Identification of Androgen Metabolic Pathways in the Brain of Little Brown Bats (Myotis lucifugus): Sex and Seasonal Differences

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ABSTRACT

During the reproductive cycle of the little brown bat (Myotis lucifugus), the display of sexual behavior is out of synchrony with maximal gonadal development, suggesting that changes in plasma hormone levels per se are not the primary determinants of neural responsiveness. In the present study, we investigated pathways of androgen metabolism in the brain of the bat and compared brain aromatase activity in spring and in autumn. Tissue homogenates were incubated with [3H]androstanedione in the presence of an NADH/NADPH-generating system, and radiolabeled products isolated and their authenticity verified by recrystallization to constant specific activity. Aromatase was identified in preoptic/hypothalamic and adjacent limbic areas, and 5α-reductase was detected in all major brain divisions. No gender-based differences in brain aromatase were seen in spring just prior to emergence from hibernation, however, activity was greater in males than in females when measured during maximal breeding activity in autumn. In addition, seasonal changes were evident in the preoptic/hypothalamic regions of both sexes (spring > autumn), although estrone yields from limbic tissues remained constant. Thus, in seasonally breeding species, the rate at which circulating androgen is converted to estrogen or other biologically active metabolites in the brain itself may be an important determinant of behavioral or feedback responsiveness.

INTRODUCTION

It is generally recognized that sex steroids acting directly on target cells in the central nervous system (CNS) influence brain sex differentiation in early development and regulate sex behavior and gonadotropin secretion in adult animals (McEwen et al., 1979). For the expression of androgen action, the brain itself, through metabolism of circulating hormone, can play an active role in initiating or modulating responses (see Callard et al., 1978; McEwen et al., 1979). In male rats, for example, both estradiol and 5α-dihydrotestosterone (DHT) are required for the complete display of copulatory behavior, and there is good evidence that these active molecules are produced in situ from circulating testosterone (Baum et al., 1974; Christensen and Clemens, 1975; Lieberburg and McEwen, 1977). In females of several species, plasma androgen has been assigned roles in triggering or suppressing sexual receptivity and in regulating gonadotropin secretion (Beyer et al., 1970; Noble and Alsum, 1975; Perez-Palacio et al., 1975). As in males, it is likely that some of these actions are mediated by central conversions.

Aromatization and 5α-reduction are two pathways common to brain tissues of all vertebrates, including numerous laboratory and domestic mammals (for review, Callard et al., 1978, 1979, 1980, 1982). Although the Chiroptera have not been investigated, androgen conversions in the brain of the little brown bat (Myotis lucifugus) may be particularly interesting. In males of this species, the time of year when breeding behavior is maximal (September) is not synchronized with the peak of circulating androgen (August) (Gustafson and Shemesh, 1976). Moreover, high basal levels of plasma testosterone (5–10 ng/ml) are found throughout the year, although copulatory behavior is restricted to the autumn and periods of arousal.

Accepted February 3, 1983.
Received August 2, 1982.
1 This work was supported by NSF PCM78-23214 and HD-33066.
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during hibernation in winter. A parallel exists in female bats where there is a temporal disparity between the occurrence of receptivity (autumn) and final maturation of the follicle destined for ovulation (spring) (see Oxberry, 1979). Although neither estrogen nor androgen levels have been reported in female Myotis lucifugus, plasma estrogen does indeed peak at ovulation in the hibernating bat Antrozous pallidus (Oxberry, 1979). Paradoxically, estrogen levels are much lower during the time when the animals are receptive. One possible explanation for this unusual asynchrony between gonadal development and the display of sexual behavior is that changes in the activity of central pathways leading to estradiol or DHT, rather than changes in circulating hormone per se, are the primary determinants of seasonal changes in the responsiveness of the neural substrate. No seasonally breeding mammals have been studied, however, gender-based and seasonal changes in brain aromatase appear to be key to the breeding cycle in a teleost (Callard et al., 1981). The primary purpose of the studies reported here was to identify the aromatase and to determine the major metabolic pathways for androgen in the brain of male and female Myotis lucifugus. We also compared brain aromatization in wild-caught animals at two points in the annual cycle: in April prior to emergence from hibernation and at the onset of the active breeding season in September.

MATERIALS AND METHODS

Animals

For each experiment, 12 adult male and 12 adult female bats (Myotis lucifugus) were collected from hibernacula in upstate New York (Experiments 1 and 2, October and April, respectively) and Vermont (Experiment 3, September). These two sites are less than 50 miles from each other and individuals are known to engage in movements between them. Furthermore, since some of the bats which hibernate at these sites disperse to and from the same maternity (summer) roost, we regard our samples as being derived from the same gene pool. Animals were sacrificed by decapitation on the morning of capture or after maintenance at 4°C for no longer than 2 days.

Dissection

Using a binocular dissecting microscope and a procedure adapted from McEwen et al. (1969), the following brain regions were sampled: preoptic area and hypothalamus (POA/HTH), ventromedial cortex adjacent to the hypothalamus and including the amygdala and anterior hippocampus (LIM), frontal cerebral cortex (CC). In Experiments 1 and 2, one of each pair of gonads (GON) was taken for enzyme analysis. In Experiment 3, both gonads from each animal were assayed. For each determination, like tissues from 4 animals were pooled by sex and maintained on ice until homogenized (3 determinations per tissue type).

Incubation

The procedure used for incubation has been described elsewhere (Callard et al., 1977). Briefly, tissue homogenates (<180 mg wet wt. per tube) were incubated for 60 min at 37°C in 2 ml phosphate/sucrose buffer with [3H]-17β estradiol (206 nM final concentration, 11 or 15 Ci/mmol, Amersham), purified just before use by thin-layer chromatography, and an NADH/NADPH-generating system. In Experiment 1, a radioinert estrone "trap" (2 μg/ml) was present during incubation to reduce possible subsequent metabolism of formed [3H]estrone and to maximize yields for the purposes of identification. In Experiments 2 and 3, which were designed to make a quantitative comparison of estrogen accumulation in spring and autumn, the estrone "trap" was omitted; however, an antioxidant (dithiothreitol, 10 mM) was incorporated into the buffer as a precaution during the lengthy dissection and incubation procedure. These reagents have been tested in aromatizing systems previously (Schwarz et al., 1973; Reed and Ohno, 1976; Reddy et al., 1972). Neither altered estrogen yields when incubated alone or together in aliquots of the same brain forebrain pool (all = 2.37 fmol per mg tissue homogenate). Controls were tissue-free tubes containing substrate and cofactors and substrate-free incubates which received [3H]estrone (∅ 100,000 cpm) after incubation in order to monitor losses during analysis. Samples were stored frozen (-20°C) until assay.

Product Analysis

Radiolabeled steroids present in the final incubates were analyzed by methods described previously (Callard et al., 1979, 1980). In Experiment 1, ether extracts were chromatographed on thin-layer silica gel plates in ether/hexane (3:1), twice in one direction, followed by chloroform/methanol (97:3), once in a second dimension. Areas corresponding to estrone, estradiol, testosterone, DHT, and 5α-androstane-3,17-dione were eluted with methanol (90%) and radioactivity sampled. For verification of estrone and estradiol-17β, aliquots from like tissues were pooled, mixed with the appropriate radioinert steroid (20 mg), and crystallized repeatedly (no fewer than 3 times) until the specific activities of crystals and mother liquors were in agreement (∅ 5% of mean). For the androgens, representative samples from each tissue type or pooled replicates were recrystallized (Table 1). Product yields were corrected and procedural losses through elution (based on 63% recovery of estrone) and also individually adjusted for yields through the crystallization step (Table 1). Values were expressed as fmole per mg of tissue incubated. Radioactivity eluted from the androstenedione area of chromatoplates was not recrystallized, although procedural losses were taken into account (88% recovery in tissue-free controls). For each incubation, percentage of added substrate recovered in the androstenedione fraction or accounted for by the sum of the three metabolites.
ANDROGEN METABOLISM IN THE BRAIN OF RATS

was detected in cerebral cortex of either sex or in testicular homogenates. All tissues displayed 17β-oxidoreductase activity as measured by the production of testosterone. In brain samples, however, only 35–60% of the radioactivity in the testosterone fractions co-crystallized with testosterone. The remaining product in these fractions did not co-crystallize with testosterone-17α (epi-testosterone), and definitive identification of this contaminant was not achieved. One product of 5α-reduction (5α-androstane-3,17-dione) was identified in all brain areas, but not in gonadal homogenates of either sex. No radioactivity in these fractions co-crystallized with the corresponding 5β-reduced metabolites. Radioactivity corresponding to DHT in the ether/hexane system separated into three components having chromatographic mobilities similar to DHT, 5α-androstane-3β-hol,17-one, and 5α-androstane-3β-hol,17-one in benzene/ethanol (9:1), but these metabolites were not further identified or quantified in any sample.

Yields of the 3 identified metabolites (estrone, testosterone and 5α-androstane-3,17-dione) from different tissues of male and female rats, corrected for recovery through the crystallization step, are given in Table 2. At this time of year (October), differences in estrone yields were not significant when corresponding brain regions were compared in males and females; however, in males preoptic/hypothalamic tissues synthesized more estrogen than limbic tissues. High but variable amounts of

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Estrone</th>
<th>Testosterone</th>
<th>5α-Androstane-3,17-dione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoptic/hypothalamic</td>
<td>1–6</td>
<td>78</td>
<td>64/65</td>
</tr>
<tr>
<td>Limbic</td>
<td>7–12</td>
<td>88</td>
<td>95/99</td>
</tr>
<tr>
<td>Cortical</td>
<td>13–18</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Testis</td>
<td>19–21</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Ovary</td>
<td>22–24</td>
<td>99</td>
<td>512/534</td>
</tr>
</tbody>
</table>

No 17β, testosterone-17α, or 5β-androstane-3,17-dione was detected.

*No 17β, testosterone-17α, or 5β-androstane-3,17-dione was detected.

Percentage of eluted radioactivity found to be authentic after crystallization.

Specific activities of final crystals (C) and mother liquors (ML) in dpm per mg authentic steroid.

ND = Nondetectable.
TABLE 2. Product yields from \(^{3}H\)androstenedione in the brain and gonads of the bat (Myotis lucifugus): sex and regional differences (Experiment 1).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Sex</th>
<th>Pool no.</th>
<th>(mg) Tissue incubated</th>
<th>Estrone</th>
<th>Testosterone</th>
<th>5α-A-3,17-dione</th>
<th>Identified metabolites</th>
<th>Recovered substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoptic area/</td>
<td>Male</td>
<td>1-3</td>
<td>28.39 ± 2.24(^a)</td>
<td>0.412 ± 0.076(^bc)</td>
<td>25.910 ± 1.530(^b)</td>
<td>35.721 ± 3.880(^b)</td>
<td>0.33 ± 0.02</td>
<td>74.59 ± 0.60</td>
</tr>
<tr>
<td>hypothalamus</td>
<td>Female</td>
<td>4-6</td>
<td>26.36 ± 1.42(^a)</td>
<td>0.333 ± 0.015(^bc)</td>
<td>22.457 ± 2.627(^a)</td>
<td>20.381 ± 3.162(^a)</td>
<td>0.22 ± 0.03</td>
<td>79.99 ± 5.23</td>
</tr>
<tr>
<td>Limbic</td>
<td>Male</td>
<td>7-9</td>
<td>53.29 ± 2.53(^b)</td>
<td>0.214 ± 0.018(^bc)</td>
<td>18.408 ± 1.897(^ab)</td>
<td>11.084 ± 1.265(^a)</td>
<td>0.30 ± 0.02</td>
<td>81.65 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10-12</td>
<td>45.08 ± 4.51(^b)</td>
<td>0.282 ± 0.049(^bc)</td>
<td>15.373 ± 1.377(^a)</td>
<td>11.114 ± 1.443(^a)</td>
<td>0.23 ± 0.02</td>
<td>85.73 ± 1.16</td>
</tr>
<tr>
<td>Cortex</td>
<td>Male</td>
<td>13-15</td>
<td>57.37 ± 6.39(^b)</td>
<td>ND(^c)</td>
<td>24.105 ± 0.715(^b)</td>
<td>19.172 ± 3.844(^a)</td>
<td>0.46 ± 0.02</td>
<td>86.17 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>16-18</td>
<td>51.26 ± 5.63(^b)</td>
<td>ND(^c)</td>
<td>21.862 ± 1.786(^ab)</td>
<td>16.655 ± 1.143(^a)</td>
<td>0.37 ± 0.05</td>
<td>86.61 ± 1.75</td>
</tr>
<tr>
<td>Gonads</td>
<td>Male</td>
<td>19-21</td>
<td>22.11 ± 1.76(^a)</td>
<td>ND(^c)</td>
<td>19,420 ± 1282.350(^c)</td>
<td>ND(^c)</td>
<td>79.89 ± 2.43</td>
<td>10.61 ± 3.96</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>22-24</td>
<td>1.95 ± 0.36(^c)</td>
<td>66.002 ± 50.431(^d)</td>
<td>407.949 ± 40.787(^d)</td>
<td>ND(^c)</td>
<td>0.19 ± 0.07</td>
<td>96.10 ± 0.89</td>
</tr>
</tbody>
</table>

\(^a-d\) For each product measured, means that bear different superscript letters are significantly different (P<0.05).

\(^c\) ND=not detectable.
FIG. 1. Sex and seasonal differences in brain and gonadal aromatase activity in the little brown bat (Myotis lucifugus). Adult males and females were collected from hibernacula in April prior to emergence from hibernation and in September during maximal mating activity. Combined preoptic area/hypothalamus (POA/HTH), amygdala and overlying cortex (LIM) and gonads (GON) were assayed for aromatase. No estrogen was synthesized in cerebral cortex or testis. Values are based on 3 determinations, each comprised of pooled tissues from 4 animals. Significant differences shown are *P < 0.05. **P < 0.01. In addition, sex differences were highly significant (P < 0.01) in September in both POA/HTH and in LIM.

Estimated estrogen were produced in the three ovarian pools.

Compared to the products of aromatization, C19 metabolites of [3H] androstenedione were found in 50- to 100-fold greater amounts, regardless of tissue type incubated. With one exception, there were no significant sex or regional differences in yields of 5α-androstane-3,17-dione or testosterone in brain incubates (Table 2). In the preoptic/hypothalamic area of males, however, the quantity of 5α-androstane-3,17-dione exceeded that in the same region of the female brain and was also greater than that formed in limbic and cerebral cortical samples of males. Conversion of androstenedione to testosterone was extensive in the testis, and smaller amounts of authentic testosterone were formed by ovarian samples.

In the testis, androstenedione utilization was nearly complete, but in all other incubates the majority of the added radioactivity could be recovered as unmetabolized substrate. This does not exclude the possibility that other metabolites were formed, since a fraction of the [3H] androstenedione added per incubation was unaccounted for.

Experiments 2 (April) and 3 (September)

Because identification of 5α-reduced products was known to be incomplete (see above), measurements in these experiments were restricted to estrone (Fig. 1). In April, male-female differences were not significant when estrone synthesis in corresponding brain regions was compared; however, in September, males had greater aromatase activity than females, and this was true for both preoptic/hypothalamic and limbic areas. In addition, when animals of each sex were compared by season, differences in estrone yields were evident in the preoptic area/hypothalamus (April > September), although yields from limbic tissues were not different at the same time points. This seasonal pattern was seen in both males and females. Neuroanatomic differences were not significant in female brain at any time or in male brain in September; however, in April male preoptic/hypothalamic samples produced more estrone than adjacent limbic tissues.

Mean ovarian estrone yields were much higher in April than in September; however, because of extreme variability among the three pooled samples in April, a statistical comparison indicated differences were not significant.

At no time did cerebral cortex (not shown) or testicular homogenates synthesize measurable estrone.

DISCUSSION

As in numerous other vertebrates, aromatase, 5α-reductase, and 17β-oxidoreductase activities are present in the brain of the little brown bat Myotis lucifugus. Although aromatase is restricted to the preoptic area/hypothalamus and adjacent limbic areas, 5α-reductase and 17β-oxidoreductase are present in all major brain divisions. No evidence was obtained for 5β-reduction in the bat CNS, although this pathway is characteristic of other seasonal breeders, for example, hamsters (Callard et al., 1979) and birds (Nakamura and Tanabe, 1975).

Aromatase activity in the brain of the little brown bat is in the same range as that reported for rats and rabbits (Reddy et al., 1972, 1974), although somewhat lower than values measured...
in hamsters using similar methodology (Callard et al., 1979). In common with other species, \(5\alpha\)-reduced metabolites in bats are formed in much greater amounts than estrogen products. Indeed, since \(5\alpha\)-androstan-3,17-dione was the only \(5\alpha\)-reduced metabolite fully identified and quantified despite presumptive evidence for other, \(5\alpha\)-reduced products, it is likely that the activity of the \(5\alpha\)-reducing pathway was underestimated. The present study confirms earlier reports of gender-based differences in brain estrogen synthesis in common laboratory mammals (male > female) (Reddy et al., 1972, 1974) and further demonstrates that sex differences are seen only at certain times in the annual cycle of wild-taken animals. In 3 experiments in which males and females were assayed simultaneously, sex differences were significant only in September but, at this time, were seen in both preoptic/hypothalamic and limbic tissues (males > females). Moreover, when tissues of each sex were assayed by the same methods at two points in the annual cycle, a seasonal pattern was apparent, and it was the same for both males and females: aromatase in combined preoptic/hypothalamic fragments was higher in spring prior to emergence from hibernation than in autumn when breeding activity was maximal. At the same time points, values for limbic aromatase remained constant. Despite differences in assay methods and possible environmental influences other than photoperiod in these wild-taken animals, values in Experiment 1 (October) and 3 (September) more closely resembled each other than those obtained in Experiment 2 (April). It may be relevant to note here that blinding reduces aromatase activity in both male and female hamsters (Callard et al., 1979). Furthermore, in male hamsters exposed to short days, hypothalamic aromatase is lower than in long-day controls, an effect of photoperiod that is independent of the gonads (Callard and Solomon, 1979). Hence, the onset of shorter days in the autumn might account for observed decreases in enzyme activity in the bat hypothalamus. Whether these seasonal changes in aromatizing potential of the brain have any relevance for reproduction or sex behavior is purely conjectural at this point. Moreover, no single interpretation of these results will suffice for both species, since hamsters are long-day breeders, whereas little brown bats mate principally in autumn (Thomas et al., 1979). It is known, however, that in hamsters short days are associated with decreased behavioral responsiveness to androgen but increased sensitivity to the negative feedback effects of the same hormone (Ellis and Turek, 1979; Campbell et al., 1978). Experimental data on bats are sparse, but in field studies of Antrozous pallidus it was observed that females, even when artificially aroused during hibernation, tended to ovulate spontaneously only toward the end of the winter period (Oxberry, 1979). Based on pituitary cytology, it was suggested that, despite the presence of a mature Graafian follicle throughout hibernation, luteinizing hormone (LH) or some LH-releasing signal is insufficient until spring (Oxberry, 1979). Since estrogen is an effective agent for induction of ovulation in bats (Racey, 1976), it is possible that changes in estrogen biosynthesis within the brain are responsible for seasonality of this parameter.

On the other hand, the ovaries of bats are also active with respect to estrogen synthesis. Variability in yields from the different pooled samples in Experiments 1 and 2 deserves comment. In these two experiments, only one of each pair of gonads was sampled for biochemical analysis. Since in Myotis lucifugus a single follicle is designated for ovulation each year, there is a marked asymmetry in ovarian development (Wimsatt, 1979). Presumably, an unequal number of “active” versus “inactive” ovaries were assigned to different samples in these two experiments. Nevertheless, mean estrogen yields were highest just prior to ovulation (April). This is the same time in the cycle when circulating estradiol peaks in Antrozous pallidus (Oxberry, 1979).

Despite our understanding of the seasonal pattern of reproduction in Myotis lucifugus, there is too little information about its basic neuroendocrinology to draw any firm conclusions regarding the physiological relevance of the data reported here. Furthermore, its small size, which precludes the possibility of further subdividing the brain and necessitates the pooling of tissues, reduces the useful information which can be obtained from any one experiment. The data reported here and in a previous study in a teleost (Callard et al., 1981), open the possibility however, that changes in brain aromatase or other androgen-metabolizing enzymes have a key role in seasonal patterns of CNS responsiveness.
REFERENCES


