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Site-specific effects of aromatase inhibition on the activation of male sexual behavior in male Japanese quail (*Coturnix japonica*)

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ABSTRACT

Aromatization within the medial preoptic nucleus (POM) is essential for the expression of male copulatory behavior in Japanese quail. However, several nuclei within the social behavior network (SBN) also express aromatase. Whether aromatase in these loci participates in the behavioral activation is not known. Castrated male Japanese quail were implanted with 2 subcutaneous Silastic capsules filled with crystalline testosterone and with bilateral stereotaxic implants filled with the aromatase inhibitor Vorozole targeting the POM, the bed nucleus of the stria terminalis (BST) or the ventromedial nucleus of the hypothalamus (VMN). Control animals were implanted with testosterone and empty bilateral stereotaxic implants. Starting 2 days after the surgery, subjects were tested for the expression of consummatory sexual behavior (CSB) every other day for a total of 10 tests. They were also tested once for appetitive sexual behavior (ASB) as measured by the rhythmic cloacal sphincter movements displayed in response to the visual presentation of a female. CSB was drastically reduced when the Vorozole implants were localized in the POM, but not in the BST nor in the VMN. Birds with implants in the BST took longer to show CSB in the first 6 tests than controls, suggesting a role of the BST in the acquisition of the full copulatory ability. ASB was not significantly affected by aromatase blockade in any region. These data confirm the key role played by the POM in the control of male sexual behavior and suggest a minor role for aromatization in the BST or VMN.

1. Introduction

Estrogens, such as estradiol (E_2), exert numerous physiological and behavioral effects on various functions, such as neurogenesis (Barha et al., 2009), memory (Sheppard et al., 2018), neuroprotection (Siddiqui et al., 2016) and reproduction (Adkins-Regan, 2012). Classically, estrogens mediate these effects through their nuclear receptors, estrogen receptor (ER) α and β. When occupied, these receptors act as transcription factors by binding to specific sites of the DNA, called estrogen response elements, and modulating the transcription of target genes (Vasudevan and Pfaff, 2007). Since this mode of action is transcriptional, behavioral or physiological effects are usually observed after relatively long latencies (several hours to days).

Estrogens are derived from androgens, through a multi-step process catalyzed by the enzyme aromatase (Boon et al., 2010; Osawa et al., 1997). Within the brain, the hypothalamus is one of the major sites of estrogen synthesis (Biegon et al., 2010; Coumailleau and Kah, 2014; Foidart et al., 1995a; Menuet et al., 2003; Roselli et al., 1998; Roselli and Resko, 2001; Sasano et al., 1998; Stanić et al., 2014). In many vertebrate species, the central aromatization of testosterone (T) is a limiting step involved in the long-term control of male sexual behavior by steroid hormones. The medial preoptic area (mPOA) is a key site of steroid action on male sexual behavior in all vertebrates species studied (Hull and Rodriguez-Manzo, 2009; Panzica et al., 1996). Implantation of T or estradiol in this brain area is able by itself (without any systemic hormone) to activate male sexual behavior in castrated males from several mammalian and avian species (Alward et al., 2013; Balthazart and Surlemont, 1990a; Barfield, 1971, 1969; Davis and Barfield, 1979; Hutchison, 1971; Lisk, 1967; Matochik et al., 1994; Moralí et al., 1986; Phillips and Barfield, 1977; Riters et al., 1998; Watson and Adkins-Regan, 1989a, 1989b). Conversely, blocking aromatase in this area or lesion of this area drastically impairs the expression of male sexual behavior (Balthazart et al., 1990a; Balthazart and Surlemont, 1990a, 1990b; Brackett and Edwards, 1984; Clancy et al., 1995; Ginton and Merari, 1977; Hansen and af Hagelsrum, 1984; Hart, 1986; Kondo et al., 1990; Krohmer and Crews, 1987; Lloyd and Dixson, 1988; Macey

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et al., 1974; Slimp et al., 1978; Watson and Adkins-Regan, 1989b; Wheeler and Crews, 1978). Finally, a local treatment with E_2 in the mPOA reverses the inhibition induced by a systemic aromatase administration and leads to the activation of male sexual behavior (e.g., (Clancy et al., 2000)).

Male sexual behavior, as is the case for most motivated behaviors, can be divided in two distinct components: appetitive sexual behavior (ASB) and consummatory sexual behavior (CSB). ASB is composed of various behaviors, which serve to anticipate and prepare the animal to engage in CSB, which consists of copulatory behavior *per se*. CSB consists of a highly stereotyped behavioral sequence that ends with the gamete transfer, whereas ASB includes more variable behaviors that are indicative of the motivation of the animal to engage in CSB (Ball and Balthazart, 2008; Pfaus, 1996). In Japanese quail (*Coturnix japonica*), both ASB and CSB are drastically reduced after castration and are restored after a chronic treatment with T (Adkins, 1977; Adkins and Adler, 1972; Balthazart et al., 1998, 1997, 1995; Schumacher and Balthazart, 1983). Although some aspects of ASB mostly depend on circulating levels of androgens *sensu stricto* (Domjan, 1987), ASB and CSB are controlled by a synergistic action of androgens and estrogens (Adkins et al., 1980; Balthazart et al., 1997, 1995; Schumacher and Balthazart, 1983; Seredynski et al., 2013; Taziaux et al., 2004).

Social behaviors, such as sexual behavior, are controlled by a network of interconnected brain regions, called the Social Behavior Network (SBN) (Newman, 1999; O'Connell and Hofmann, 2011). This network is highly conserved across vertebrates (Goodson, 2005; O'Connell and Hofmann, 2011). The mPOA is part of this network, but several other loci within the SBN also express aromatase and ER, including the bed nucleus of the stria terminalis (BST), the medial amygdala (MeAMY), the ventromedial nucleus of the hypothalamus (VMN) and the periaqueductal gray (PAG) (Balthazart et al., 1989; Coumailleau and Kah, 2014; Li et al., 1993; Menuet et al., 2003; Sasano et al., 1998; Stanić et al., 2014). However, the contribution of the estrogens produced in these nuclei of the SBN to the control of male sexual behavior has not been investigated. Therefore, the aim of this study is to test if aromatization in the BST and in the VMN plays a role in the activation of male sexual behavior by T using the Japanese quail as an animal model. One additional group was included to compare within the same experiment the role of aromatase in the mPOA, and more specifically in the sexual dimorphic nucleus of the mPOA called the medial preoptic nucleus (POM) where aromatase action is known to play a critical role in the control of male sexual behavior (Panzica et al., 1996).

2. Material and methods

2.1. Animals

All experiments were conducted with Japanese quail, a well described animal model in behavioral neuroendocrinology (Ball and Balthazart, 2010). A total of 42 male quail were used as experimental subjects, whereas 29 females were used as stimuli. Birds were obtained from our colony (Animalerie Centrale de l'ULiège, GIGA Neurosciences, agreement number: LA1610002). From their birth until the age of 10 weeks, animals were raised in mixed-sex groups. They were then housed in individual cages. During their entire life they were provided with food and water *ad libitum*. Birds were raised in environmental conditions mimicking long summer days (16 h of light, 8 h of dark). They were regularly weighed to confirm a good recovery after surgeries and to insure that they maintained good health over the duration of the experiment. All procedures performed in this study were in agreement with the Belgian laws for the use of animals in scientific research and were approved by the ethical committee of the University of Liège (protocol #1442).

2.2. Surgical procedures

At the age of 3 weeks, males were castrated as previously described (Schumacher and Balthazart, 1984) under gas anesthesia. Birds were first given 4% isoflurane (in oxygen; Isovet, Verdifarm) through a mask, until a deep anesthesia was induced, and then concentration was lowered for anesthesia maintenance (1% in oxygen). Both testes were removed through a single lateral incision on the left side.

At the age of 10 weeks, birds were stereotaxically implanted with a bilateral guide cannula (PlasticsOne, reference: c232G-1.6 Spc, 7.8 mm below pedestal) filled with the aromatase inhibitor Vorozole (Vor, graciously provided by Dr. R. DeCoster (Janssen Research Foundation, Beerse, Belgium)) or left empty as a control (Ctl). Implants were prepared as previously described (Balthazart et al., 1990a) by tamping them repeatedly (20 times) into powdered Vorozole, then a thin wire was inserted in the cannula to ensure that the powder had entered the cannula to about 1 mm height from its tip. Under deep isoflurane anesthesia birds were placed in a stereotaxic apparatus (Kopf Instruments; Tujunga, CA, USA) with the beak holder aligning the horizontal axis of the brain with the horizontal axis of the stereotaxic apparatus. After the skull was opened, the cannula was inserted into the brain targeting the junction between the POM and the BST and attached to the skull using dental cement. This target location was selected because we anticipated based on previous experience that the random errors in cannula placement would end up producing subjects with implants located in the three main nuclei expressing aromatase in quail, namely the POM, BST and VMN. Coordinates of the tip of the left arm of the bilateral cannula were 0.0 mm anterior (X coordinate), 3.5 mm dorsal (Y coordinate) and 0.5 mm lateral (Z coordinate) to the zero-reference point (center of inter-aural axis). Birds were randomly assigned to the control or treated group. On the same day, birds were also implanted subcutaneously in the neck region with two 20 mm long subcutaneous Silastic™ capsules (Dow Corning, 1.57 mm I.D., 2.41 mm O.D.) filled with crystalline T (Sigma-Aldrich), a procedure that was previously shown to activate male sexual behavior to the same level as in gonadally intact males (Balthazart et al., 1990b). Before implantation, implants were pre-incubated for 24 h in saline in order to initiate the diffusion of T in the tube walls.

2.3. Behavioral procedures

Starting two days after the stereotaxic surgery and the onset of T treatment, birds were tested every week on Monday, Wednesday and Friday for the expression of male consummatory sexual behavior (CSB) for a total of 10 tests. Birds were placed in a testing arena (60 [length] × 40 [width] × 50 [height] cm) with a female for 5 min. CSB was quantified by an experimenter who was blind to the treatments as previously described (Adkins and Adler, 1972; Hutchison, 1978), by measuring the frequency and latency of the first occurrence of neck grabs (NG), mount attempts (MA), mounts (M) and cloacal contact movements (CCM) which represent copulation *sensu stricto*. Neck grab and mount frequencies were similar to and provided similar results as frequencies of mount attempts and cloacal contact movements, respectively. To avoid redundancy, only results concerning mount attempts and cloacal contact movements are presented here. One day after the 6th copulatory test, males were also tested once for appetitive sexual behavior (ASB) by recording the frequency of rhythmic cloacal sphincter movements (RCSM) when the males were given visual access to a female. This measure was previously shown to reflect sexual motivation (Seiwert and Adkins-Regan, 1998; Thompson et al., 1998). This behavioral test was performed in a small glass arena (40 [length] × 20 [width] × 25 [height] cm) during 2.5 min following a baseline period during which the male did not see the female for the same duration (see (Balthazart et al., 1998; Seiwert and Adkins-Regan, 1998) for details of procedures).

2.4. Brain collection and histological staining

On the afternoon of the last test day, brains were collected and the presence of powdered Vorozole in the implant was confirmed; all implants were still filled with Vorozole at the end of the experiment. After rapid decapitation, brains were soaked in 5% acrolein (Sigma-Aldrich; in phosphate buffered saline [PBS]) for 2.5 h. After two 30 min rinses in PBS, they were cryopreserved by soaking them in sucrose (30%, in PBS) until they had sunk. They were then stored at −80 °C until cryosectioning. Brains were cut in the coronal plane in four series of 30 μm thick sections from the level of the tractus septomesencephalicus (TSM) to the caudal end of the hypothalamus marked by the appearance of the optic nerve (Kuenzel and Masson, 1988).

One series (in which successive sections are separated by 120 μm) was then Nissl-stained. Briefly, sections were first rehydrated in successive baths of decreasing alcohol concentration (ddH_2O, Isopropanol 90%, 70%, 20%) for 1 min each. They were then soaked in toluidine blue (200 mg/100 ml of Walpole buffer) for 1 min, followed by 2 rinses in Walpole buffer for 15 min. Staining was then fixed in a solution of molybdate (5 g/100 ml) for 2.5 min. Sections were then dehydrated using 6 successive baths of increasing alcohol concentration (ddH_2O, Isopropanol 20%, 70%, 90%, 100%, 100%) for 1 min each. Sections were finally cover-slipped using Eukitt (Sigma-Aldrich, 03989).

Another series of sections was immunostained for the enzyme aromatase with a rabbit immunoglobulin raised against recombinant quail aromatase (QR2/05), which was previously validated for aromatase staining in quail brains (Carere et al., 2007; Foidart et al., 1995b). Immunohistochemical labeling was carried out by the avidin–biotin technique on free-floating sections, as previously described for the acrolein fixation (Bardet et al., 2010). Briefly, sections were incubated successively in sodium borohydride (0.1%, in Tris Buffered Saline [TBS]) for 15 min, in hydrogen peroxide (0.6%, in TBS) for 30 min, and saturated in 5% Normal Goat Serum (NGS; in TBS with 0.1% of Triton X-100 (Sigma-Aldrich) [TBST]) for 1 h, respectively to block the activity of endogenous peroxidases and limit the non-specific staining. Each step, but the last, was followed by three 5 min rinses in TBS. Sections were then left 2× overnight in the primary antibody (QR02/05, 1:3000, in TBST) along with 5% NGS on a rotating plate at 4 °C. After 3 rinses, they were incubated with a goat anti-rabbit biotinylated antibody (Jackson 111-065-003, 1:400, in TBST) for 2 h. After 3 rinses, the antibody–antigen complex was localized by the avidin–biotin complex method performed with the Vector Elite Kit (Kit ABC Vectastain Elite PK-6100, Vector Laboratories PLC, Cambridge, UK). After a final series of rinses, the peroxidase was visualized with diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride, Sigma-Aldrich, 0.04% along with H_2O_2 0.012%, in TBS) as chromogen.

The localization of the stereotaxic implants was determined based on the regions defined by both aromatase and Nissl staining as well as anatomical landmarks, such as the TSM, the anterior commissura (CA), the tractus occipitomesencephalicus (OM) and the decussatio supraoptica dorsalis (DSD; see Fig. 1). To be considered as being within a target brain region the tip of the lesion left by the implant had to reside at the dorsal edge of, at the lateral boundary or within the targeted region. In all cases, the cannula on both sides of the brain had to meet these criteria for the subject to be considered as having bilateral cannulae within the target region since aromatase inhibition must be bilateral to have a significant effect on behavior.

2.5. Statistical analyses

The statistical analyses were performed using GraphPad Prism 7.04. The effects of implant location on behavior were tested in control males by one-way analyses of variance (ANOVA) of the sum of behavioral frequencies measured during the last three tests (when the behavioral frequencies and latencies had reached a plateau). The activation of copulatory behavior (MA or CCM) was analyzed by a two-way ANOVA, with the behavioral tests as a repeated measure and the treatment (all Ctl-empty vs. Vor in different sites) as an independent factor. They were followed when significant by Dunnett's multiple comparison tests to analyze the significant interaction, comparing all groups to controls. To determine the overall effect of treatment at the end of the experiment, the sum of behavioral frequencies measured during the last three tests (when the behavioral frequencies and latencies had reached a plateau) were also compared between Vor in different brain regions and Ctl-empty using one-way ANOVAs with treatments as independent factor. RCSM frequencies tested during one single test conducted between the 6th and 7th copulatory tests were analyzed similarly. They were followed when significant by Dunnett's post-hoc tests. The presence or absence of CCM during the last three tests was also analyzed by Fisher's exact probability tests, comparing Vor in different brain regions to all Ctl-empty birds. Eta squared (η^2) or partial eta squared (η_p^2) were calculated based on the sums of squares provides by the ANOVAs. Effects were considered significant when $p < 0.05$. All data are presented by their mean ± S.E.M.

3. Results

As expected for stereotaxic placements attempted on small brains, the tip of implants was slightly scattered around the target site. Analysis of the implants localization resulted in the following groups: BST (Ctl, n = 7; Vor, n = 6), POM (Ctl, n = 3; Vor, n = 6), and VMN (Ctl, n = 4; Vor, n = 3), as well as a group of males whose cannula was located outside of any nucleus expressing aromatase (OUT: Ctl, n = 7; Vor, n = 6). The detailed localization of individual implants is provided in Fig. 1 and representative implant tip locations are illustrated in Fig. 2. Note also in Fig. 2 the presence of aromatase-immunoreactive cells (presumably in glial cells; Peterson et al., 2001) induced around the cannula tracts. This induced expression of the enzyme is unlikely to contribute to any of the reported effects since it is present in areas that do not contribute to the activation of sexual behavior.

One-way ANOVA first compared the effect on behavior, as measured by the MA frequency averaged over the last three tests, of empty implants located in different brain regions expressing aromatase (POM, BST or VMN) or outside any aromatase expressing region. The results confirmed the overall absence of difference between these different conditions ($F_{3,17} = 2.407$, $p = 0.1029$, $\eta^2 = 0.298$). Therefore, all males with an empty implant were pooled together as a control group (Ctl-empty) to be compared to the effect of treatment with Vor in four different locations: POM, BST, VMN and outside aromatase-expressing sites. This also demonstrated that there was no effect on behavior of the potential lesion due to an implant in a target nucleus.

As previously described (Balthazart et al., 1990b), T treatment activated copulatory behavior, as all birds in the Ctl-empty group (n = 21) exhibited active male sexual behavior within a few days after initiating testosterone treatment. Local aromatase inhibition impaired the behavioral activation in a site-specific manner as illustrated in Fig. 1. The qualitative analysis of the behavioral activation over time confirmed that chronic aromatase blockade in the POM prevented the acquisition of the full copulatory sequence but it also revealed a delay in behavior activation when the implant was in the BST as illustrated by the slower increase in MA frequency and slower decrease of MA latency in this group (Fig. 3). The same effect was observed when looking at CCM frequency and latency (data not shown).

These observations were confirmed by two-way ANOVAs with the repetition of tests and treatments as repeated and independent factors respectively. These analyses detected a main effect of tests (MA frequency: $F_{9,333} = 9.152$, $p < 0.0001$, $\eta_p^2 = 0.198$; MA latency: $F_{9,333} = 27.12$, $p < 0.0001$, $\eta_p^2 = 0.423$; CCM frequency: $F_{9,333} = 5.251$, $p < 0.0001$, $\eta_p^2 = 0.124$; CCM latency: $F_{9,333} = 7.711$, $p < 0.0001$, $\eta_p^2 = 0.172$) and treatments (MA frequency: $F_{4,37} = 5.604$, $p = 0.0012$, $\eta_p^2 = 0.377$; MA latency: $F_{4,37} = 30.09$, $p < 0.0001$, $\eta_p^2 = 0.765$; CCM frequency: $F_{4,37} = 3.806$, $p = 0.0109$,

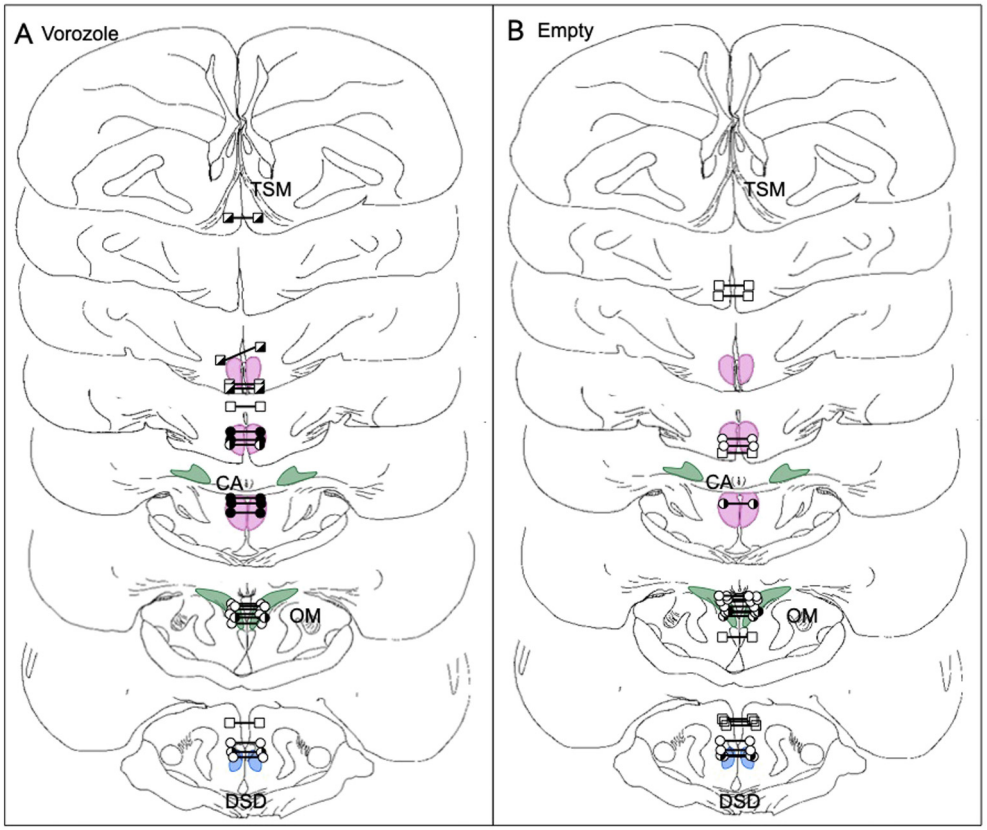

Fig. 1. Schematic representation of the localization of individual bilateral implants and their effect on behavior. Panel A shows the distribution of Vorozole implants, whereas panel B shows the distribution of empty implants. A straight line connects both sides of a given bilateral cannula. Squares represent implants that were outside of any population expressing aromatase, whereas circles represent cannulas that were in an aromatase positive population. Empty shapes represent implants that did not have any effect on behavior, whereas black shapes represent implants that had a strong inhibitory effect on sexual behavior (birds that showed < 10 MA and no CCM during the last 3 tests). Half-filled shapes represent implants that had a slight inhibitory effect on behavior (> 10 MA but < 3 CCM during the last 3 tests). Regions expressing aromatase are represented in color shapes. Pink: POM, Green: BST and Blue: VMN. Landmarks used for implant localization are noted as followed: TSM, tractus septomesencephalicus; CA, commissura anterior; OM, tractus occipitomesencephalicus; DSD, decussatio supraoptica dorsalis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$\eta_p^2 = 0.292$; CCM latency: $F_{4,37} = 6.598$, $p = 0.0004$, $\eta_p^2 = 0.416$) in all measures. A significant interaction between the two factors was also detected in the analysis of MA frequency and latency, as well as CCM frequency (MA frequency: $F_{36,333} = 1.848$, $p = 0.0030$, $\eta_p^2 = 0.167$; MA latency: $F_{36,333} = 2.601$, $p < 0.0001$, $\eta_p^2 = 0.219$; CCM frequency: $F_{36,333} = 1.554$, $p = 0.0258$, $\eta_p^2 = 0.144$) but the interaction did not reach statistical significance for CCM latency ($F_{36,333} = 1.441$, $p = 0.0537$, $\eta_p^2 = 0.135$). Dunnett's post-hoc analyses on these interactions indicated that males with Vor in the POM displayed fewer MA and took longer to attempt mounting than controls as early as tests 3 and 2, respectively (see Fig. 3 for details). They also showed fewer CCM than Ctl-empty birds from test 6 to 10, except on test 8 (data not shown). Males in which aromatase was inhibited in the BST showed lower MA frequency than controls on test 5 only. They were also slower to display this behavior than controls during tests 3 to 6 but were no longer different thereafter (see Fig. 3 for details).

The effects of treatments at the end of the experiment were also analyzed by comparing the sum of behavioral occurrences displayed during the last three tests when behavioral frequencies had reached a plateau (Fig. 4A and B). The impairment of copulation by aromatase inhibition was clearly dependent on the site of inhibition. Indeed, only 3 out of 6 males bearing an implant in the POM displayed at least one CCM during these last three tests, while all Ctl-Empty males (n = 21) and males implanted with Vor in the BST (n = 6), VMN (n = 3) or outside (n = 6) did copulate at least once. Statistical analysis of these data by Fisher's exact probability tests showed a significant effect of treatment compared to the Ctl-empty birds when Vor was in the POM ($p = 0.0068$), but not elsewhere (BST: $p = 0.9999$; VMN: $p = 0.9999$; Out: $p = 0.2222$). A one-way ANOVA of both MA and CCM frequencies during the last 3 tests with treatment (Ctl-empty vs. Vor in different brain regions) as independent factor revealed a significant effect of treatment (MA: $F_{4,37} = 4.906$, $p = 0.0028$, $\eta^2 = 0.347$; CCM: $F_{4,37} = 4.788$, $p = 0.0033$, $\eta^2 = 0.341$). Post-hoc analyses of these effects indicated that birds with a Vor implant in the POM showed significantly fewer MA and CCM during the 3 last tests compared to birds with an empty implant (Fig. 4A and B). By contrast, birds with an implant in the BST or VMN did not show any decrease in behavior frequency compared to the controls (Fig. 3A and B). Somewhat surprisingly, in birds with an implant outside nuclei expressing aromatase, Vor-treated birds also showed lower CCM frequency than controls. This

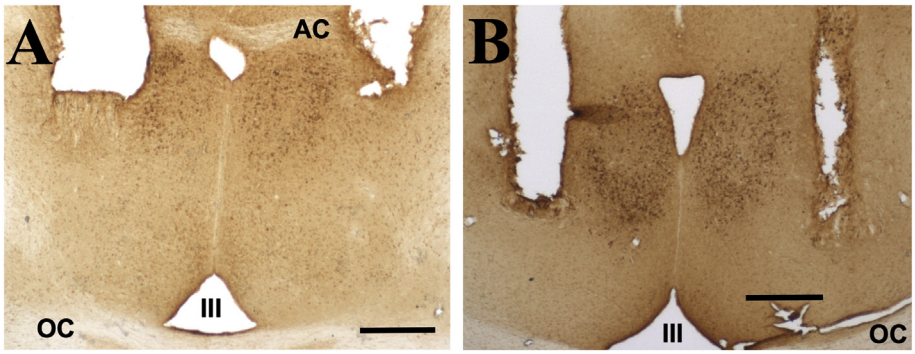

Fig. 2. Photomicrographs illustrating the location of an implant in POM (A) or outside POM (B). Note the presence of aromatase-immunoreactive cells (presumably glia), induced along the cannula tracts in panel B. The magnification bar is equal to 400 µm. Abbreviations: AC, anterior commissure; III, third ventricle; OC, optic chiasm.

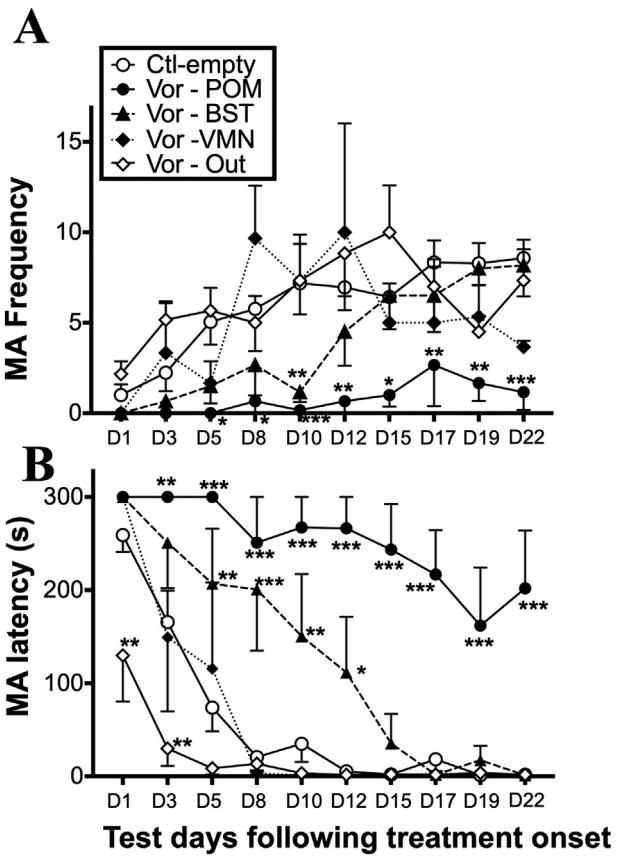

Fig. 3. Frequency of MA (A) and Latency of first MA (B) across tests depending on the treatment. *, **, ***: p < 0.05, < 0.01, < 0.001 vs Ctl-empty, respectively as revealed by Dunnett's tests following significant interaction in a 2 way ANOVA with treatment and the repetition of tests as factors. Graphs represent mean data ± SEM.

presumably reflects the diffusion of the inhibitor to the adjacent aromatase-expressing areas.

Finally, despite an apparent decrease observed in males treated with VOR in the POM (Fig. 4C), no significant effect of treatment was observed on appetitive sexual behavior as measured by RCSM frequency during one single test performed between the 6th and 7th copulatory tests ($F_{4,37} = 1.719$, $p = 0.1666$, $\eta^2 = 0.157$). Due to the presence of 3 groups unaffected by the treatments, the effect size in this ANOVA is quite small so that a total sample size of 490 subjects would be needed to demonstrate an effect at p = 0.05 with a power of 80%. Note however that a planned *t*-test comparing RCSM frequencies in the Ctl-Empty versus POM-Vor group would detect a significant difference ($t_{25} = 2.470$, $p = 0.0207$).

4. Discussion

The aim of this study was to determine the contribution of local estrogen production in nuclei that are part of the SBN to the activation of male sexual behavior by T in castrated males and compare these results with an inhibition of aromatase in the POM. Together the results confirmed the key role of preoptic aromatase in the long-term control of male sexual behavior by T. Indeed, CSB was drastically impaired when the aromatase inhibitor was implanted in the POM. Interestingly, ASB also decreased in frequency though this drop was not statistically significant in the general ANOVA. The inhibition of aromatase in the BST had no lasting effect on the frequency of CSB, but delayed the full expression of the behavior. By contrast, the inhibition of aromatase in the VMN was without effect on these behavioral responses. These conclusions and their implications require further discussion.

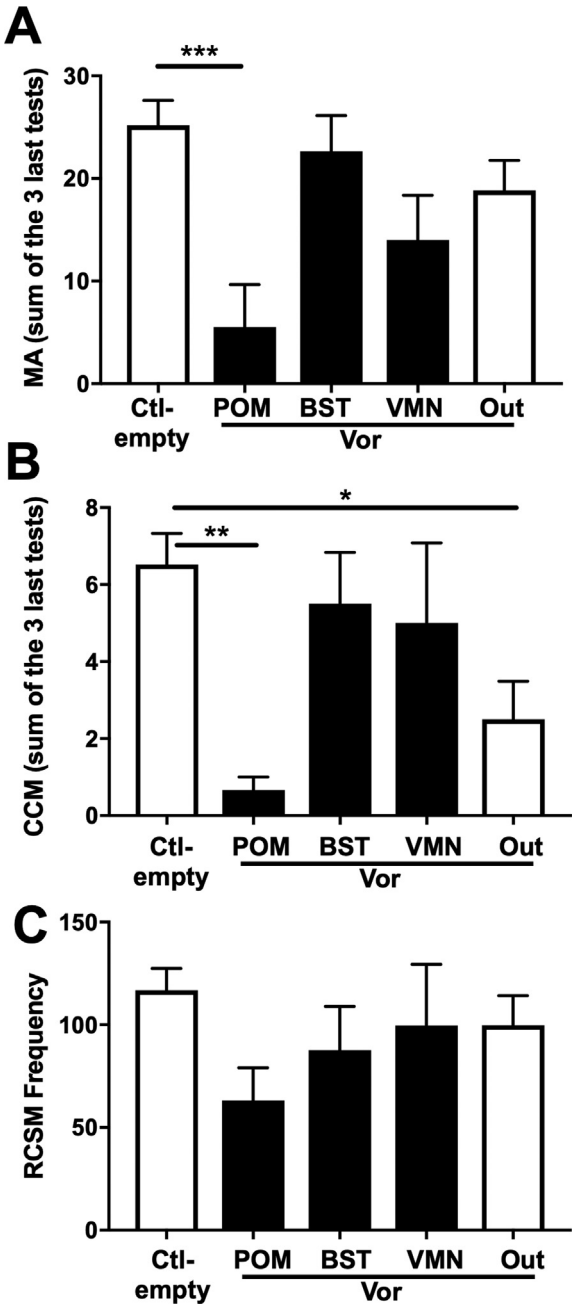

Fig. 4. Effect of aromatase inhibition depending on the brain region targeted on cumulative MA (A) and CCM (B) frequencies during the last 3 tests and on RCSM frequency during the test performed between sexual behavior tests 6 and 7. *** p < 0.001 vs Ctl-empty as revealed by Dunnett's tests following significant interaction in a one way ANOVA with treatments (Ctl-empty and Vor in different regions) as factors. Graphs represent means data ± SEM.

4.1. POM

The production of estrogens in the POA was previously shown to play a critical role in the long-term control of male CSB. Multiple studies on different animal models support this conclusion. In rats (*Rattus norvegicus*), lesion or inactivation of the POA was shown to completely impair the expression of male sexual behavior (Arendash and Gorski, 1983; Hurtazo et al., 2008; Paredes et al., 1993). Moreover, infusion of the aromatase inhibitor Fadrozole in the POA of intact male rats decreases the expression of male sexual behavior (Clancy et al., 1995). Conversely, the implantation of T or E_2 in this brain region leads to the activation of sexual behavior in castrated or intact non-copulating male

rats (males who had not copulated when presented several times to a receptive female) (Antonio-Cabrera and Paredes, 2014; Davis and Barfield, 1979; Moralí et al., 1986). The same type of experiments were also conducted in birds, leading to the same conclusion as in rats: lesions of the mPOA drastically reduces the expression of male sexual behavior in quail (Balthazart and Surlemont, 1990b), whereas T or E_2 implants in this area activate CSB in a variety of species (Balthazart and Surlemont, 1990a, 1990b; Barfield, 1971; Hutchison, 1971; Riters et al., 1998; Watson and Adkins-Regan, 1989a).

In Japanese quail particularly, it was shown that blocking estrogen synthesis or action within the POM leads to a drastic impairment of the activation of CSB (Balthazart et al., 1990a; Balthazart and Surlemont, 1990a). Interestingly, a previous study in quail reported a functional topography in the organization of POM related to the control of CSB and ASB: the rostral POM seemed to be specifically involved in sexual motivation, whereas the caudal part of the POM was implicated specifically in the control of copulation (Balthazart et al., 1998). A similar conclusion was reached based on the analysis of neural activation associated with the expression of ASB and CSB as assessed based on the expression of the immediate early gene c-fos (Taziaux et al., 2006). However, whether preoptic aromatization plays a role in the activation of ASB and whether this activation exhibits a functional topography within the POM has not been tested. The present results provide additional evidence for a role of aromatization specifically in caudal POM related to the activation of CSB. Indeed, as illustrated in Fig. 1, all implants that resulted in a drastic and significant reduction in copulatory behavior were placed in the caudal portion of POM. Unfortunately, none of the implants were located in the rostral part of this nucleus thus preventing us from testing the idea of a sub-regional specificity. It is nevertheless interesting to notice that preoptic aromatase blockade in the caudal POM failed to significantly reduce RCSM frequency despite a trend toward a decrease (50%) which would be consistent with a control of this behavior more prominently by the rostral POM (Fig. 4C). The present data thus confirm the key role of testosterone aromatization in the POM for the expression of male sexual behavior. In particular, estrogen synthesis in the caudal POM is necessary for the expression of CSB and could also be important for the expression of ASB but further investigations are required to confirm this notion.

4.2. BST

Most of the previous studies investigating the link between BST and male sexual behavior analyzed the number of activated cells (i.e. cells expressing immediate early genes [IEG]) after ASB or CSB). These studies performed on mammals, birds as well as lizards revealed an increased activation of the BST after mating or visual/olfactory exposure to a sexually relevant stimulus (Syrian Hamsters, *Mesocricetus auratus*: (Fiber et al., 1993; Kollack-Walker and Newman, 1997; Westberry and Meredith, 2003); Rat: (Coolen et al., 1997, 1996; Robertson et al., 1991); Brown anole, *Anolis sagrei*: (Kabelik et al., 2013); Sheep, *Ovis aries*: (Borja and Fabre-Nys, 2012); Chicken, *Gallus gallus*: (Xie et al., 2011); Gerbils, *Meriones unguiculatus*: (Heeb and Yahr, 1996); Japanese Quail: (Iyilikci et al., 2014; Taziaux et al., 2006; Tlemçani et al., 2000)). The analysis of the co-expression of ER with IEGs revealed that ER positive cells were activated in this cell population after male sexual behavior in rats (Gréco et al., 1998). Whether IEGs are expressed in aromatase positive cells following CSB seems to be less clear. Indeed, following CSB, the two markers were found to be co-expressed in the BST of chickens (Xie et al., 2011) but not in Japanese quail (Ceuleers et al., 2014; Foidart and Meddle, 1999). However, in one published experiment, T implants placed in the BST were not sufficient to activate male sexual behavior of castrated male Japanese quail suggesting that T and local estrogen synthesis in this region is not sufficient to activate this behavior (Riters et al., 1998). The present study showed that aromatase inhibition in this brain area does not impair CSB in an enduring manner, but clearly delays the initiation of copulation.

Interestingly, the rise in neuronal activation induced by copulation in the BST of Japanese quail was shown to decrease in magnitude after males gained sexual experience (Can et al., 2007). An experience-dependent role of the posterior subdivision of the BST was also suggested in Syrian Hamsters in which lesions of the BST reduce the preference for female odors and delay the initiation and completion of the copulatory sequence but only if males were sexually naive prior to lesion (Been and Petrulis, 2010). After this lesion, males showed a lack of preference for female's odor, which was not due to a general inability to discriminate volatile odors. In this context, the delay to initiate copulation observed here following chronic aromatase inhibition in the BST could thus reflect a decrease in sexual motivation due to a reduced attraction to female cues in sexually naive males. This delay would thus suggest an experience-dependent role of aromatization in the BST in the control of sexual motivation and/or attraction to sex specific cue.

A role for brain estrogen synthesis in sexual motivation is also supported by the observation that the enzymatic activity of aromatase in the BST is rapidly changed 2 or 5 min after a visual interaction with a female (de Bournonville et al., 2013). Estrogens were also previously shown to be implicated in the control of reward processes in females (Parada et al., 2012; Tonn Eisinger et al., 2018). Interestingly, BST is part of both the SBN and the mesolimbic reward system (O'Connell and Hofmann, 2011). Estrogens synthesized in the BST could thus be important to process sexually relevant information, mediate the rewarding aspect of sexual behavior and/or acquire sexual experience explaining why in the present experiment the delay in copulation initiation is strong in the first tests when males are naive but is no longer present in the latest tests, when males have gained sexual experience.

4.3. VMN

The role of VMN in the context of the activation of male sexual behavior has been investigated less than the POM and BST. Lesions of this area did not impair CSB in cats (*Felis catus*) (Leedy and Hart, 1985) and even facilitated copulation in male rats (Christensen et al., 1977), suggesting an inhibitory role of the VMN on male sexual behavior. Moreover, the role of estrogens in this brain region on the control of male sexual behavior is not clear: implants of T or E_2 were found to partially restore sexual behavior of castrated male mice (Nyby et al., 1992) but not of castrated male rabbits (Melo et al., 2008). Interestingly, a recent study, employing stereotaxic injection of a virus containing a small hairpin RNA targeting ERα to knock down its expression in the VMN of male mice reported a decrease in the rate of sexual behavior compared to controls (Sano et al., 2013). In the present study, we found no effect of aromatase inhibition in the VMN on the initiation or expression of male sexual behavior, suggesting that estrogens derived from testosterone aromatization in the VMN do not seem to be involved in the activation of male sexual behavior in Japanese quail. This conclusion must however be considered cautiously given the limited number of subjects with a Vor implant in this nucleus.

5. Conclusions

Together the present results confirm that estrogens locally synthesized in the brain play a site-specific role in the control of male sexual behavior. The major site of estrogen production required for the activation of male sexual behavior is clearly the POM, as illustrated by the drastic impairment of male sexual behavior following aromatase inhibition in this area. The aromatization of T in other brain areas seems to play a less important role in the control of male sexual behavior, as copulatory performance was not affected in birds treated with an aromatase inhibitor in the VMN or BST. However, the delay in copulation initiation observed in males for which aromatase was inhibited in the BST suggests a role of aromatization in this area in the sexual motivation or processing of sexual relevant stimuli.

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