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Rapid Action on Neuroplasticity Precedes Behavioral Activation by Testosterone

Thierry D. Charlier^{1,*}, Gregory F. Ball², and Jacques Balthazart¹

¹University of Liège, Center for Cellular and Molecular Neurobiology, Research Group in Behavioral Neuroendocrinology, Liège, Belgium

²Department of Psychological and Brain Sciences, Johns Hopkins University, Baltimore, MD 21218, USA

Abstract

Testosterone has been shown to increase the volume of steroid-sensitive brain nuclei in adulthood in several vertebrate species. In male Japanese quail the volume of the male-biased sexually dimorphic medial preoptic nucleus (POM), a key brain area for the control of male sexual behavior, is markedly increased by testosterone. Previous studies assessed this effect after a period of 8–14 days but the exact time-course of these effects is unknown. We asked here whether testosterone-dependent POM plasticity could be observed at shorter latencies. Brains from castrated male quail were collected after 1, 2, 7 and 14 days of T treatment (CX+T) and compared to brains of untreated castrates (CX) collected after 1 or 14 days. POM volumes defined either by Nissl staining or by aromatase immunohistochemistry increased in a time-dependent fashion in CX+T subjects and almost doubled after 14 days of treatment with testosterone while no change was observed in CX birds. A significant increase in the average POM volume was detected after only one day of testosterone treatment. The optical density of Nissl and aromatase staining were also increased after one or two days of testosterone treatment. Activation of male copulatory behavior followed these morphological changes with a latency of approximately one day. This rapid neurochemical and neuroanatomical plasticity observed in the quail POM thus seems to limit the activation of male sexual behavior and offers an excellent model to analyze features of steroid-regulated brain structure and function that determine behavior expression.

Keywords

brain plasticity; medial preoptic nucleus; Japanese quail; sexual behavior; fast steroid action; aromatase

Introduction

Several brain nuclei have been identified in vertebrate species that exhibit either a male or female size bias (De Vries et al., 2002; Morris et al., 2004). These differences emerge during development either in response to a differential steroid hormone environment or based on sex

Correspondence concerning this article should be addressed to: Jacques Balthazart, University of Liège, Center for Cellular and Molecular Neurobiology, 1, avenue de l'Hôpital, B-4000 Liège, Belgique, Phone: 32 43665970-----Fax: 32 43665971---Email: jrbalthazart@ulg.ac.be.

*Current address: University of British Columbia, Department of Psychology 2136 West Mall Vancouver, B.C. Canada, V6T 1Z4

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differences in the expression of specific but generally unidentified genes (Arnold and Gorski, 1984; Arnold et al., 2004). Only a few of these brain regions have been found to continue to exhibit neural plasticity into adulthood. A single example has been described in mammals. The posterodorsal nucleus of the medial amygdala has a larger volume in male than in female rats, but adult male castration shrinks this nucleus to a size in the range of female values whereas androgen treatment enlarges its volume in females to male-typical levels (Cooke et al., 1999).

More widespread steroid-induced plasticity of this sort has been observed in adult avian species. For example, in canaries several key song nuclei are larger in males than in females (Nottebohm and Arnold, 1976) corresponding with more elaborate male song. Treating adult females with testosterone (T) induces male-typical song as well as increases in song nuclei volume (Nottebohm, 1980). The medial preoptic nucleus (POM) in quail is another well-documented example of neural plasticity specifically related to the control of behavior. POM volume is larger in males than in females and this difference is maintained by sex differences in the secretion of steroid hormones in adulthood. Castration decreases POM volume in males and treatment with exogenous T increases this volume in both sexes to male-typical values (Panzica et al., 1991).

The POM is a key brain site mediating the activation by T of male sexual behavior (Balthazart and Ball, 2007). T implantation into the POM fully restores sexual behavior in castrated males and lesions of the nucleus inhibit the expression of the behavior (Balthazart and Ball, 1998; Balthazart and Ball, 2007). T must be aromatized into an estrogen within POM itself to exert its behavioral effects and accordingly the POM is characterized by high aromatase expression and activity. Aromatase expression in the POM is also T-dependent (Balthazart et al., 2004). Recent experiments showed that the behavioral and neurochemical effect of T and its metabolite estradiol in the POM depend on the presence of the steroid receptor coactivator-1 (SRC-1). Inhibition of the expression of SRC-1 by intracerebroventricular injection of antisense oligodeoxynucleotides markedly blocks the steroid-dependent activation of male sexual behavior, as well as the expression of aromatase and vasotocin. In parallel, the POM volume is significantly decreased in birds treated with SRC-1 antisense (Charlier et al., 2005). The behavioral, neurochemical and morphological effects of the antisense were no longer apparent 2 days after the cessation of the antisense injections and the volume of the POM actually increased by more than 60 percent within these two days, suggesting the existence of an unexpectedly rapid neuroplasticity (Charlier et al., 2005). These data led us to consider whether this rapid neurochemical change was specifically linked to the SRC-1 modulation or reflected the normal rate of T-induced effects. In previous work, the effects of T on the POM volume were quantified after exposure to the steroid for one or two weeks (Panzica et al., 1996b) which is within the range of time courses widely employed to investigate steroid hormone effects on brain plasticity. However, evidence has been accumulating indicating that T can influence brain morphology much more rapidly (Ball, 1999; Thompson et al., 2007; Tramontin et al., 2000). We investigated here whether T could rapidly increase POM volume within even one day and how this change in brain morphology might be related to changes in T-dependent sexual behaviors.

Material and Methods

Animals and in vivo treatments

Male Japanese quail (*Coturnix japonica*) were purchased at the age of approximately 3 weeks from a local breeder in Belgium. Birds were exposed to a long photoperiod (16L:8D) and had food and water available *ad libitum*. All experimental procedures were approved by the Ethics Committee for the Use of Animals at the University of Liège.

One week after their arrival, all males were castrated (Balthazart et al., 1983), 2 weeks later they received two subcutaneous 20 mm-long SilasticTM capsules (SilclearTM Tubing, Degania Silicone Ltd, DeganiaBet, 1513, Israel; 1.57 mm i.d.; 2.41 mm o.d.) filled with crystalline testosterone (CX+T; Sigma, St Louis, MO) or left empty as control (CX). These T implants restore physiological concentrations typical of sexually mature males (Balthazart et al., 1983). CX or CX+T birds were matched for body weight ($t=0.108$, $df=34$, $p=0.7448$). Before implantation, all SilasticTM capsules were incubated overnight in isotonic saline solution to initiate steroid diffusion and avoid an initial surge in steroid release.

Until brain collection, animals were tested every day (5 min) for sexual behavior in the presence of a female. Sexual behaviors were quantified by an observer blind to the treatment of the birds. These behaviors include neck grabs (NG), mount attempts (MA), mounts (M), and cloacal contact movements (CCM; see Hutchison, 1978 for description).

Subjects were killed in six groups, at four different times: two CX groups of similar body mass ($t=0.415$, $df=9$, $p=0.688$) killed on day 1 ($n=5$) and 14 ($n=6$), and four CX+T groups killed on day 1 ($n=7$), 2 ($n=7$), 7 ($n=6$) and 14 ($n=5$). The body mass of the CX+T males killed on day 1 was similar to that of the remaining birds ($t=0.122$, $df=23$, $p=0.904$). Allocation to the three other CX+T groups was performed progressively based on the number of CCM performed by the birds on their last day. Day 2 birds did not differ on this variable from the remaining males ($t=0.039$, $df=16$, $p=0.969$) and day 7 birds did not differ from day 14 group ($t=0.678$, $df=9$, $p=0.514$). In this way, the groups of T-treated males were matched for behaviorally relevant variables as accurately as possible.

Approximately 5 hours after their last behavioral test, birds were weighed and their cloacal gland area, an androgen-dependent structure (Sachs, 1967), was measured with calipers. Birds were anesthetized with Hypnodil (15 mg/kg) and transcardially perfused with 4% paraformaldehyde, 0.2% glutaraldehyde, 15% picric acid in 0.01 M phosphate buffer. Brains were dissected out of the skull, cryoprotected in 20% sucrose, frozen on dry ice and stored at -75°C .

Tissue processing and staining

Thirty μm -thick coronal sections were cut on a cryostat starting at the rostral end of the brain. The plane of the sections was adjusted to match as closely as possible the plane of the quail brain atlas (Baylé et al., 1974). Sections located between the tractus septopallio-mesencephalicus (TSM) and the locus coeruleus were collected in four sets and stored in a cryoprotectant solution (PBS 0.05 M, 1% Polyvinyl pyrrolidone, 30 % sucrose, 30 % ethylene glycol) at -20°C until used. Sections were then stained either for Nissl bodies or for aromatase by immunohistochemistry as previously described (Charlier et al., 2005). Briefly, one set was mounted on gelatin-coated slides, allowed to dry for a minimum of 24 hours and dipped overnight at room temperature into 0.0014 % toluidine blue in distilled water. The slides were then quickly rinsed in distilled water, dehydrated in ethanol baths of increasing concentration, from 20 % to 100 % and rinsed twice in xylene before coverslipping with EukittTM.

For aromatase staining, sections were incubated sequentially in 0.6 % hydrogen peroxide for 20 minutes to inhibit endogenous peroxidases, followed by 5 % normal goat serum for 3 hours to decrease non-specific binding. Tissue was then incubated overnight at 4°C in the aromatase rabbit antibody (1:3000, generous gift of Professor Harada, Fujita Health University Toyoake, Aichi 470-11 Japan). Endogenous biotins were blocked by the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA), the sections were then left for 2 hours in secondary biotinylated goat anti-rabbit antibody (1:400, Dako A/S, Goslstrup, Denmark) and finally incubated in ABC Vectastain elite Kit PK-6100 (Vector Laboratories). All reagents were in Tris-buffered saline 0.05 M containing 0.1% triton X-100 (TBST) and several rinses

were performed between each step. The peroxidase enzymatic activity was then visualized with 20 mg of 3, 3' diaminobenzidine tetrahydrochloride in 50 ml TBST containing 0.012 % hydrogen peroxide. Reaction was terminated by several rinses in TBS and the sections were mounted in an aqueous gelatin medium and coverslipped.

Image analysis

Images were digitized through a CCD camera attached to a microscope and connected to a MacIntosh computer (software: Image J version 1.34s, Wayne Rasband, NIH, Bethesda, MD, USA). POM volume was measured in the aromatase- and Nissl-stained sections (objective 10X). The area of the nucleus was measured in all sections containing the nucleus throughout its rostro-caudal extent. Areas were summed and multiplied by the sampling interval to derive an estimate of volume.

The percentage of surface covered by aromatase-immunoreactive (ARO-ir) structures was also measured in the dorso-lateral, medial and ventro-lateral part of the POM at the level of the anterior commissure (objective 40X). For these analyses, digitized images were made binary and manual thresholding was used to discriminate the labeled material from the background. The number of pixels corresponding to immunoreactive structures was divided by the total number of pixels covering the surface being analyzed and defined as the fractional area.

Under the 10x and 40X objectives, we also measured the optical density of Nissl and aromatase staining. The area that was analyzed encompassed the entire POM surface observed at the largest extension of the anterior commissure. The relative optical density (ROD) was defined as the difference between of the optical density (gray levels) measured (after calibration) within the POM and in an equivalent adjacent, more lateral, area (background). A more detailed analysis of the Nissl and aromatase ROD was performed at higher magnification (objective 40 X) on the same section (POM at the maximal extent of the anterior commissure) separately in the dorso-lateral, medial and ventro-lateral part of the POM. ROD measures were collected for the entire computer screen.

Data Analysis

Data were analyzed by Student's *t*-tests and one- or two-way analyses of variance (ANOVA) with the experimental groups as independent factor and the successive behavioral tests or anatomical locations as repeated factor. ANOVAs were followed where appropriate by post hoc Fisher protected least significant difference (PLSD) tests. Differences were considered significant for $p < 0.05$. In five cases, one brain measure could not be collected due to technical problems and the number of subjects is decreased by one unit in the corresponding analyses, as reflected in the listed degrees of freedom. All data are presented as means $\pm$ SEM.

Results

Student's *t*-tests revealed no significant difference between the two CX groups (day 1 and 14) for all variables considered. Results from these two groups were thus pooled in all statistical analyses but the two sets of data are presented separately in the figures to illustrate the lack of difference between these two groups.

Behavioral tests performed on every day beginning when T capsules were implanted showed that male copulatory behavior began to appear two days after the beginning of the T treatment and progressively increased to reach a plateau on day 6. As expected, this behavior was never observed in untreated castrated males. Results of these daily tests are however not presented in detail and analyses were focused on the means of NG and CCM frequencies recorded on the day birds were killed (see Figure 1A). MA and M frequencies showed a similar pattern and

are not presented. One-way ANOVAs revealed significant differences between groups in all cases (NG: $F_{4,31}=11.061$, $p<0.0001$; MA: $F_{4,31}=7.988$, $p<0.0005$, M: $F_{4,31}=8.762$, $p<0.0001$ and CCM: $F_{4,31}=9.282$, $p<0.0001$). T already activated NG significantly on day 2 of treatment ($p<0.05$ vs. pooled CX groups; see Fig. 1 for detail of post hoc tests) but a significant increase in CCM frequencies was only observed on day 7. Only 17% of the birds showed the full copulatory sequence on day 2 whereas 100% of the CX+T birds showed the full sequence including CCM on day 7 and 14.

T also progressively increased the size of the cloacal gland (Fig. 1B) resulting in significant differences between groups on the day birds were killed ($F_{4,31}=129.024$, $p<0.005$). A significant increase was already present on day 1 (CX: 41.6 ± 1.2 mm², CX+T Day 1: 94.3 ± 5.7 mm², $p<0.005$ Fisher PLSD). The gland size continued to grow for the entire duration of the experiment. The body weight measured on the day of sacrifice was not affected by the experimental treatments ($F_{4,31}=2.052$, $p=0.11$).

Five hours after the last behavioral test, separate groups of birds were killed and their brain was processed to assess changes in POM organization (CX+TD1 and CXD1 on day 1, CX+TD2 on day 2, CX+TD7 on day 7, CX+TD14 and CXD14 on day 14). The histological analyses revealed that the increase of male sexual behavior frequencies was paralleled and even preceded by major neuroanatomical changes (see Figure 2 for representative pictures).

The volume of the POM defined by both Nissl staining and aromatase immunoreactivity was significantly affected by the treatments (Nissl: $F_{(4, 31)}=29.848$, $p<0.0001$; aromatase: $F_{(4,30)}=22.444$, $p<0.0001$) and progressively increased to reach its maximal value (81% increase compared to castrates, from 0.186 ± 0.01 mm³ in CX to 0.337 ± 0.014 mm³ in CX+TD14 for volumes defined by Nissl) after 14 days of T treatment (see Figure 3A). The post-hoc Fisher's PLSD tests comparing the different groups (symbols above the bars in Figure 3A) indicated that the POM volume defined by Nissl staining significantly increased already two days after the beginning of the T treatment (24.4%, $p<0.0001$, Figure 3A). A slight numerical increase of this volume was already detected on day 1 (8%) but was not statistically significant.

The analysis of POM defined by aromatase immunoreactivity revealed a similar volume increase but with this more accurate marker, the volume was already significantly larger than in castrates 24 hours after the beginning of the T treatment (25% increase, $p<0.005$ by Fisher's PLSD; Figure 3B). After 14 days of T treatment, the POM volume defined by aromatase immunoreactivity was 104.6% larger than in castrated controls (0.193 ± 0.010 mm³ in CX vs. 0.395 ± 0.032 mm³ in CX+TD14). The volume of the control nucleus rotundus, which is not androgen-sensitive, did not vary between the different groups ($F_{(4, 30)}=0.572$, $p=0.658$), indicating the specificity of the effects of T on the POM volume (data not shown).

The relative optical density (OD) of the Nissl staining measured in the entire surface of the POM at low magnification (10X; see material and methods) was not different between the groups ($F_{(4, 31)}=0.016$, $p=0.999$, Figure 4A). However, the more detailed analysis of three separate regions (the dorso-lateral, ventro-lateral and medial periventricular areas, see inserts in figure 2 for representative pictures) analyzed at higher magnification (40X, see Figure 4C) revealed significant changes of the Nissl OD. Analysis of these data by two-way ANOVA with the 5 experimental groups (treatments) as independent factor and three POM regions as repeated factor identified significant effects of the treatments ($F_{(4, 31)}=4.617$, $p<0.005$) and of the region ($F_{(2, 62)}=10.665$, $p=0.0001$) but no interaction between these factors ($F_{(8, 62)}=0.768$, $p=0.632$). The separate analysis of each region by one-way ANOVA confirmed the treatment effect on the OD ($p<0.05$ for each region). Groups were compared two by two with post-hoc Fisher's PLSD tests which indicated that significant effects of T on the Nissl OD were already present after 24 hours of T treatment in the ventro- and dorso-lateral parts of the POM but this

effect became significant only one day later (CX+TD2) in the medial periventricular part of the nucleus. It is also interesting to note that no further increase of the OD was observed and the four groups of CX+T showed a similar level of OD.

T also significantly affected the relative optical density of the aromatase staining as measured at low magnification (10X, $F_{(4,29)}=7.718$, $p<0.0005$, Figure 4B). Significant increases were however observed only after 7 and 14 days (See Figure 4B for detail of post-hoc tests). The detailed analysis at higher magnification (40X, see Figures 4D) of the three separate regions that had been studied for Nissl staining was also carried out on sections stained for aromatase. Analysis of these results by a two-way ANOVA (groups and regions as factors) identified a significant effect on the aromatase OD of the group factor ($F_{(4,30)}=10.960$, $p<0.0001$), of the region ($F_{(2,60)}=26.441$, $p<0.0001$) and of their interaction ($F_{(8,60)}=3.353$, $p<0.005$). The separate analysis of each region by one-way ANOVA confirmed the group effect on the OD ($p<0.0005$ in each area) and further comparisons of the different groups two by two with post hoc Fisher's PLSD tests indicated that increases in aromatase OD became significant after 2 days of T treatment in the ventro-lateral and in the medial periventricular region but only after 7 days in the dorsolateral zone (see Figure 4D for detail of these post-hoc tests).

The T treatment also significantly affected the fractional area (FA) covered by aromatase-immunoreactive structures in the POM. The analysis by two-way ANOVA of FA measured at high magnification (40X) in three sub-regions of the POM revealed a significant effect of the treatments ($F_{(4,31)}=18.661$, $p<0.0001$), of the POM region ($F_{(2,62)}=41.101$, $p<0.0001$) and of their interaction ($F_{(8,62)}=2.856$, $p<0.01$) on FA. The one-way ANOVA of the data concerning each region separately confirmed the significant effect of the T treatment on the aromatase FA ($p<0.001$ in each case) and post hoc analysis revealed a significant effect already 24 hours after the beginning of the T treatment in all three zones (see detail in Figure 5).

Discussion

One of the most important developments in the field of functional neuroendocrinology in the past 20 years is the observation that steroid hormones can act with a time course much faster than previously realized (Ronnekleiv and Kelly, 2002; Schumacher, 1990; Vasudevan and Pfaff, 2007). This is most often recognized for certain types of membrane actions of steroids that can result in non-genomic effects on cellular and behavioral processes with a temporal resolution on par with what happens as the result of the action of neuromodulatory transmitters (Balthazart and Ball, 2006). In the present study we address the question as to whether the anatomical effects of steroids that are presumably genomic in nature can also occur with a faster time course than previously thought. The data reported here indicate that, preceding its effects on male copulatory behavior, T rapidly changes the volume and neurochemical characteristics of the POM. The term “rapid” in this context refers to the normal time course one expects for the induction of morphological changes detectable at the light microscopic level. Typically steroid hormones or other modulatory chemical messengers are not thought to be able to induce measurable neuroanatomical changes within 24 hours (McEwen and Alves, 1999). Aromatase expression in POM and the volume of this nucleus were both found to significantly increase already at the 24 hour time point after the beginning of the treatment of castrated males with T. Relatively rapid (i.e., within 24 hr) steroid-induced neuroanatomical plasticity in the vertebrate brain was previously reported only at the synaptic level based on the assessment of changes in synapse density in, for example, the hippocampus (e.g., Woolley and McEwen, 1992). Two previous reports have identified a significant volume increase of the song control nucleus HVC after only one week of exposure to long days (Ball, 1999) or treatment with T (Tramontin et al., 2000) but these studies did not test for shorter treatments. A fast decrease of HVC volume has also been observed in white-crowned sparrows 12 hours after castration and removal of a T subcutaneous implant (Thompson et al., 2007). Although

the authors suggest that the effect relates to T withdrawal, the stress induced by the surgery cannot be totally ruled out. None of these changes can thus compare with the significant volume increase observed here in POM within 24 hours.

Interestingly, the increase in POM volume observed here preceded the activation of male sexual behavior. This observation supports the idea that neuroanatomical and neurochemical changes in POM are (one of) the limiting step(s) in behavioral activation. Within one day, T augmented the optical density of the POM as measured in Nissl stained material. Nissl substance reflects the amount of ribosomal RNA and number of ribosomes (LaVelle, 1956) and T increases the volume of neuronal organelles (rough endoplasmic reticulum and polyribosome) supporting protein synthesis in POM neurons (Panzica et al., 1996a). It is therefore likely that changes in Nissl staining observed here mirror changes in transcriptional activity in POM. It is not novel to postulate that the behavioral effects of steroids must first induce changes in the brain to be effective. What is surprising is that this can all happen within 24 hours.

It was previously established that T increases preoptic aromatase activity within 16 hours and blockade of this enzymatic activity prevents activation of copulatory behavior (Balthazart et al., 1990). The present study demonstrates that this enzymatic induction is reflected at the cellular level by an increase of the optical density and fractional area covered by aromatase staining suggesting that aromatase is one of the multiple behaviorally relevant (Balthazart et al., 2004) proteins regulated by T at the transcriptional level.

The time course of the T-induced changes in Nissl and aromatase optical densities was region specific. Additionally, Nissl staining increased to its maximum density within one-two days and was not affected to any further degree later on while a progressive increase in aromatase optical density was observed throughout the experiment. The mechanisms controlling this regional specificity, the difference between Nissl and aromatase staining and their physiological significance remain to be understood.

Increases in global volume of steroid-sensitive brain nuclei have been assigned to changes in neuronal size, size of the non-neuronal compartment (glial cells, blood vessels), cell spacing, volume of the neuronal dendritic arborization, and in the case of the song control nucleus HVC in oscine songbirds with the incorporation of new neurons (Alvarez-Buylla et al., 1988; Smith et al., 1997). No neurogenesis has to date been reported in the diencephalon of birds (Ling et al., 1997), including songbirds (Alvarez-Buylla and Kirn, 1997) except following localized lesions (Chen et al., 2006). It is thus unlikely that the T-induced increase in POM volume reflects incorporation of new neurons, although this hypothesis has never been directly tested. The time-course of the observed effect is also incompatible with such a mechanism. Furthermore, although T is known to affect glial cell morphology in the rat hypothalamus (Mong et al., 1999), very few interspersed glial cells, including oligodendrocytes and astrocytes, can be observed in the quail POM (Cameron-Curry et al., 1991; Castagna et al., 2003). Their contribution to POM volume is thus probably limited. The contribution of changes in the vascular arborization, which is known to be T-dependent at least in the HVC of songbirds (Louissaint et al., 2002), is unknown in POM.

The modification of the POM structure is thus probably related to an increase in cellular volume and to the development of the dendritic tree. T is known to act directly on the neuronal morphology at least in part by modifying the cytoskeleton. Gonadal steroids can indeed increase the expression of the growth-associated membrane protein (GAP-43) in the neonate and adult rat limbic system (Lustig et al., 1991; Shugrue and Dorsa, 1994) and estradiol increases neurite extension in hypothalamic cell cultures (Toran-Allerand, 1976), presumably through the modulation Tau (Ferreira and Caceres, 1991) and class II-b-tubulin (Rogers et al., 1993) protein expression. The modification of the cytoskeleton by steroid action is therefore

a likely mechanism responsible for volume change observed here in the POM. Castration or the specific reduction of steroid-dependent transcription by the inhibition of steroid receptor coactivator expression (SRC-1) has been shown to significantly reduce the peptidergic innervation in the POM while T treatments fully restore this innervation (for data concerning the vasotocinergic system, see for example: Charlier et al., 2005; Viglietti-Panzica et al., 1994). It is broadly accepted that steroids affect the expression of the neuropeptide itself (Panzica et al., 1999) but it is also likely that the branching of these peptidergic neurons is also affected (Castagna et al., 1999). In line with this hypothesis, the present results demonstrate a significant increase of the fractional area covered by aromatase-immunoreactive structures in the POM supporting the notion of an increased neurite outgrowth of aromatase-expressing cells following T treatment.

In parallel to the neurite extension, previous studies also reported an increase of the volume of neurons in the POM of T treated quail compared to untreated castrates (Aste et al., 1991; Panzica et al., 1996b; Panzica et al., 1991). No quantitative studies of neuronal size have been performed in this work and we do not know whether the cellular volume and architecture can be rapidly affected by T after only one or two days of treatment. The increase in POM volume observed here likely results from a combination of both neurite outgrowth development and neuronal volume augmentation. More detailed investigations should be performed to determine the relative importance of each of these parameters on the overall growth of the nucleus and to define the potential differential time frame of all structural changes affecting POM neurons.

We also demonstrated recently by *in vivo* diffusion-weighted magnetic resonance imaging (DW-MRI) on a 7 Tesla-system the existence of complex changes in total water content and ratio of extra- to intra-cellular water in the POM of castrated quail treated for one or two days with T (Van der Linden et al., 2006). The physiological mechanisms underlying these observations remain to be investigated but suggest a two-step model in which 1) cells first migrate away from each other (increase in extracellular water) to allow 2) neurite extension and increased vascularization (increase overall water content in the nucleus).

In conclusion, we report here the existence of T-induced structural changes affecting the volume of an entire brain nucleus that condition the activation of male sexual behavior by T with a time course far faster than is generally assumed to be necessary to implement such changes. This is to our knowledge the most rapid increase ever reported that affects the global volume of a brain nucleus. We have also demonstrated a close sequential temporal association between these morphological effects and the activation of male-typical sexual behavior. This is significant because T action in the quail POM is necessary and sufficient for the activation of these behavioral effects (Balthazart and Ball, 2007). The quail POM thus represents a very useful model to analyze mechanisms of steroid-regulated adult brain plasticity and test their causal connection to the activation by steroids of a stereotyped motivated behavior.

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Figure 1.

Bar graphs illustrating A. the frequencies of neck grabs and cloacal contact movements (CCM) and B. the size of the cloacal gland recorded just before brain collection in castrated birds killed on day 1 (CXD1) and day 14 (CXD14) and in castrated birds treated with exogenous testosterone for 1 day (CX+T D1), 2 days (CX+T D2), 7 days (CX+T D7) or 14 days (CX+T D14). In this and other bar graphs, the bars relative to the two CX groups are partly overlapping to indicate that data for these two groups are illustrated separately but they were pooled for all statistical analyses. Results of Post Hoc Fisher's PLSD tests are presented as follows: a: $p < 0.05$ vs pooled CX birds; b: $p < 0.05$ vs CX+T D1; c: $p < 0.05$ vs CX+T D2; d: $p < 0.05$ vs CX+T D7.

Figure 2.

Photomicrographs illustrating the POM as defined by Nissl staining (A–C) and ARO-ir cells (D–F) in castrates (A, D) and castrates treated with exogenous testosterone for 1 day (B, E) or for 14 days (C, F). The inserts represent higher magnification photomicrographs in the dorso-lateral part of the POM. CA: anterior commissure. The scale bar located in F represents 200 μm in the 6 main panels and 50 μm in the 6 inserts at higher magnification.

Figure 3.

Bar graphs illustrating the POM volume defined by Nissl staining (A) and aromatase immunoreactivity (B) in castrated birds killed on day 1 (CXD1) and day 14 (CXD14) and in castrated birds treated with exogenous testosterone for 1 day (CX+T D1), 2 days (CX+T D2), 7 days (CX+T D7) or 14 days (CX+T D14). Results of Post Hoc Fisher's PLSD tests are presented as follows: a: $p < 0.05$ vs CX; b: $p < 0.05$ vs CX+T D1; c: $p < 0.05$ vs CX+T D2; d: $p < 0.05$ vs CX+T D7.

Figure 4.

Bar graphs illustrating the relative optical density of Nissl staining (**A**) and aromatase immunoreactivity (**B**) measured in the POM at the objective 10X in castrated birds killed on day 1 (CXD1) and day 14 (CXD14) and in castrates treated with exogenous testosterone for 1 day (CX+T D1), 2 days (CX+T D2), 7 days (CX+T D7) OR 14 days (CX+T D14). Measures at objective 40X were also performed in the ventro-lateral, dorso-lateral and medial periventricular regions of the POM in Nissl- (**C**) and aromatase-stained (**D**) sections. Results of Post Hoc Fisher's PLSD tests are presented as follows: a: $p < 0.05$ vs CX; b: $p < 0.05$ vs CX +T D1; c: $p < 0.05$ vs CX+T D2; d: $p < 0.05$ vs CX+T D7.

Fractional area covered by ARO-ir structures

Figure 5.

Bar graphs illustrating the fractional area covered by aromatase-immunoreactive structures measured at objective 40X. The analyses were performed in the ventro-lateral, dorso-lateral and medial periventricular regions of the POM in castrated bird killed on day 1 (CXD1) and day 14 (CXD14) and in castrated birds treated with exogenous testosterone for 1 day (CX+T D1), 2 days (CX+T D2), 7 days (CX+T D7) or 14 days (CX+T D14). Results of Post Hoc Fisher's PLSD tests are presented as follows: a: $p < 0.05$ vs CX; b: $p < 0.05$ vs CX+T D1; c: $p < 0.05$ vs CX+T D2; d: $p < 0.05$ vs CX+T D7.