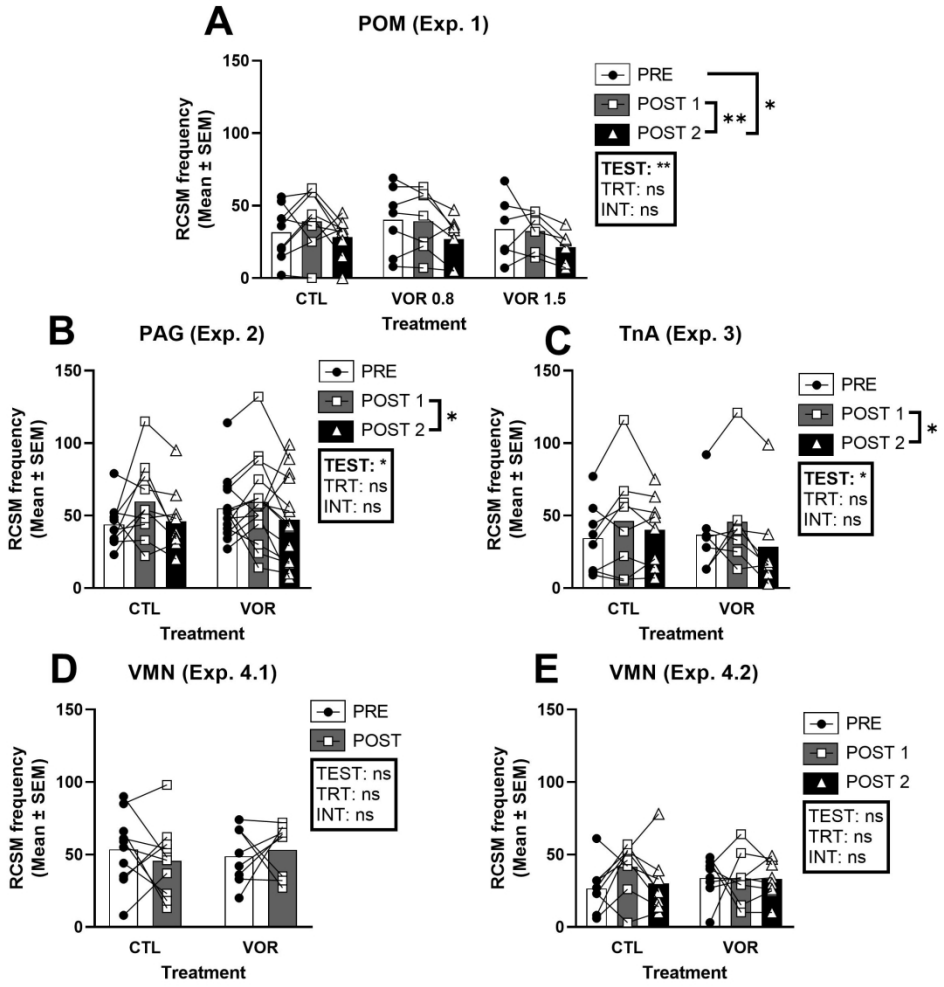


Figure 2. Site-specific inhibition of aromatization does not affect the frequency of rhythmic cloacal sphincter movement (RCSM) produced by male quail in response to the view of a female. RCSM frequency was quantified before (pre-test phase, PRE), approximately one week (POST 1) and several weeks after the cannula was implanted (POST 2; see methods for detailed timing). Three groups of birds were used to target the medial preoptic nucleus (POM; A): control quail implanted with a bilateral cannula filled with cholesterol as control (CTL) and two groups of quail treated with the aromatase inhibitor vorozole (VOR) administered through a bilateral cannula characterized by either a 0.8 mm (VOR 0.8) or a 1.5 mm (VOR 1.5) center-to-center (c-c) distance between the tips. Experiments targeting the periaqueductal gray (PAG; B), the nucleus taeniae of the amygdala (TnA; C) and the ventromedial nucleus of the hypothalamus (VMN; two experiments Exp.4.1 in fig. D and Exp.4.2 in fig. E) included control (CTL) and vorozole (VOR) groups of male quail. Data from each brain nucleus (A to E) were analyzed by separate two-way repeated measures ANOVAs (boxed text) with the successive tests (TEST) as a repeated measure and the treatments (TRT) as an independent factor followed by Sidak's post-hoc tests when the effect of repeated testing was significant. *p<0.05, **p<0.01. INT= interaction; ns= not significant.

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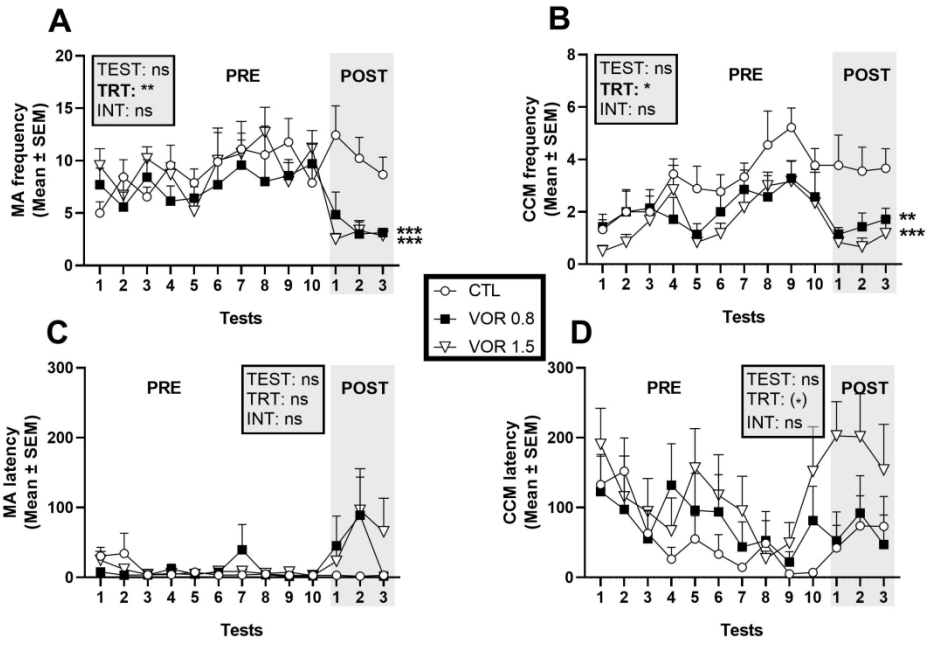


Figure 3. Effect of the inhibition of aromatization in the medial preoptic nucleus (POM) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 10 pretests (pre-test phase, PRE) and the 3 tests performed after cannula implantation (post-test phase, POST, gray square), but statistical results are only presented for the POST phase (for the PRE phase, see the main text). Three groups of male quail received cannulas filled either with cholesterol or with the aromatase inhibitor vorozole (VOR) with a center-to-center distance between the tips of 0.8 mm (VOR 0.8) or 1.5mm (VOR 1.5). The statistical boxes present the results of the analyses on POST data. The data were analyzed by two-way repeated measures ANOVA followed by Sidak post-hoc tests: (*)p<0.10, *p<0.05, **p<0.01, ***p<0.001. TRT= treatment; INT= interaction; ns= not significant.

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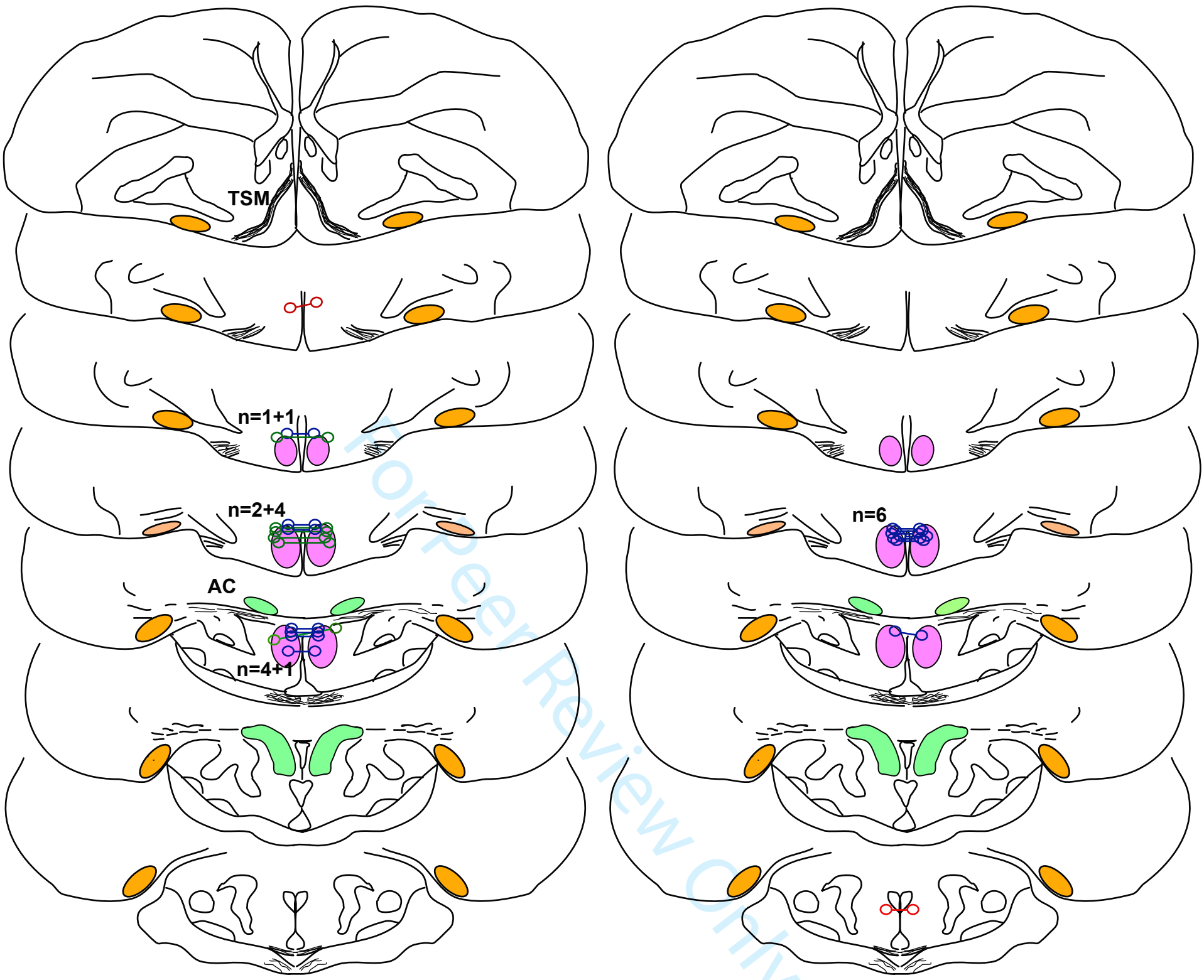

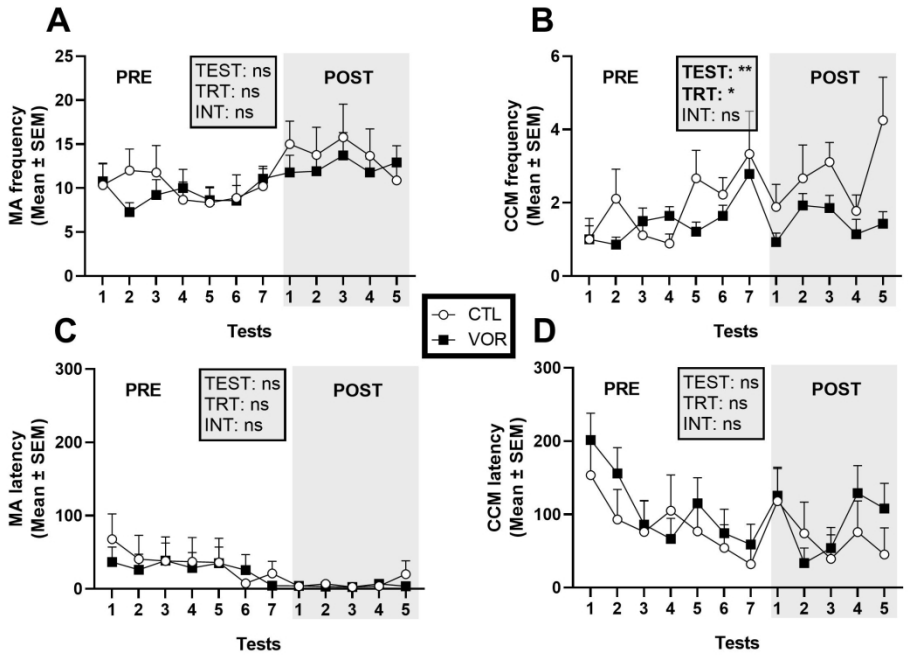


Figure 5. Effect of the inhibition of aromatization in the periaqueductal gray (PAG) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 7 pretests (pre-test phase, PRE) and the 5 tests performed after cannula implantation (post-test phase, POST; gray square), but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA: (*)p<0.10, *p<0.05, **p<0.01, ***p<0.001. Sidak's post-hoc tests for the test effect on CCM frequencies were not significant. TRT= treatment; INT= interaction; ns= not significant.

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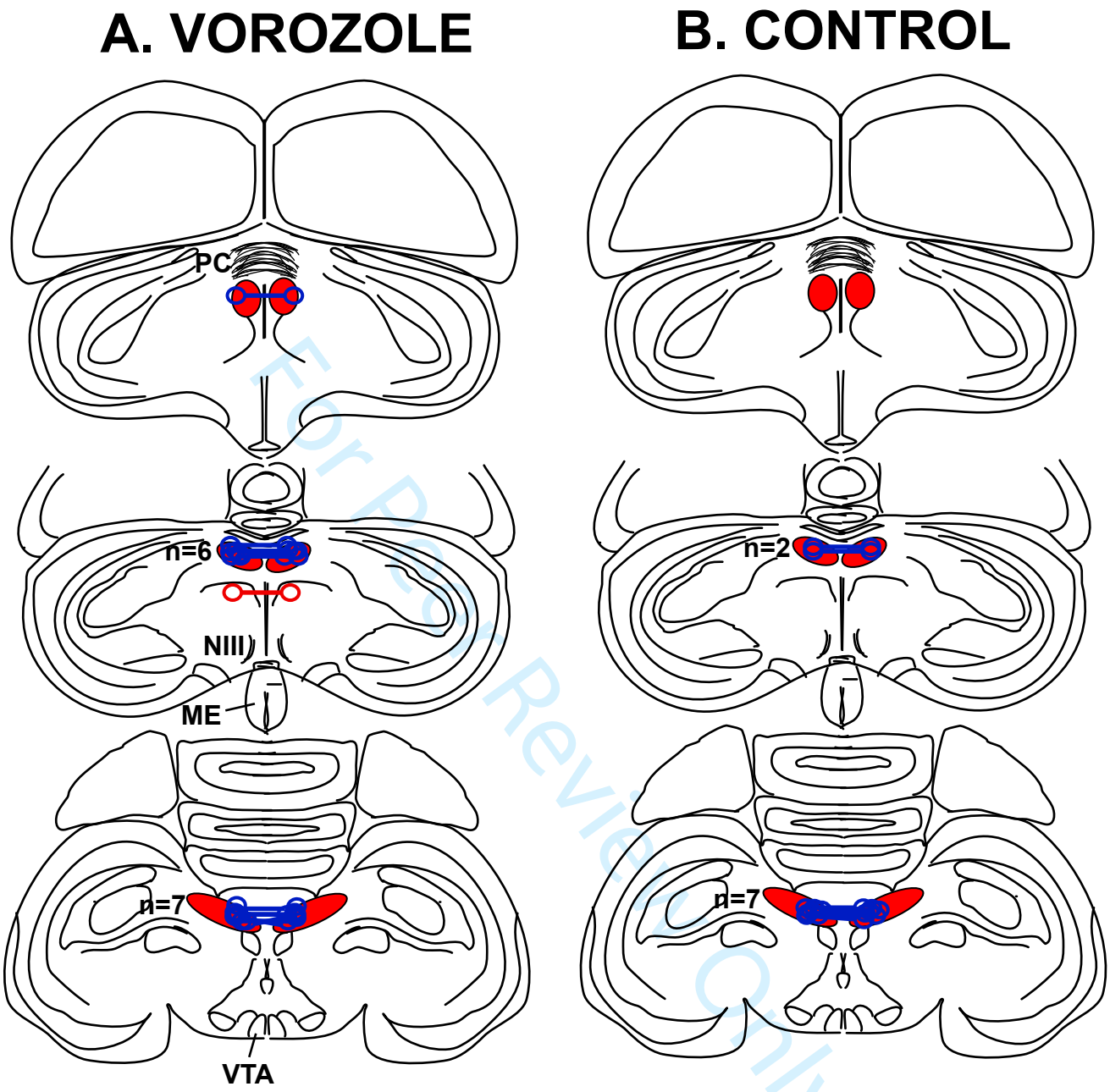

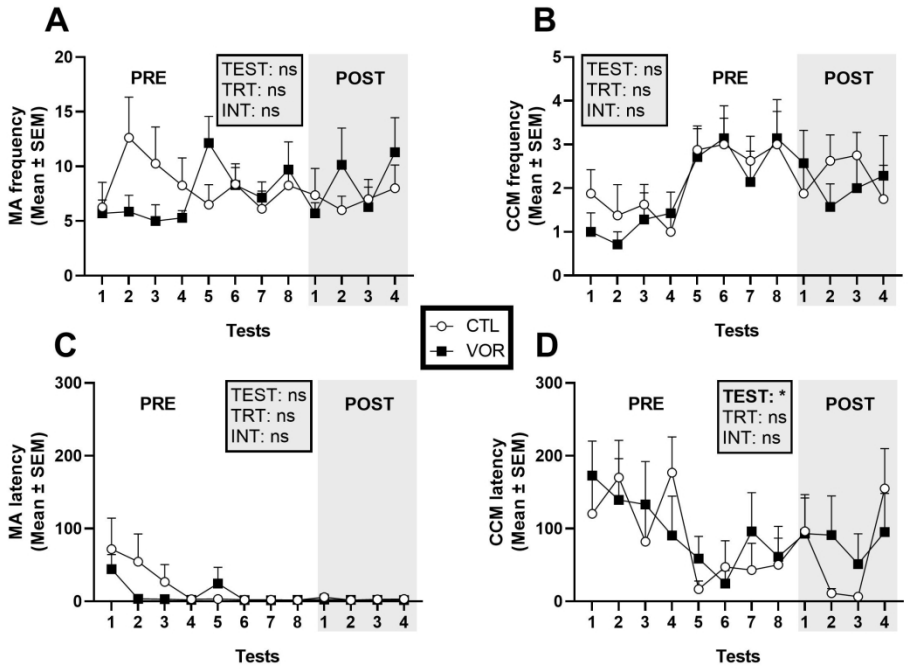


Figure 7. Effect of the inhibition of aromatization in the nucleus taeniae of the amygdala (TnA) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 8 pretests (pre-test phase, PRE) and the 4 tests performed after cannula implantation (post-test phase, POST) but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA followed by Sidak post-hoc tests. TRT= treatment; INT= interaction; ns= not significant.

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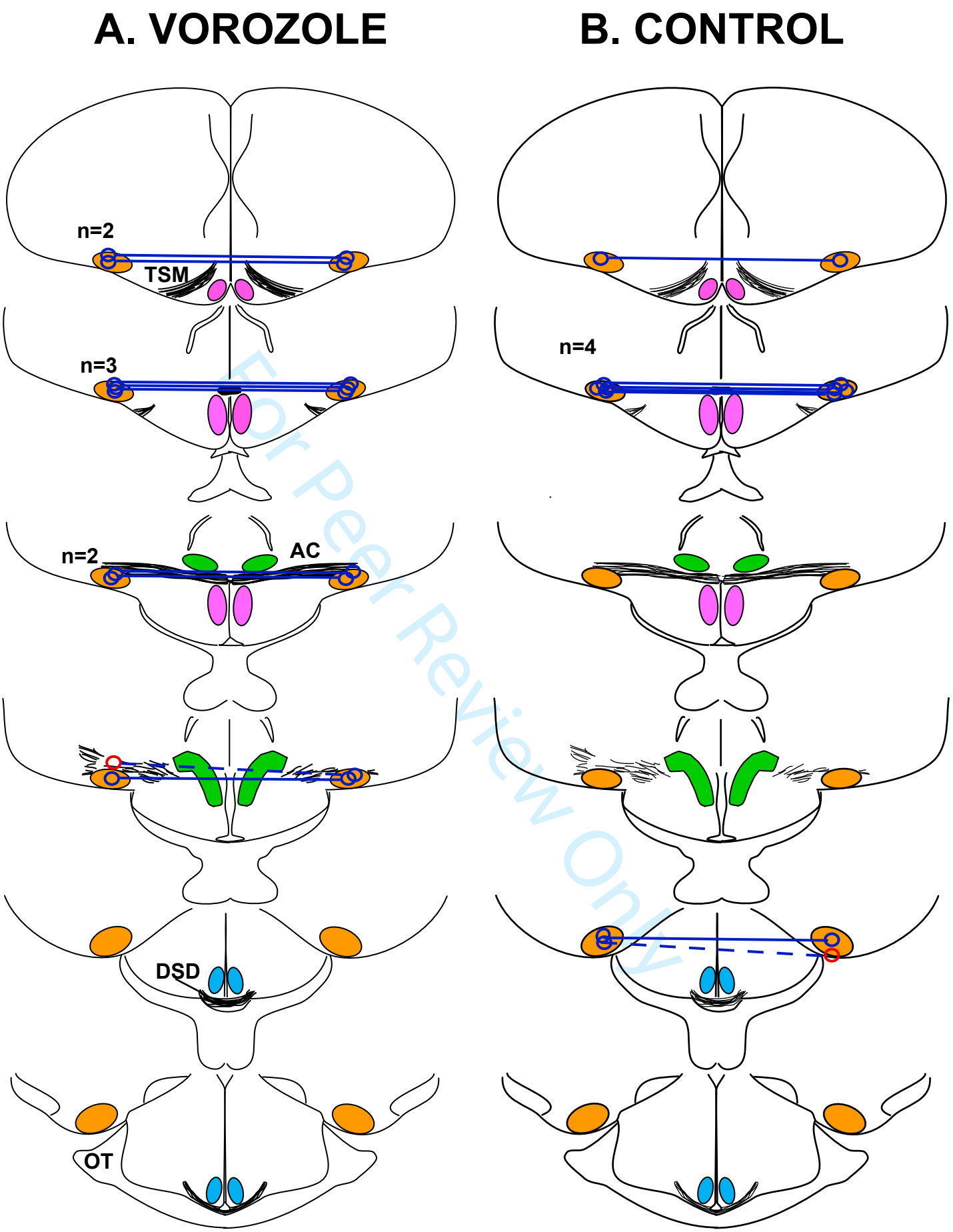

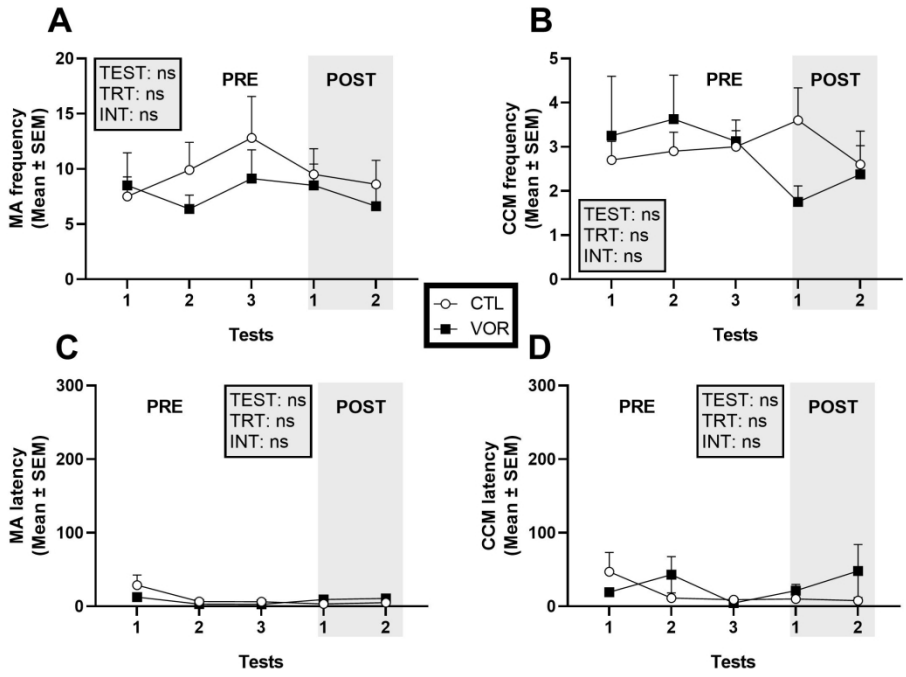


Figure 9. Effect of the inhibition of aromatization in the ventromedial nucleus of the hypothalamus (VMN; Exp. 4.1) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 3 pretests (pre-test phase, PRE) and the 2 tests performed after cannula implantation (post-test phase, POST), but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA: TRT= treatment; INT= interaction; ns= not significant.

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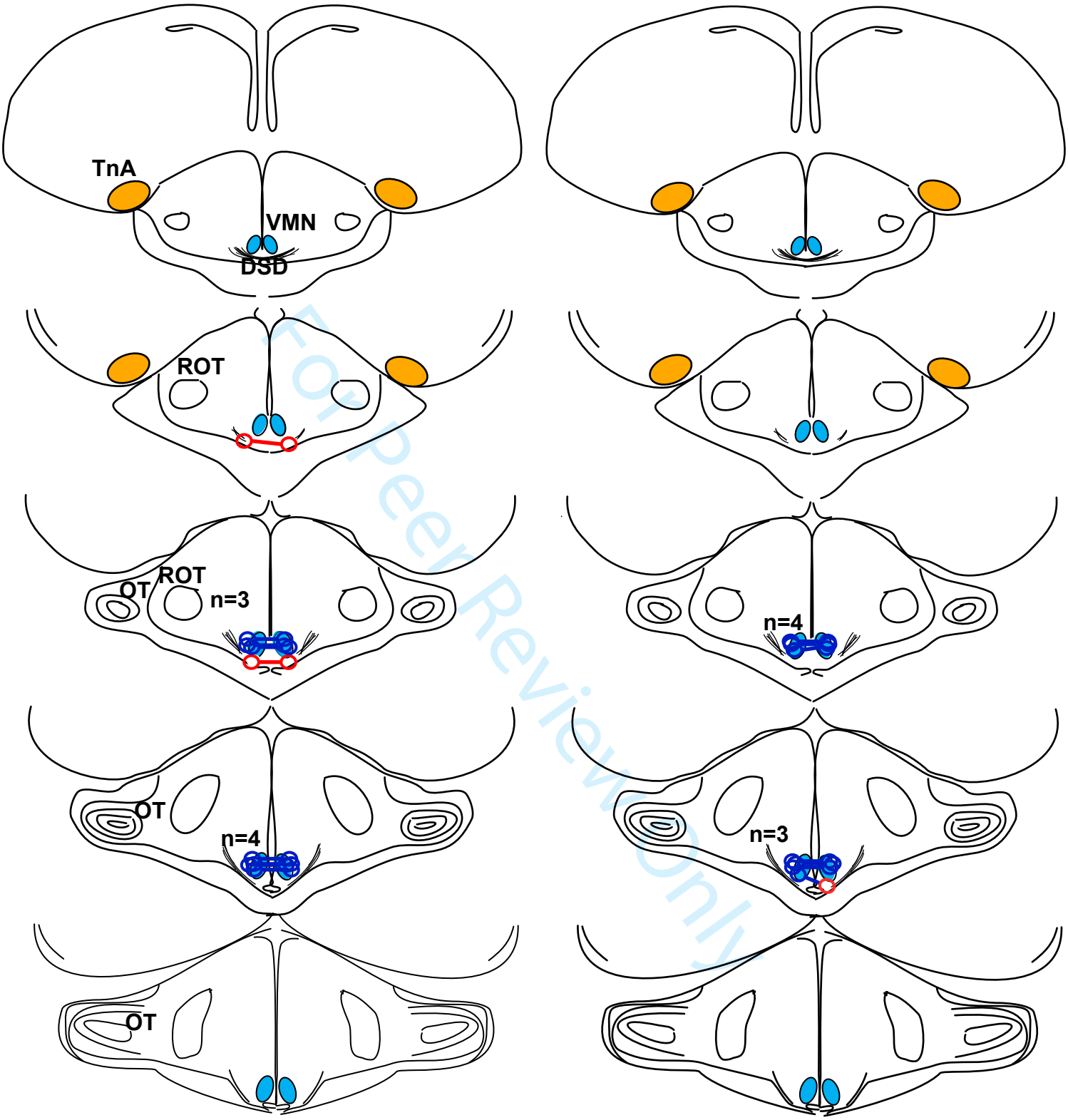

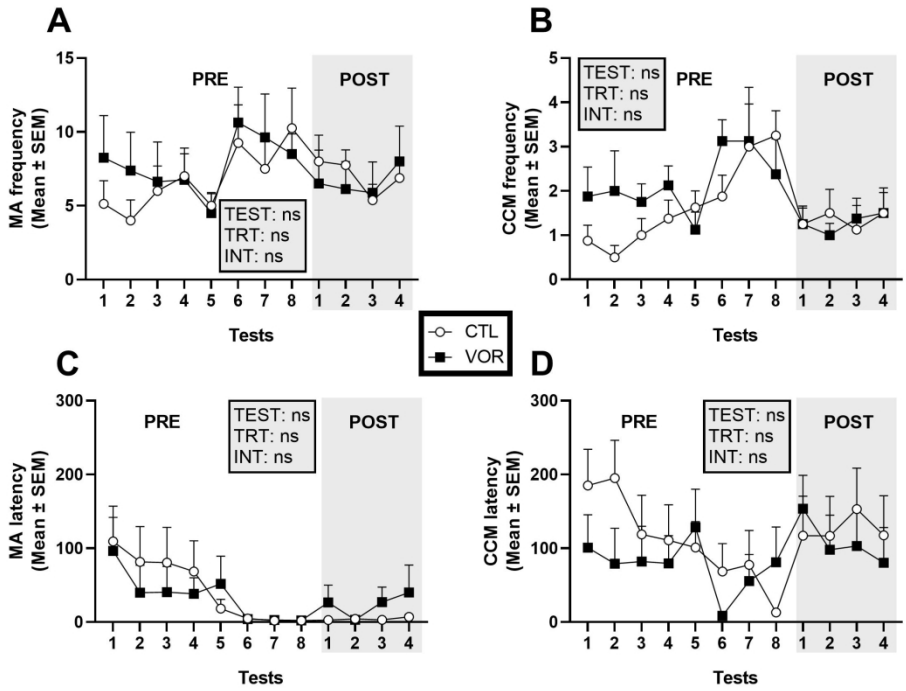


Figure 11. Effect of the inhibition of aromatization in the ventromedial nucleus of the hypothalamus (Exp. 4.2) on the expression of copulatory behavior in sexually experienced males chronically treated with testosterone. Frequencies and latencies of mount attempts (MA) or cloacal contact movements (CCM) are presented for the 8 pretests (pre-test phase, PRE) and the 4 tests performed after cannula implantation (post-test phase, POST), but statistical results are only presented for the POST phase (gray square; for the PRE phase, see the main text). The data were analyzed by two-way repeated measures ANOVA: TRT= treatment; INT= interaction; ns= not significant.

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