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Embryonic Differentiation of Sexual Dimorphism in Vasotocin and Mesotocin Levels in Chickens

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ROBINZON, B., N. SAYAG, T. I. KOIKE, S. L. KINZLER AND P. A. MARKS. *Embryonic differentiation of sexual dimorphism in vasotocin and mesotocin levels in chickens.* PHARMACOL BIOCHEM BEHAV 42(4) 823-829, 1992.—Chicken embryos of both sexes were injected on the tenth day of incubation with either estradiol benzoate (EB), aromatase inhibitor [1,4,6-androstatrien-3, 17-dione (ATD)], antiestrogen [tamoxifen (TAM)], antiandrogen [flutamide (FLU)], or the oil vehicle as control (C). At adulthood, at the age of 26 weeks, 10 chickens of each sex were killed and the amounts of immunoreactive arginine vasotocin (AVT) and mesotocin (MT) in the anterior hypothalamus (AHA), posterior hypothalamus (PHA), neurohypophysis (NHP), and pineal gland (PNL) were determined. Control hens had significantly more AVT in PNL and less MT in AHA and NHP than the corresponding roosters. This sexual dimorphism was affected by the embryonic treatments; TAM increased AVT in AHA of cockerels but not of hens. In both sexes, TAM and FLU increased AVT content in NYP. In males, but not in females, ATD also increased AVT content in the NHP. TAM and FLU administration to the female embryo reduced PNL AVT to the amount present in normal males. None of the treatments effected AHA MT in hens, while in cockerels TAM increased it. In females, TAM and FLU significantly increased NHP MT to the level of C males. In roosters, ATD, TAM, and FLU increased NHP MT further. In hens, but not roosters, FLU reduced MT in PNL. These results indicate that embryonic differentiation of the MT and AVT systems is affected by gonadal steroids in chickens.

Chicken Embryo Gonadal steroids Tamoxifen Flutamide ATD Aromatase inhibitor
Arginine vasotocin Mesotocin Hypothalamus Neurohypophysis Pineal gland Sexual differentiation

THE distribution of arginine vasotocin (AVT) and mesotocin (MT) in the White Leghorn chicken is sexually dimorphic. In the pineal gland, there is four times more AVT in hens than in cockerels either intact or castrated and supplemented with estradiol or testosterine. On the other hand, intact cockerels have twice the amount of MT in the neurohypophysis than females. Castrated males treated with either testosterone or estradiol have similar levels of neurohypophysial MT, which are significantly lower than in intact males but higher than in females (48).

Sexual dimorphism in AVT immunostaining was observed in both lizard and canary brains (57,63,64). In the canary, males have a denser AVT innervation of the lateral septum and higher number of AVT-immunoreactive cells in the dorsal diencephalon (63,64). In these birds, testosterone administration to the female increased the AVT immunostaining in both areas to that of the male, while castration of the male decreased AVT immunoreactivity to that of the female (63,64). Testosterone increases the number of AVT binding sites in the brain of female canaries, where their density is normally lower than in males (62). Thus, one may suggest that the sexual dimorphism in brain AVT in canaries is the result of circulating androgen levels and not of an early differentiation process. However, this does not seem to be the case with the chicken because castration and estradiol administration did not abolish the sexual dimorphism in pineal AVT and neurohypophysial MT (48).

In chickens, embryonic administration of either estradiol, aromatase inhibitor [1,4,6-androstatrien-3, 17-dione (ATD)], antiestrogen [tamoxifen (TAM)], or antiandrogen [flutamide (FLU)] cause a certain degree of demasculinization but no feminization of male sexual behavior. None of these treatments cause any change in female sexual manifestation (52-54). These results are in contrast to the suggested model for

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differentiation of sexual behavior in Aves, which was developed based upon studies with the quail (1-3). According to this model, the male is the neutral sex and male sexual behavior develops in the absence of gonadal steroids. Ovarian estrogen, produced in the female embryo, eliminates the capacity for displaying masculine sexual behavior. In the male, a slight demasculinization of sexual behavior occurs normally by endogenous embryonic androgen, which is aromatized to estrogen.

The present study was designed to verify if embryonic manipulations that affect differentiation of sexual behavior in cockerels can similarly affect the sexual dimorphism in AVT and MT distribution.

METHOD

Animals and Embryonic Injections

Fertile White Leghorn chicken eggs were incubated in a rotating forced-air incubator at 37°C and 60% relative humidity. On the tenth day of incubation, prior to the end of the critical period for differentiation of sexual behavior (67), eggs were injected with the test drug dissolved in 0.05 ml sesame oil. Injections were performed via a hole drilled at the small end of the egg into the albumen. Hot paraffin was used to seal the injection site. Each fertile egg was injected with either 0.5 mg estradiol benzoate (EB), 0.3 mg of the *trans*-isomer (TAM), 0.3 mg FLU, 0.6 mg ATD, or sesame oil only for the control (C). TAM is a nonsteroidal antiestrogen (42) obtained from Sigma Chemical Co. (St. Louis, MO) FLU, a compound with a pure antiandrogen activity (45), was kindly supplied by Schering Co. (Kenilworth, NJ) ATD, an efficient aromatase inhibitor (35), was obtained from Steraloids, Inc. (Wilton, NH). The doses used were found previously to affect differentiation of sexual behavior in the male White Leghorn (54).

After hatching, all chicks were sexed, identified with small wing bands, and placed in a heated pen. During the whole experimental period, they were exposed to a 16 L : 8 D cycle. Free access to water and standard commercial mash was provided. At 3 months of age, chickens were transferred into individual wire cages for the est of the experimental period.

Tissues Collection and Extraction

At 26 weeks of age, 10 cockerels and 10 hens from each embryonic treatment group were killed by cervical dislocation and the anterior hypothalamic area (AHA), posterior hypothalamic area (PHA), neurohypophysis (NHP), and pineal gland (PNL) were immediately removed (47), cleaned of adhering tissues, and homogenized in 10 vol cold (4°C) 0.2 M acetic acid. The tissues were extracted by the method described previously (47), and the extracts were stored at −20°C pending determination of AVT and MT.

Radioimmunoassay

Tissue AVT and MT were measured in single assays by methods described previously (47). The minimal detectable dose for AVT was 0.44 pg/tube with an intraassay coefficient of variation of 5.4%, and the minimal detectable dose for MT was 1.4 pg/tube with an intraassay coefficient of variation of 5.0%.

Statistical analysis of data was carried out using the Mann-Whitney *U*-test.

RESULTS

The AVT and MT contents in the AHA, PHA, NHP, and PNL of hens and cockerels of the various embryonic treatments are given in Tables 1 and 2.

A significant sexual dimorphism in PNL AVT and in NHP and AHA MT contents were found. The PNL of C hens contained about three times more AVT than that of C cockerels. The amount of MT in AHA of C males was over twice that in C females. The latter had only one third the amount of NHP MT observed in C cockerels.

Embryonic administration of the various drugs had remarkable effects on AVT and MT contents in some of the organs tested. Administration of TAM increased AVT in the AHA of cockerels but not of hens; thus, TAM-treated males had significantly more AVT in the AHA than corresponding hens. Levels of AVT in the PHA were not significantly affected by either treatment or gender. In the NHP of chickens of both sexes, embryonic administration of either TAM or FLU induced a significant increase in AVT content. In males, but not in females, ATD administration produced the same effect. TAM and FLU administration to the female embryo reduced significantly the PNL AVT to the amount present in males and thus removed the sexual dimorphism normally observed. None of the treatments had any effect on PNL AVT in males.

In hens, none of the embryonic treatments had any effect on the AHA content of MT and, thus, regardless of treatment all females had significantly less AHA MT than males. In cockerels, embryonic TAM increased the AHA MT to a level significantly higher than that of embryonic EB-treated roosters. The variability in PHA levels of MT was large and might mask effects of embryonic manipulations. However, in hens ATD and FLU caused nonsignificant decreases, while in cockerels ATD and TAM caused nonsignificant increases, in MT levels in the PHA. Thus, TAM- and ATD-treated males had significantly higher levels of PHA MT than ATD- and FLU-treated females. In females, embryonic TAM and FLU caused a significant increase in NHP MT to a level not significantly different from that of C males but still significantly lower from any of the treated cockerels. In roosters, embryonic ATD, TAM, and FLU increased NHP MT significantly over controls. The elevation due to ATD was significantly greater than following EB and TAM, while the increase in the FLU-treated group was significantly higher than in the EB-supplemented one. In hens, but not in roosters, embryonic FLU reduced the amount of MT in the PNL.

DISCUSSION

Sexual dimorphism of neurohypophysial peptides has been examined mainly in mammalian species. In several rodent species, dimorphism has been reported for oxytocin (OT) levels in various hypothalamic sites (26), the content of propressophysin mRNA in the bed nucleus of the stria terminalis (39), arginine vasopressin (AVP) and neurophysin-II pathways in the limbic system, and structures in the medial preoptic area (11,18,21,29,36,61). In humans, the distribution of AVP-producing cells in the suprachiasmatic nucleus is also dimorphic (58). Sex differences in basal and stimulated rates of AVP and OT secretion have been observed in adult rats and humans (8,13,56,66).

There is considerable evidence indicating that gonadal steroids are linked to sexual dimorphism of neurohypophsial peptides. OT and AVP levels in the PVA and supraoptic nucleus (SON), the main source of hormones in the NHP, fluctu-

TABLE 1

AVT-LIKE IMMUNOREACTIVITY IN THE AHA, PHA, NHP AND PNL OF 26-WEEK-OLD HENS (HEN) AND COCKERELS (COC) INJECTED ON THE TENTH DAY OF INCUBATION WITH EITHER 0.5 mg EB, 0.3 mg TAM, 0.3 mg FLU, 0.6 mg ATD, OR 0.05 ml SESAME OIL, THE VEHICLE, AS CONTROL (C)

	AHA (ng/organ)	PHA (ng/organ)	NHP (μg/organ)	PNL (pg/organ)
HEN-C	26*	7.6	1.6†	936‡
	(14–48)	(2.5–13.0)	(0.1–2.6)	(260–3,424)
HEN-EB	39*‡	6.3	1.8†	623‡
	(36–42)	(0.5–12.0)	(0.7–2.2)	(284–1,580)
HEN-ATD	28*	4.4	2.2†	615*‡
	(26–32)	(1.5–10.0)	(1.0–2.9)	(56–2,380)
HEN-TAM	19*	3.7	4.7*‡	238*†
	(14–28)	(1.5–7.6)	(1.7–8.9)	(64–664)
HEN-FLU	28*‡	3.6	6.5‡	105†
	(14–38)	(1.0–11.0)	(4.6–8.8)	(10–322)
COC-C	38*‡	4.3	1.8†	230*†
	(10–66)	(1.4–7.8)	(1.4–2.6)	(56–650)
COC-EB	26*‡	3.4	4.0*	351*†
	(12–38)	(0.5–10.2)	(1.0–6.6)	(56–1,690)
COC-ATD	31*‡	12.9	4.8*‡	257*†
	(16–46)	(2.4–24.9)	(3.3–7.1)	(2–1,857)
COC-TAM	65‡	13.8	4.9*	219*†
	(34–98)	(3.2–54.9)	(1.3–5.9)	(2–838)
COC-FLU	37*‡	11.7	6.4†	293*†
	(22–54)	(1.1–39.8)	(3.5–8.4)	(60–918)

Mean and range; n = 10/group.
*†‡Different symbols indicate significant differences ($p < 0.05$).

TABLE 2

MT-LIKE IMMUNOREACTIVITY IN THE AHA, PHA, NHP AND PNL OF 26-WEEK-OLD HENS (HEN) AND COCKERELS (COC) INJECTED ON THE TENTH DAY OF INCUBATION WITH EITHER 0.5 mg EB, 0.3 mg TAM, 0.3 mg FLU, 0.6 mg ATD, OR 0.05 ml SESAME OIL, THE VEHICLE, AS CONTROL (C)

	AHA (ng/organ)	PHA (pg/organ)	NHP (μg/organ)	PNL (pg/organ)
HEN-C	0.9*	202*†	0.4‡	374§
	(0.4–1.5)	(0–324)	(0.1–0.6)	(38–1,032)
HEN-EB	0.6*	157*†	0.6‡	347§
	(0.4–0.9)	(28–286)	(0.2–0.8)	(78–1,088)
HEN-ATD	0.5*	27*	0.5‡	299§
	(0.3–0.6	(0–54)	(0.3–0.7)	(52–1,284)
HEN-TAM	0.6*	179*†	1.0¶	119†§
	(0.1–0.9)	(8–320)	(0.6–1.3)	(0–310)
HEN-FLU	0.4*	44*	1.2¶#	57†
	(0.1–0.6)	(0–208)	(0.6–1.8)	(0–170)
COC-C	2.4†§	480*†§	1.3¶#	227†§
	(1.5–3.8)	(0–1,280)	(0.8–1.9)	(14–578)
COC-EB	2.0†	265*†	1.8*#	283§
	(1.6–2.4)	(0.672)	(0.8–4.6)	(86–1,160)
COC-ATD	2.4†§	812†§	3.9§	401§
	(1.3–3.3)	(144–1,265)	(1.9–8.6)	(74–3,144)
COC-TAM	7.4§	1580§	1.8*†	174†§
	(2.8–20.0)	(846–2,932)	(1.1–2.8)	(0–552)
COC-FLU	2.7†§	763*†§	3.4†§	360§
	(1.7–3.4)	(0–2,900)	(1.7–10.2)	(90–1,284)

Mean and range; n = 10/group.
*†‡§¶#Different symbols indicate significant differences ($p < 0.05$).

ate during the estrus cycle in rats (25). Gonadectomy reduces the OT content in the NHP of both female and male rats, whereas administration of estrogen or progesterone to intact female rats increases OT levels (23). The density of OT immunoreactive fibers and OT binding sites in the ventromedial hypothalamus diminishes following ovariectomy and increases following administration of estradiol and progesterone in gonadectomized male and female rats (16,17,32,33,55). Sexual dimorphism in the distribution and secretion of NHP peptides is affected by gonadectomy and sex steroid administration in a manner that suggests that it is due to a direct action of circulating steroids rather than to a primary differentiation process (4,5,8,9,15,18,20-22,24,30,31,36,37,43, 56,60,61,66).

Magnocellular neurons that contain neurophysin and OT and concentrate estradiol are presented in the hypothalamic PVN of female rats (46) and guinea pigs (65). Binding of estradiol also occurs in neurophysin-I/AVP-containing neurons of the PVN and SON of female mice (51). In female rats, estradiol increases the amount of OT mRNA in the lateral subcommisural nucleus (12), magnocellular neurons that project to the NHP, and parvocellular neurons that innervate several structures within the brain (38). In castrated male rats, estrogen receptors are localized in the AVP neurons in the bed nucleus of the stria terminalis and the medial amygdala (7). Binding sites for dihydrotestosterone are presented in the nuclei of pituicytes in the NHP of male guinea pigs (28) and baboons of both sexes (27). In the latter, uptake of the steroid by the NHP is greater in males than in females.

Sexual dimorphism in the quantities of AVT in the PNL and MT in the NHP in C chickens observed in this study is in agreement with earlier findings (48). The results of this study also demonstrate dimorphism in the content of MT in the AHA but not in the PHA. This is reasonable because the AHA is the main source of the peptides in the NHP. In canaries, sexual dimorphism in AVT immunostaining and AVT binding sites in the brain is believed to be the result of circulating androgen levels rather than an early differentiation process (62-64). Similarly, in the newt binding of AVT to the amygdala is reduced by gonadectomy (10). Thus, sexual dimorphism in the distribution of NHP peptides is found in mammals, birds, and reptiles and may be a common feature in vertebrates. This dimorphism does not appear to be the result of early events during embryogenesis, but rather is a response to the amount and types of gonadal steroids presented in circulation.

In adult chickens, however, sexual dimorphism in PNL AVT is not affected by orchidectomy or administration of estradiol or testosterone (48). In castrated roosters treated with either estradiol or testosterone, NHP MT levels are less than in intact controls but greater than in hens (48). Furthermore, the type of steroid administered in adult chickens has no effect on NHP MT. These observations indicate that in chickens early embryonic differentiation of the MT and AVT systems occurs and the results of the present study support this conclusion. In agreement with this suggestion, gonadectomy followed by testosterone administration does not abolish sexual dimorphism in AVP immunoreactivity in several limbic structures of mature Long-Evans rats (19).

In chickens, the AVT content of the PNL is greater in hens than in cockerels (48). The critical period for differentiation of male sexual behavior in this species occurs before the thirteenth day of embryonic development (67). The results of the present study demonstrate that administration of either antiestrogen (TAM) or antiandrogen (FLU) during the critical period of differentiation (tenth day of embryonic development) reduces PNL AVT in adult females to the level present in C males. These results suggest that TAM and FLU causes defeminization or masculinization in the hen's PNL AVT level.

According to the model suggested for differentiation of avian sexual behavior, the male is the neutral sex and differentiation occurs in the absence of gonadal steroids (1-3). Ovarian estrogen, produced in the female embryo, causes feminization and prevents masculinization. The effect of TAM on PNL AVT in females, but not in males, is in agreement with this model because blocking estrogen activity alters differentiation. However, FLU, which is considered a pure antiandrogen, causes similar effects in hens but has no effect in males. Furthermore, EB administration to male embryos does not cause significant demasculinization or feminization of PNL AVT levels.

Although we found no sex difference in AVT level in the NHP or AHA, some of the embryonic treatments affected AVT levels in these organs at adulthood. TAM and FLU increased AVT levels in the NHP of both sexes. FLU was more effective than TAM. In males only, EB and ATD also increased NHP AVT. Thus, sexual dimorphism is apparent in the responses to ATD and EB but not to TAM and FLU. In the AHA, the main source for NHP AVT, only administration of TAM to males increased AVT content. AVT levels in the NHP can be influenced by both the rate of synthesis and secretion of the hormone. Our data do not allow an assessment of either of these processes.

In agreement with previous observations (48), a clear sexual dimorphism was found in the NHP content of MT, males having twice the content of hormone compared with females. A similar dimorphism was also observed for MT in the AHA. Embryonic administration of both TAM and FLU in females increased the content of MT in the NHP to the level observed in C cockerels, which could be considered either masculinization or defeminization. However, these treatments also increased the MT content of the NHP in roosters so that TAM- and FLU-treated males had higher NHP MT than corresponding females. In males, but not in females, embryonic ATD increased NHP MT. Because ATD prevents the aromatization of androgens into estrogens, one interpretation might be that ATD causes hypermasculinization of NHP MT in males. EB treatment to male embryos, however, did not cause demasculinization or feminization of NHP MT content. TAM and FLU increased MT content of NHP in females but had no detectable effect on MT levels in the AHA. Because the AHA is the site of synthesis of AVT and MT whereas the NHP is the site of hormone accumulation, it is possible that moderate changes in rate of synthesis of hormone are not detected in the AHA because levels are relatively low in this organ compared with the NHP.

In roosters, TAM treatment caused an increase in AHA MT to a level significantly higher than in the EB-treated group although hormone levels in the NHP were similar in the two groups. In TAM-treated males, however, MT levels in the PHA were much higher than in EB-treated ones. In mammals, AVP and OT neurons from the AHA innervate both the neurohypophysis and posterior neural structures (6,44,59). Neurons of the avian PVN have similar projections (34,40,41). One possibility is that TAM treatment of male embryos changes the proportion of MT fibers or the transport of MT to the NHP and PHA. Androgenization of female rat pups results in masculinization of the OT response to stress in adulthood (14), suggesting that, as in chickens, the organizational

basis for sexual dimorphism in NHP peptides exists in both classes of vertebrates.

The effects of embryonic manipulations on sexual differentiation in the distributions of MT and AVT in chickens do not correlate with their effects on differentiation of sexual behavior. When manifestation of sexual activity is observed, all drugs tested in this study cause some degree of demasculinization in roosters but have no effect on feminization in hens (52-54). However, with respect to the distribution of AVT and MT some of the treatments caused defeminization or masculinization of the hen (e.g., PNL AVT and NHP MT). In cockerels, however, none of the treatments (including EB) caused demasculinization or feminization; some even caused hypermasculinization. Because EB did not demasculinize AVT and MT distribution in roosters, the differentiation of these peptides does not fit the model suggested for differentiation of sexual behavior in quails (1-3). Thus, in chickens the sexual differentiation of behavior and distribution of MT and AVT appear to be independent events although both are affected by gonadal steroids.

Embryonic administration of EB demasculinizes comb growth in male chicks, while embryonic treatment with testosterone masculinizes comb growth in females (52). Both steroids cause demasculinization of behavior in male chicks without affecting female sexual behavior (52). In broilers, embryonic treatment with TAM defeminizes female adiposity but has no effect on body fat in males (49,50). On the other hand, in this breed embryonic androgen sensitizes the male, but not the female, to the fat-reducing effect of testosterone during the stage of rapid growth (50). It would thus appear that the morphogenesis of each of the sexual dimorphic characteristics has its own mechanism of differentiation in responses to the steroidal milieu.

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