

Aromatization of Testosterone in the Preoptic Area and Control of Male Sexual Behavior in the Japanese Quail

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In Japanese quail (*Coturnix coturnix japonica*), as in most vertebrates, male sexual behavior is activated by testosterone. It is clearly established that castration suppresses copulatory behavior within one week or two after surgery (1) and that a treatment with testosterone in the form of daily injections or subcutaneous implantation of silastic capsules filled with the steroid readily restores this behavior (2, 3). The neuroanatomical and neurochemical bases of this activation are, however, poorly understood and no coherent picture of the events which lead from the uptake of testosterone by the brain to the activation of the behavior can be presented. In this chapter I shall review the research conducted in my and in other laboratories which is aimed at understanding the actions of testosterone in the quail brain which underlie the activation of male reproductive and, in particular, copulatory behavior.

I. THE SEXUALLY DIMORPHIC MEDIAL PREOPTIC NUCLEUS

The preoptic area (POA) in vertebrates is one major site of testosterone action on behavior (4). Assuming that this would also be the case in quail, we have concentrated our investigations on the anatomy and neurochemistry of this region. In collaboration with the group of Carla Viglietti and Gian Carlo Panzica at the University of Torino, we first carried out a detailed cytoarchitectural study of the POA in male and female quail. A sexually dimorphic structure was identified in this region. A dense group of cells located periventricularly and extending throughout most of the preoptic region was heavily stained by toluidine. Its volume was measured using a computer-assisted planimeter. This nucleus appeared to be about 25% larger in males than in females (5). It was called medial preoptic nucleus (POM) by reference to a similar structure described in the pigeon by Berk and Butler (6). Other nuclei in the same brain region did not show a corresponding dimorphism in volume. The volume of the POM is androgen-dependent (Fig. 1).

A decrease of about 25% is observed following castration and the

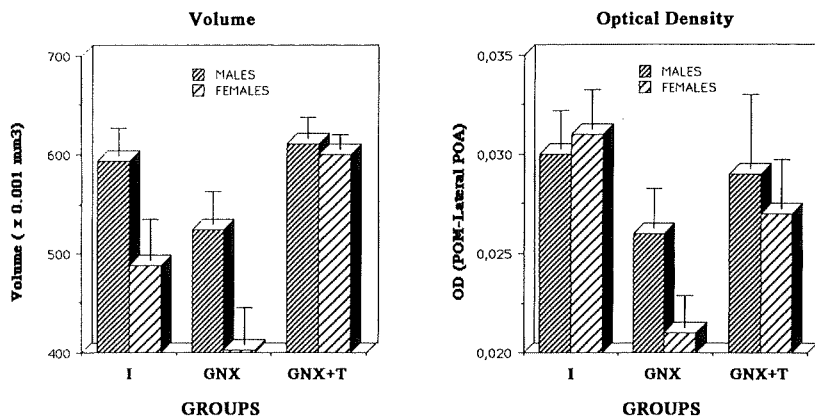


Fig. 1. Effect of gonadectomy (GNX) and testosterone replacement therapy (GNX+T) on the volume and optical density of the medial preoptic nucleus (POM) in male and female quail. The optical density was measured by microdensitometry on an IBAS system after staining of the Nissl bodies with toluidine. The values reported are differences between the density in POM and in the lateral POA just adjacent to it. All values are means $\pm$ standard errors.

nucleus size can be restored to the level seen in intact males by a treatment with testosterone (7). The optical density of the POM measured by microdensitometry following staining of the Nissl bodies by toluidine blue shows the same variations as the POM volume following castration and treatment with testosterone (Fig. 1). The POM volume seen in gonadectomized testosterone treated birds, however, is not sexually dimorphic which suggests that it is directly controlled by the circulating levels of testosterone and does not present a true sex dimorphism, *i.e.*, is not differentiated by the perinatal hormones. In support of this interpretation, the injection of estradiol benzoate (EB) into quail embryos on day 9 of incubation, a treatment which is known to alter profoundly the sexual differentiation of copulatory behavior (8, 9), does not affect the volume of the POM in the adult birds grown from treated eggs (7).

The POM can thus be considered as a good marker of androgen action in the POA. Its optical density in Nissl stain is modified in the presence of testosterone which suggests that the steroid induces in this nucleus an active synthesis of proteins. The increased density of staining probably explains, at least in part, the increase in volume. It is indeed unlikely that testosterone increases POM size by the addition of new neurons. The change in volume thus probably reflects either an increase in cell size (true volume increase for the nucleus) or an enhancement of the detectability of the nucleus at its edges as a result of the stimulated protein synthesis (apparent volume increase). Experiments are now in progress to research which of these (not mutually exclusive) interpretations is correct.

The action of testosterone on the sexually dimorphic POM appears to be directly related to the activation of copulatory behavior. Stereotaxic implantation in the POM of 27 gauge needles filled with crystalline testosterone indeed restored full copulatory behavior in castrated male quail (10). Implants localized in all other positions within the POA were behaviorally ineffective. Diffusion from such testosterone implants was probably limited and had no physiological relevance because implants that were located as close as 200–300 μ m lateral to POM were also behaviorally ineffective. This clearly suggests that the POM is the active site of testosterone action within the POA for the activation of copulatory behavior.

II. THE PREOPTIC AROMATASE

Having precisely identified a site of testosterone action, it then became possible to begin detailed investigations of the neurochemical mechanisms underlying the behavioral activation. We had shown previously that the POA contained an active aromatase (11), the enzyme which transforms testosterone into estradiol (E_2). This is a critical step in the production of the behavioral effects of testosterone (12-16). The aromatase activity in POA was, like the sexual behavior, testosterone-dependent: it was dramatically reduced by castration and

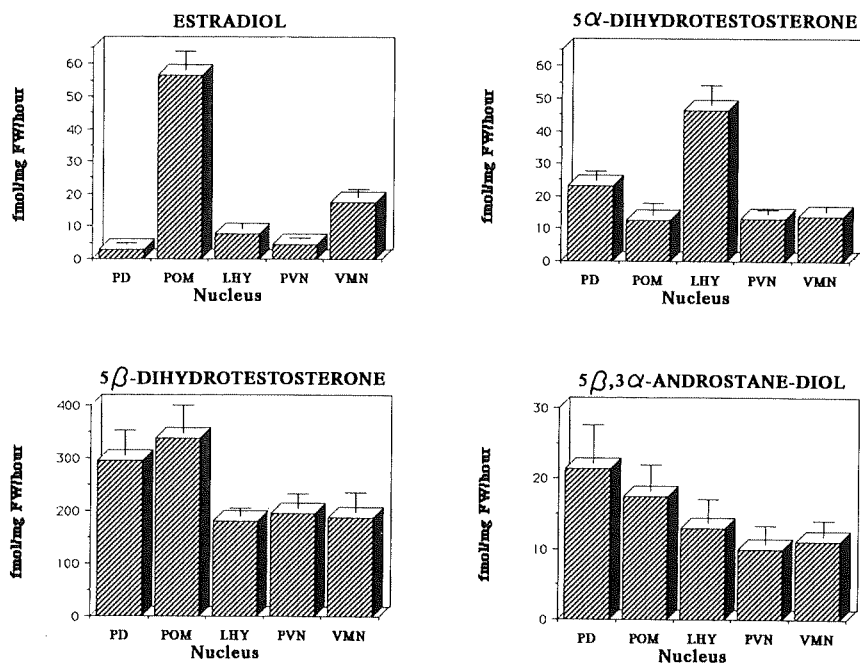


Fig. 2. Production of estradiol, 5 α - and 5 β -dihydrotestosterone and of 5 β -androstane-3 α , 17 β -diol (5 β , 3 α -androstane-diol) by different preoptic and hypothalamic nuclei in the male Japanese quail. Nuclei were dissected by the Palkovits punch technique (18, 19) and the activity of the enzyme was measured by *in vitro* radioenzyme assays using tritiated testosterone as a substrate. All data are means $\pm$ standard errors. PD, nucleus preopticus dorsolateralis; POM, nucleus preopticus medialis; LHY, area lateralis hypothalami; PVN, nucleus paraventricularis; VMN, nucleus ventromedialis hypothalami.

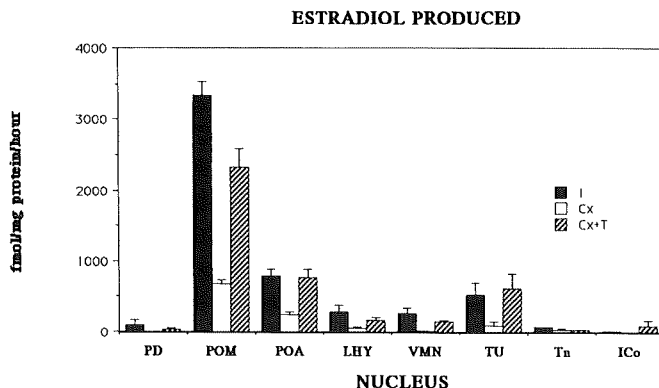


Fig. 3. Effect of castration and testosterone replacement therapy on the aromatase in different telencephalic, hypothalamic and mesencephalic nuclei of the male quail brain. See also Fig. 2 for details on the methods. POA, preoptic area excluding the POM; TU, tuberal hypothalamus; Tn, nucleus taeniae; ICo, nucleus intercollicularis.

restored by a treatment with subcutaneous silastic capsules filled with testosterone (17). Additional experiments were recently performed to define more precisely the neuroanatomical localization of the aromatase activity within the POA. Using the Palkovits punch technique (18, 19) in combination with *in vitro* radioenzyme assays, we first analyzed the distribution of aromatase activity and of other testosterone-metabolizing enzymes in a series of preoptic and hypothalamic nuclei (20). This revealed that each of these enzymes had a preferential localization (Fig. 2).

In particular, the highest level of aromatase activity was found in the sexually dimorphic POM. This enzymatic activity in POM was testosterone-dependent as had been seen previously in the entire POA. Castration produced a 4-fold decrease in the aromatase activity of POM which was reversed by treatment with testosterone (Fig. 3).

Similar effects were seen in other hypothalamic nuclei and in the rest of the POA but the magnitude of the changes and the absolute levels of enzyme activity were much smaller (see figure). The activity of other testosterone-metabolizing enzymes such as the 5α - and 5β -reductase did not change in response to the variation of testosterone levels.

A subsequent series of experiments demonstrated that the induction by testosterone of aromatase activity in the POA is a close

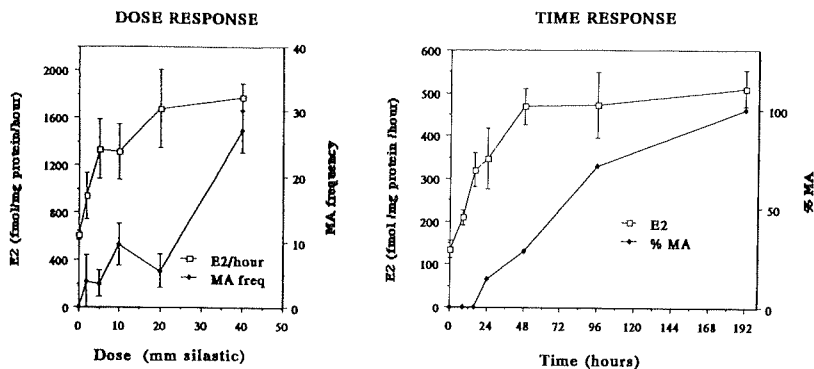


Fig. 4. Effects of testosterone on the aromatase activity in the POA and on the sexual behavior in male quail: dose and time response curves. Castrated males were treated with silastic implants filled with testosterone (2.5 to 40 mm for the dose response; 40mm for the time response) and sacrificed after 2 weeks (dose response) or after various time between 0 and 8 days (time response). Sexual behavior (frequency of mount attempts (MA) or percentage of birds showing the behavior) was measured during standardized presentations to receptive females and aromatase activity was measured in the POA by radioenzyme assays. Data are means $\pm$ standard errors.

correlate of the behavioral effects of the steroid in this region. The induction of aromatase activity and of sexual behavior by testosterone are both dose-dependent (21). Treatment of castrated male quail with testosterone filled silastic implants in the range 2.5 to 40 mm in length produced a gradual increase in aromatase activity from about 600 to about 2,000 fmol/mg protein/hr. The minimal dose of testosterone which reliably restored copulatory behavior in treated males was 10 mm of silastic implant corresponding approximately to a doubling of the enzyme activity in the POA (Fig. 4)

The activation of the sexual behavior and of the aromatase activity are also correlated in time: in castrated male quail, the implantation of 40 mm silastic capsules filled with testosterone significantly increased the aromatase activity of the POA within 8 hr (see Fig. 4). The maximal increase (approximately 4-fold) in enzyme activity was seen after approximately 48 hr. The induction of the aromatase activity preceded the activation of sexual behavior by 24 to 48 hr.

The highest levels of aromatase activity are thus seen in the POM of the POA which is the site where testosterone acts to activate behavior. Aromatase activity is increased by testosterone in a dose and

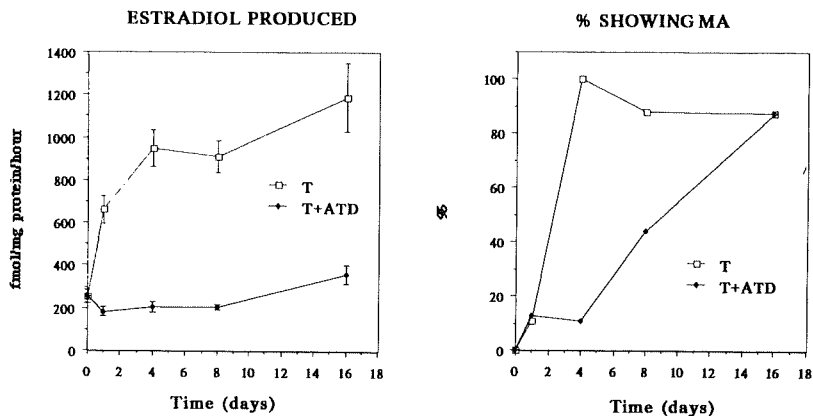


Fig. 5. Effects of androstatrienedione (ATD), an aromatase inhibitor on the induction by testosterone (T) of aromatase activity in the POA and on the activation of copulatory behavior (% of birds showing mount attempts (MA)). Castrated male quail were implanted with 20 mm silastic capsules filled with testosterone and birds in the ATD group received in addition ten 20mm capsules filled with the inhibitor. Birds were sacrificed after various times between 0 and 16 days just after being tested for sexual behavior. Aromatase activity was measured in the POA by radioenzyme assays.

time-dependent manner which parallels the activation of the copulatory behavior.

These neuroanatomical, dose, and time correlations reveal true causal links as can be demonstrated by the use of specific inhibitors. If castrated males are treated with silastic implants filled with testosterone and in parallel with implants containing androstatrienedione (ATD), a specific aromatase inhibitor, the activation of copulatory behavior is delayed by about one week. In parallel, the increase of aromatase activity in the POA is completely suppressed (Fig. 5).

It thus seems that the aromatization of testosterone into E_2 is a necessary step in the chain of biochemical processes which lead to the activation of the copulatory behavior. This aromatization of testosterone has probably to take place in the POA and more specifically in the POM, because systemic treatment with ATD blocks the activation of behavior produced by stereotaxic implantation of testosterone in the POM (22) and conversely implantation of ATD in the POM blocks the behavioral effects of systemic treatment with testosterone (J. Balthazart and C. Surlmont, unpublished data). The exact role played by the E_2 produced in this way, however, remains unclear at present.

III. ESTROGEN ACTION IN THE BRAIN

The most widely accepted hypothesis assumes that E_2 derived from testosterone aromatization directly activates behavior by interacting with nuclear estrogen receptors and promoting the synthesis of new proteins. In support of this interpretation is the fact that nuclear estrogen receptors can be detected in the POM both by autoradiography following *in vivo* injection of tritiated E_2 (23) and by immunocytochemistry using the monoclonal antibody H222SP directed against estrogen receptors from a human mammary tumor (24). By the latter technique, we showed recently that about 90% of the cells in POM contain estrogen receptors. The biochemical substrate for a behavioral action of estrogen mediated through the classical intracellular receptors is thus present in the POM.

This classical view which represents the aromatization hypothesis in its strongest meaning has, however, been challenged on several occasions. Many research reports have shown the existence of a synergism between estrogen and androgens in the activation of sexual behavior, and in some cases it has even been demonstrated that androgens which are not aromatizable such as 5α -dihydrotestosterone or methyltrienolone (R1881) can by themselves activate a full copulatory behavior in castrates (25). These results lead to the reevaluation of the role of estrogens in behavioral activation and it has been proposed that the estradiol derived from testosterone aromatization was not directly activating the behavior but rather was modifying the effects of androgens by changing their central catabolism (26, 27). No physiological evidence for this interpretation could be collected, however, in quail (28) and in recent rat studies (29).

The mode of action of E_2 derived from testosterone aromatization is also questioned by the recent discovery that a substantial amount of the aromatase activity is localized in the synaptosomal fraction obtained during differential centrifugation (30, 31). This suggests that the E_2 which is produced is available at the synaptic level and could thus potentially play a direct role in the neurotransmission. It has indeed been shown using *in vitro* preparations that estrogens can modify the properties of neuronal membrane within seconds of their application (for review see refs. 31, 32). Future research will have to examine what the mechanism is by which E_2 affects the reproductive behavior in quail

and in other vertebrates. However, it is very clear at present that the production of E_2 by aromatization of testosterone is finely regulated and the aromatase activity is, in each situation tested so far, correlated with the behavioral effects of testosterone. The changes in the activity of the aromatase thus modulate at the level of the target organ the physiological effects of testosterone.

Additional modulation is also potentially provided by the changes in estrogen receptor concentration which were recently detected in the preoptic area of the quail (33). The total concentration of estrogen receptors (free plus bound) is indeed increased in castrated quail by treatment with testosterone, and interestingly, this effect is not mediated by testosterone aromatization because it is also observed in birds simultaneously treated with an aromatase inhibitor. This recent finding provides an interesting biochemical substrate for the known synergism between androgens and estrogens in the activation of behavior. Androgens would increase the concentration of estrogen receptors which would then bind the estrogens coming either from the periphery or from central aromatization of androgens. Future experiments should determine the importance of this mechanism in the control of copulatory behavior.

IV. STEROID-INDUCED NEUROCHEMICAL CHANGES

At present, we thus have a fairly good understanding of the steroid biochemistry which underlies the activation of behavior. The subsequent neurochemical mechanisms implicated in this process are by contrast not so clear.

It can *a priori* be ascertained that a change in behavior which is brought about by the presence of a steroid must implicate some changes in the neurotransmission. If E_2 derived from testosterone aromatization acts through the classical nuclear estrogen receptors to promote the synthesis of new proteins, one can then suggest that some of these new proteins are enzymes controlling the synthesis or the catabolism of neurotransmitters or receptors to these molecules. Several lines of evidence implicate the catecholamines in the control of reproductive physiology and, in particular, in the activation of male copulatory behavior in mammals. We have consequently initiated a series of experiments to determine whether these transmitters also control copu-

lation in quail and to analyze how steroids control the catecholaminergic activity in the POA. Special interest was given in this context to the noradrenergic transmission because earlier studies using a spectrofluorimetric assay had shown that norepinephrine (NE) was extremely concentrated in the POA of quail, contrary to dopamine which was only present in minimal amounts (34). In addition, this study had demonstrated that NE was more concentrated in the POA of females than in males.

At the same time, a series of studies carried out in ring doves (*Streptopelia risoria*) demonstrated that NE, acting probably through the alpha adrenergic receptors was inhibiting some aspects of the reproductive behavior (35, 36). If NE was also inhibiting copulatory behavior of quail, then the higher concentration in the POA of females could have been responsible, at least in part, for the behavioral sex dimorphism in this species (testosterone activates copulatory behavior in males but not in females (3, 37)). Several types of data have been collected since then which support this hypothesis.

Using a more sensitive and more specific assay technique (high performance liquid chromatography (HPLC) combined with electrochemical detection), we have confirmed that NE is more concentrated in the POM of females than in the POM of males. In this experiment, the nucleus was dissected by the Palkovits punch method (18, 19) so that the localization of this neurochemical dimorphism is known with precision (J. Balthazart *et al.*, unpublished data). The concentrations of NE which were assayed in the POM are also extremely high (around 200 ng/mg protein) which suggests that the noradrenergic transmission plays a key role in this nucleus. In addition, some evidence was collected suggesting that the turnover of NE in the POA is controlled by steroids (38).

In collaboration with Dr. G. F. Ball from Rockefeller University, we also initiated a series of studies using quantitative autoradiography to localize and quantify various subtypes of noradrenergic receptors. This showed that the POM is specifically labeled by an extremely high density of α_2 noradrenergic receptors (39) but contains only minute amounts of the α_1 receptor subtype (40). The α_2 receptors (binding sites for *para*-amino-clonidine), in fact, outline the entire nucleus POM through its rostral to caudal axis and define a region identical to that delimited during our cytoarchitectural studies using Nissl staining. This finding is thus in agreement with the attribution of a key role to

the noradrenergic transmission in this nucleus. Functional studies have now confirmed this suggestion.

Using a specific noradrenergic neurotoxin, N-(2-chlorethyl)-N-ethyl-2-bromobenzylamine (DSP4), it was demonstrated that the noradrenergic transmission modulates several aspects of the reproductive process including the copulatory behavior, the secretion of luteinizing hormone and the aromatase activity in the POA. One single intraperitoneal injection of DSP4 at the dose of 50 mg/kg produces a significant depletion of NE concentrations in many brain areas including the POA (41) and, more specifically, the POM (42). The depletion is fairly specific from the chemical point of view as concentrations of epinephrine, dopamine, and serotonin are usually not affected. The decrease of NE concentration has a limited amplitude (25 to 50% according to the brain area) but is nevertheless sufficient to induce clear and reproducible physiological and behavioral effects. In a series of experiments (41), it was shown that a pretreatment with DSP4 significantly enhances the activating effects of testosterone on copulatory behavior. In DSP4-treated castrates, the latency between the initiation of testosterone treatment and the first appearance of behavior was reduced and the behavioral frequencies were increased by comparison with control saline-injected birds. After a given latency, the percentage of birds showing copulatory behavior was thus higher in birds treated with testosterone and DSP4 than in birds treated with testosterone alone.

This observation could be made in four independent experiments. The behavioral stimulation was specific to the copulatory behavior and did not affect other androgen-dependent behaviors such as crowing or androgen-dependent morphological characteristics such as the growth of the cloacal gland (41). The behavioral effect of DSP4 was only detected in testosterone-treated birds but not in castrated animals which did not receive the steroid replacement therapy. This clearly suggests that in physiological conditions, the noradrenergic transmission plays an inhibitory role on the activation by testosterone of male sexual behavior. The noradrenergic receptor subtype(s) implicated in this effect is unknown at present. However, considering that testosterone activates copulatory behavior by acting mainly in the POM and that this nucleus is heavily labeled by adrenergic receptors, it is tempting to suggest that this receptor subtype mediates the inhibitory effects of NE on the sexual behavior.

How this inhibition is produced is not understood at present. One

possible mechanism is suggested by the recent finding that a treatment with DSP4 potentiates the induction by testosterone of the aromatase activity in the POA (42). If this effect is directly related to the depletion of NE by the drug, it then reveals that in intact animals the noradrenergic transmission inhibits the aromatase activity. The facilitation of behavioral activation in DSP4-treated birds could then be a consequence of the increased aromatase which is known to be a limiting step in the activation of the behavior by testosterone (see section III). The lower aromatase activity in females compared to males (11, 17) would, in this perspective, be a consequence of a higher inhibition in females due to a higher NE content of the POA (34). Experiments are now in progress to determine the importance of this mechanism in the physiological control of the behavior.

V. CONCLUSIONS

The data available at present thus provide a clear picture of the steps which separate the production of testosterone from its action on brain neurochemistry. Testosterone is aromatized in specific brain areas and the activity of the enzyme which controls this metabolism is finely regulated. The steroid and/or its aromatized metabolite then interact with specific androgen or estrogen receptors and modify specific aspects of the neurotransmission. The noradrenergic system is probably one major target at this level. It seems also likely that the activity of the steroid is modulated by a noradrenergic feedback. Studies in mammals have shown that the noradrenergic system can modify the concentration of estrogen receptors in specific brain areas and our recent research in quail suggests that NE could also control the activity of the aromatase in the POA. These links between steroid action and the neurotransmitters probably represent critical neurochemical mechanisms underlying the activation of the behavior. The POM is a central site where these interactions take place. It is sexually dimorphic and testosterone sensitive; it is characterized by a finely regulated aromatase and high concentrations of estrogen receptors; and it is specifically labeled by adrenergic receptors and contains extremely high concentrations of NE. This nucleus is also the key site of testosterone action. It is thus an excellent model structure for the study of the neurochemical mechanisms which underlie the behavioral action of the steroid.

SUMMARY

Sexual behavior in Japanese quail is activated by testosterone and the POA is one major site of action of the hormone. We review here the anatomical and neurochemical mechanisms which underlie the activation of copulatory behavior by testosterone. The POA contains a sexually dimorphic nucleus (the medial preoptic nucleus or POM) which is testosterone sensitive. Stereotaxic implantation of testosterone in but not around the POM activates copulation in castrated males. This nucleus also contains high levels of aromatase activity and most of its cells contain estrogen receptors. The aromatase activity in the POM is higher in males than in females and is androgen-dependent. The induction by testosterone of aromatase activity in the POA is correlated with the activation of sexual behavior by the steroid. If the induction of the aromatase is blocked by specific inhibitors, the activation of the behavior is suppressed or at least delayed which shows the key role played by this enzymatic transformation in the activation of behavior. The noradrenergic transmission seems to be very important in the POM and probably participates in the mechanisms which control copulatory behavior. The POM contains high concentrations of norepinephrine and is specifically labeled by noradrenergic receptors. A partial denervation produced by the specific noradrenergic neurotoxin DSP4 enhances the effects of testosterone on both behavior and aromatase. This suggests that under physiological conditions, the noradrenergic system inhibits the effects of testosterone in the POA. Research is now in progress to identify the adrenergic receptor subtypes which are implicated in these responses and what their relevance is to the control of the reproduction in intact animals.

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