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Sex- and hormone-dependent antigen immunoreactivity in developing rat hypothalamus

(sexual differentiation/sexual dimorphism/radial glia/androgen/asymmetry)

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ABSTRACT **Morphological sex differences in adults can result from differential gonadal steroid exposure during critical perinatal periods. This study describes the use of a monoclonal antibody we have developed to study mechanisms of sexual differentiation of brain structure and function. Used as a marker in immunocytochemistry, antibody AB-2 revealed subsets of cells, including radial glia, transiently during the perinatal period. Peak reactivity in radial glia was on embryonic day 19 in males and on postnatal day 1 in females. On postnatal day 1, AB-2 immunoreactivity in radial glia was 2-fold greater in females than in males. Greater activity was detected in males on one side of the brain than the other (2- to 4-fold, depending on the region). To test the hormone dependence of this sex difference, pregnant rats were injected with testosterone propionate to expose fetal females to androgen on embryonic day 18. This resulted in lower levels of AB-2 immunoreactivity in radial glia of the treated female offspring on postnatal day 1 relative to control females, and the pattern was bilaterally asymmetric, approaching that of males. Thus the difference between sexes in immunoreactivity with AB-2 as a marker was hormone dependent in a predictable manner. Whether this marker is revealing a sex difference in accessibility of antigen by immunocytochemistry or a sex difference in intrinsic antigen levels is not yet resolved. In either case these results support the hypothesis that certain hormone-dependent molecular events occur transiently during development.**

An increasing number of experiments have indicated sex differences in neural morphology discernible in adult brains in virtually all species examined (reviewed in refs. 1 and 2). In many cases, these sex differences in adult morphological characteristics arise as a result of sex differences in gonadal steroid exposure during critical perinatal periods (e.g., refs. 3–5). However, the molecular mechanisms by which gonadal steroids induce sex-specific differences in the developing brain have yet to be elucidated (6–8).

To identify and characterize developmentally important molecules during the critical periods of sexual differentiation, we are generating monoclonal antibodies against homogenates of neonatal rat hypothalamus. We are using these markers to test several hypotheses about the mechanisms of sexual differentiation of brain structure and function. One of these is that specific hormone-dependent molecular events occur transiently early in development with long-term sex-selective consequences. Antibody AB-2 (9) used in immunocytochemistry (ICC) reveals reactive cellular elements throughout the perinatal period. The antigen(s) recognized is localized in at least one cell type (radial glia) that is known to exist transiently during development (10). Therefore, we have focused attention on the time course of expression of AB-2 antigens detected by this monoclonal antibody, comparing the temporal pattern of immunocytochemical expression in males and females.

Significant sex differences in endocrine factors such as plasma testosterone concentrations (11) and brain aromatase activity (12, 13) are prominent at specific times during development. In this study we characterized the sex differences in the expression of AB-2 immunoreactivity in the hypothalamus and tested its hormone dependence. AB-2-immunoreactive radial glia were examined after exposing genetic females to testosterone on embryonic day 18 (E18), a time when males and females normally are exposed to different levels of androgen (11).

MATERIALS AND METHODS

Monoclonal Antibody AB-2. Recipient BALB/c mice (60-day-old females; Charles River Breeding Laboratories, Wilmington, MA) were injected first with homogenates of hypothalamus and temporal cortex from postnatal day 4 (P4) female Sprague–Dawley rats (Charles River Breeding Laboratories). To increase the probability of an immune response selective for antigens differing quantitatively or qualitatively in males and females, these mice were also administered the immunosuppressant cyclophosphamide [100 mg/kg of body weight; 2 mg/0.5 ml of saline (14)] 0, 24, and 48 hr after the injection of homogenate from female donors. Two weeks later these mice were immunized with homogenate from P4 male rat hypothalamus and temporal cortex. After repeating this entire 2-week sequence three times, hybridomas were produced and subcloned according to standard procedures (15) using the stimulated spleen cells and cultured NS-1 myeloma cells. Monoclonal antibodies were screened by ICC using paired cryostat sections (20 μm, sagittal) from nonperfused P4 male and female rat brains. After initial selection on the basis of presence of reaction product, the AB-2 antibody was characterized further by ICC in 50-μm cryostat sections of perfusion-fixed brains from E19 to adulthood. Hybridoma supernatants of monoclonal antibody AB-2 were precipitated with ammonium sulfate and dialyzed against phosphate buffer (0.05 M, pH 7.4)/saline (PBS) to facilitate storage and homogeneity of antibody aliquots. The precipitated antibody stock was used for ICC at a concentration (1:30) that was similar to the original supernatant. This concentration provided excess antibody, since immunoreactivity did not decline until concentrations were >1:50. A goat anti-mouse IgM was used as a secondary antibody for AB-2 since, in immunocytochemical screening, goat anti-mouse IgG secondary antibodies were ineffective.

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Abbreviations: E, embryonic day; P, postnatal day; TP, testosterone propionate; ICC, immunocytochemistry.

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Rats. Timed-bred Sprague–Dawley females were purchased from Charles River Breeding Laboratories and delivered to the Shriver Center on day 16 of gestation. The day of breeding was designated E0 and the day of birth was designated P0 (P0 = E22). The ages examined included E19, E21, P1, P4, P7, P10, P13, and adult. To obtain animals at embryonic ages, pregnant rats were anesthetized with ketamine (Vetalar; 80 mg/kg) and xylazine (Rompun; 8 mg/kg), the gravid uterus was excised, and the fetuses (E19 or E21) were placed on ice for cryoanesthesia. Ice anesthesia was also used for P1 pups. At older ages, ketamine and xylazine were injected intramuscularly. Perfusions within a given day were always alternated between males and females, and all subsequent processing occurred with males and females paired to ensure equivalent procedures for both sexes. When hormone-treated animals were sacrificed the same care for employing identical procedures for tissue handling for all groups (experimental and control) was taken.

Animals were perfused intraaortically under controlled, positive pressure [80 psi for the P4 and younger, 100 psi for older (1 psi = 6.89 kPa)] with warmed PBS (≈30°C) containing 3000–6000 units of heparin followed by 30–200 ml (depending on age) of phosphate buffer (0.1 M, pH 7.4/4% paraformaldehyde (degassed under vacuum and cooled to 4°C). Brains were removed and postfixed in the same fixative for 2 hr and then were transferred to 0.1 M phosphate buffer/2% paraformaldehyde/12.5% sucrose for overnight incubation prior to placement in 0.1 M phosphate buffer/25% sucrose. Fixation and storage procedures following brain removal were done at 4°C. Paired male and female samples were stored a maximum of 14 days during which staining intensity and distribution were stable under the conditions in the present study.

For prenatal treatments, timed-pregnant Sprague–Dawley females were obtained from Charles River Breeding Laboratories on day 16 of gestation and were injected intramuscularly with 4 mg of testosterone propionate (TP) in 0.05 ml of peanut oil as vehicle or peanut oil vehicle alone on the morning of E18. This dose was previously shown to be effective in masculinizing the volume of the sexually dimorphic nucleus of the preoptic area of rats (16). Animals were perfused as outlined above either on E21, P1, or P4. Since TP treatment caused a 1-day delay in parturition in 40% of the mothers injected, only pups that were born on E22 were used the following day for P1.

ICC. Brains from rats sacrificed at perinatal ages were compared in immunocytochemical experiments examining AB-2 immunoreactivity. To control for potential variations in immunoreactivity among experiments, brains of animals sacrificed at different ages were cut in individual experiments. Brain sections were cut in a cryostat at a thickness of 50 μm. To allow comparable analysis with separate antibodies for a given brain, sections were distributed sequentially into the required number of tissue holders [mesh-bottom boats (17)] in an iterative fashion. Antibodies used included other monoclonal antibodies that we have generated [e.g., 3D10 (18)], anti-microtubule-associated protein 2 [IgG monoclonal I2, and a rabbit serum antibody; gifts from I. Fischer (19)], anti-transferrin (rabbit serum antibody; Boehringer Mannheim), and antiglial filamentous protein [rabbit serum antibody R-62; gift from D. Dahl (20)]. Additional immunocytochemical controls consisted of sections exposed to hybridoma supernatants or alternate antibody dilution buffers in place of primary or secondary antibodies. Sections were placed in PBS (4°C) after they were cut, washed in PBS three times, and washed once in PBS with either 0.1% gelatin or 1.0% bovine serum albumin (fraction V). Primary antibodies were diluted in PBS with 0.1% gelatin. Incubations with primary antibody were overnight (18 hr) at 4°C; in one experiment incubation was 40 hr with no enhancement of staining. After washing four times in 1 hr with PBS containing 1.0% normal goat serum, sections were exposed for 2 hr to the appropriate affinity-purified horseradish peroxidase-coupled goat antiimmunoglobulin (Boehringer Mannheim). After an additional four washes in 1 hr in PBS, sections were exposed to 0.05% 3,3′-diaminobenzidine (DAB) in PBS with 0.02% hydrogen peroxide. AB-2 immunoreactivity, revealed as brown DAB reaction product, was evaluated microscopically. In some experiments, the Vectastain ABC technique [(21) Vector Laboratories, Burlingame, CA] was used with the same result.

Analysis. Cellular immunoreactivity was analyzed by using bright-field microscopy, and immunoreactivity in radial processes was analyzed by using bright-field and dark-field microscopy. Coronal sections spanning the hypothalamus and preoptic area were examined at 6 atlas levels of Paxinos and Watson (22), 500 μm apart. Atlas figures 15–20 were assigned as levels 1–6, respectively. In the perinatal brains examined, the corresponding levels were 300–400 μm apart depending on age. To ensure evaluation of a minimum of three or four sections per antibody at each rostral-caudal level, sections from the brains of animals younger than P7 were usually allowed to react with only two antibodies per experiment.

Slides were coded to preclude *a priori* determination of either sex or age. In evaluating radial glia at each rostral to caudal level, three equally spaced ventral to dorsal divisions were rated using a four-point scale following Levitt and Rakic (10) with 0 = none evident, 1 = sparse, 2 = moderate, 3 = dense, and 4 = very dense. For animals in some experiments a hole was put into the right striatum prior to sectioning so that the brain side of the sections examined was determinable later. Regional landmarks in sections from each individual animal allowed determination of side when the absolute left/right side was not available. To estimate total radial glial densities, the ratings from all six regions (3 dorsal/ventral × 2 left/right) were summed for each animal (maximum score is therefore 3 × 2 × 4 = 24). To estimate the degree of asymmetry, the ratings from each side of the brain were summed at each level and the ratio of high side rating to low side rating was calculated for each animal. A section symmetrical for AB-2 immunoreactivity would have a ratio of 1.

RESULTS

AB-2 immunoreactivity was revealed by the presence of reaction product in several morphological elements, including both neuronal and glial cell types. The extent and intensity of immunoreactivity and the regional distribution were dependent on age. One prominent type of immunopositive element observed was radial processes. These were evident at the earliest age studied (E19) and extended unbranched from the wall of the third ventricle of the pial surface at the base of the brain (Fig. 1), where conical endfeet were observed. In general, the largest number of radial processes in the hypothalamus were apparent caudal to the optic chiasm, with a consistently large number throughout the length of the bilateral arcuate nucleus in the ventral region of the third ventricle. Immunopositive radial processes were also noted in other brain regions, including the septum, striatum, and regions of cortex. From E19 to P4 these cells/processes conformed to criteria for radial glial cells (23–25). Beginning at P4, and into the second postnatal week, AB-2-immunoreactive radial processes in the hypothalamus traversed shorter distances and were more restricted in their distribution, primarily in caudal regions in the ventral half of the third ventricle around the median eminence. These processes in older animals appeared less as radial glia and more as a subset of the class of cells commonly called tanycytes (25).

A second type of immunoreactive element observed was neuronal and/or glial cell bodies. The greatest number of cell

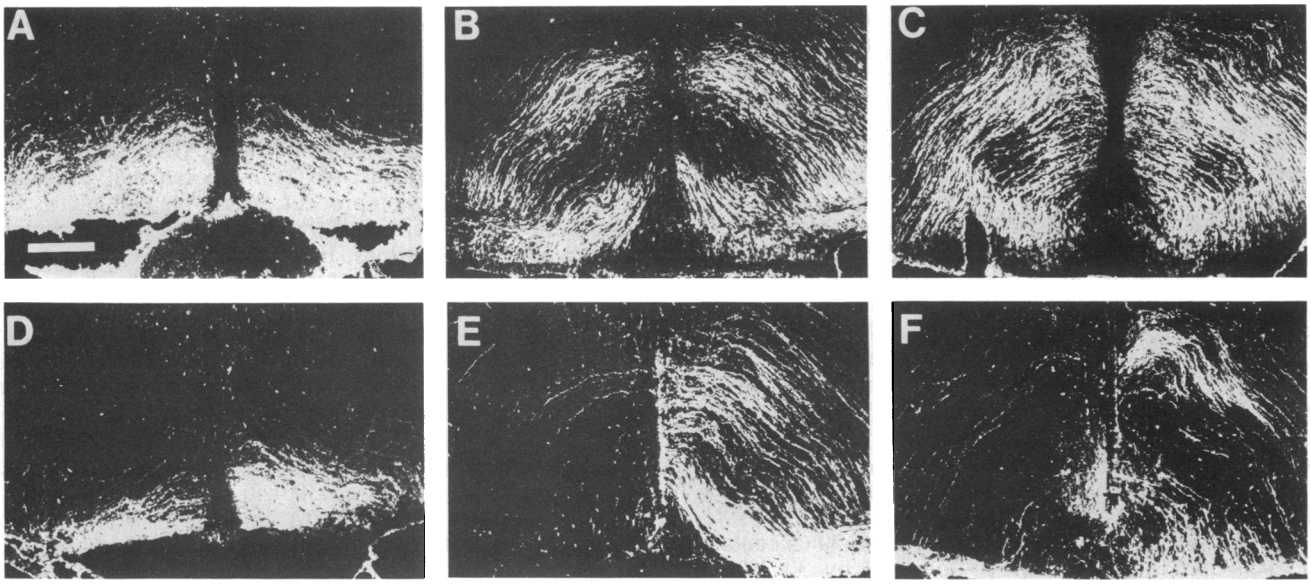

FIG. 1. Dark-field photomicrographs of coronal sections show AB-2 immunoreactivity in radial glia on P1 in the preoptic area (*A* and *D*; level 1 in subsequent graphs), preoptic area/anterior hypothalamic area (*B* and *E*; level 3 in subsequent graphs), and area around the rostral median eminence/ventromedial nucleus of the hypothalamus (*C* and *F*; level 5 in subsequent graphs). *A–C* are from a female and *D–F* are from a male. (Bar in *A* = 200 μm.)

bodies were observed on P4 in the preoptic area and hypothalamus. These immunopositive cells were on both sides of the ventral half of the third ventricle, from the rostral preoptic area to the level of the arcuate nuclei in the medial basal hypothalamus. More caudally, in the ventromedial nucleus, the region of highest cellular immunoreactivity extended beyond the ventrolateral border of the nucleus (not shown).

Within specific regions of the preoptic area and hypothalamus, the age-dependent detectability of radial glia with the AB-2 monoclonal antibody differed between males and females. Among males the maximal AB-2 immunoreactivity in radial glia was apparent on E19, whereas in females the maximal immunoreactivity was apparent on P1 (Fig. 2). The largest sex differences were apparent in regions spanning the caudal preoptic area to the rostral median eminence. This is just caudal to the sexually dimorphic nucleus of the preoptic area (26) and coincident with the rostral portion of the ventromedial nucleus of the hypothalamus. On P1, at these levels, significantly more processes were apparent in females than males (Figs. 1 and 3).

A striking feature of the sex difference observed on P1 was the frequency and extent of asymmetrical AB-2 immunoreactivity. Asymmetrical patterns of immunoreactivity were more frequent and extensive in males than females (Figs. 1 and 4). The asymmetry was restricted primarily to sections that lie caudal to the midpoint of the optic chiasm, running caudally as far as the rostral arcuate nucleus/median eminence region. The asymmetry was most apparent at middle and dorsal levels of the third ventricle rather than ventral locations, although the asymmetry was apparent at all ventral-dorsal levels in the region of the caudal optic chiasm. In instances where the side of the brain was positively identified

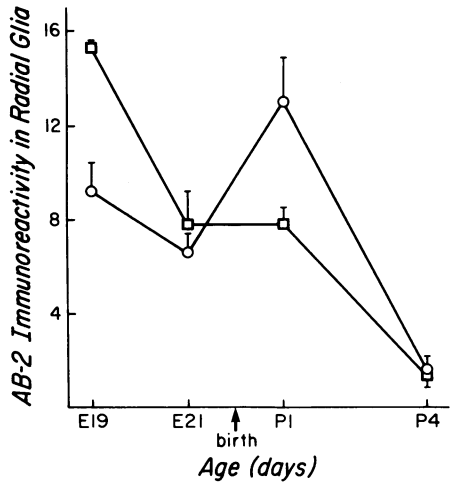

FIG. 2. Ratings of AB-2 immunoreactivity in radial glia from E19 to P4 in females (○) and males (□). Data are from the preoptic/anterior hypothalamic area and are computed from levels 3 and 4 combined for each animal. Values are mean ± SEM. The maximum score possible per datum point is 24. The number of animals per point is 2 males and 2 females at E19, 4 females and 3 males at E21, 8 females and 8 males at P1, and 10 females and 13 males at P4.

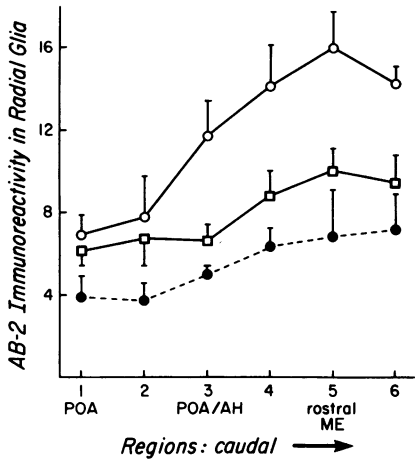

FIG. 3. Ratings of AB-2 immunoreactivity in radial glia on P1 in females (○, *n* = 8), males (□, *n* = 8), and females exposed to exogenous androgen on E18 (●, *n* = 4). Brain regions are contiguous from rostral level 1 to caudal level 6. Values are mean ± SEM. The maximum score possible per datum point is 24. POA, preoptic area; AH, anterior hypothalamic area; ME, median eminence.

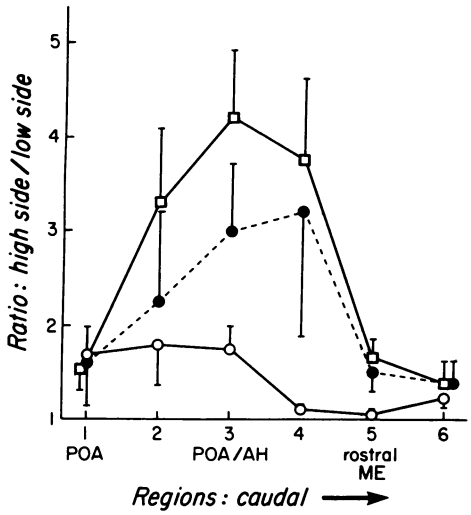

FIG. 4. Asymmetry of AB-2 immunoreactivity in radial glia in the hypothalamus on P1 in females (○, n = 8), males (□, n = 8), and females exposed to exogenous androgen on E18 (●, n = 4). Brain regions are contiguous from rostral level 1 to caudal level 6. Values are mean ± SEM. POA, preoptic area; AH, anterior hypothalamic area; ME, median eminence.

as left or right, higher densities of AB-2-immunoreactive radial glia were almost always noted on the right side of the brain (8/9 on P1; 4/4 on E21). This was also true in the few females that were asymmetric on either E21 or P1.

The asymmetric immunoreactivity seen with AB-2 in radial glia was not seen in features revealed by other antibodies. Antibody 3D10 served as one control for the secondary antibody, since no elements were immunopositive in the E19, E21, and P1 hypothalamus, the same as obtained either without primary or without secondary antibodies. However, putative tanycytes were immunopositive using 3D10 in the hypothalamus at P4 and older. Under the conditions used, glial filamentous protein immunoreactivity was restricted to a subset of radial glia in the ventral hypothalamus of male and female rats, and these were generally symmetric in both sexes. Antibodies directed against microtubule-associated protein 2 or transferrin revealed perikaryal immunoreactivity in the hypothalamus at perinatal ages and did not appear sex-dependent. Experiments with other antibodies that also recognize subsets of radial glia are necessary to determine the relationship of AB-2-immunoreactive radial glia to the total population of radial glia in the diencephalon.

Effect of Prenatal Androgen Treatment. Treatment of females with TP on E18 lowered ratings of AB-2 immunoreactivity in radial glia on P1 relative to control females (Figs. 3 and 5). Furthermore, the immunoreactive pattern was asymmetric in restricted rostral-caudal planes, approaching the male pattern (Figs. 4 and 5). In contrast, when similarly TP injected females were examined at E21, the number of AB-2-immunoreactive radial glia cells was not significantly different from that of control females or males (not shown).

DISCUSSION

Experiments performed with antibody AB-2 allow us to begin examining the timing of molecular changes during the critical period of sexual differentiation of the brain. We are testing the hypothesis that events leading to the maturation of certain sexual dimorphisms in adults involve sex-dependent expression, under gonadal hormone control, of a transient class of molecules during specific developmental periods. The first step in this approach is to determine if sex differences occur

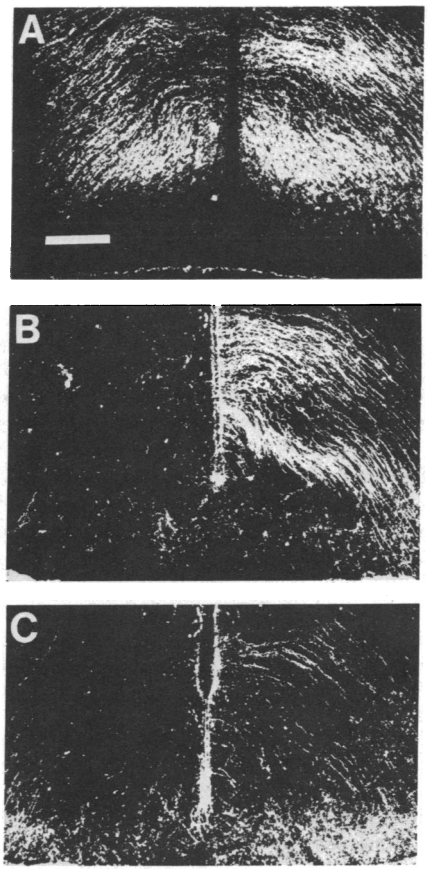

FIG. 5. Dark-field photomicrographs of coronal sections show AB-2 immunoreactivity in radial glia on P1 in a caudal aspect of the anterior hypothalamic area (level 4 from previous graphs) of a female (*A*), male (*B*), and female exposed to exogenous androgen on E18 (*C*). (Bar in *A* = 200 μm.)

during the critical period in the expression of transient markers.

The perinatal period is one of rapid and extensive change for AB-2 immunoreactivity in cells and processes in the rat hypothalamus. Ages of maximal immunoreactivity were earlier for radial glia processes (E19–P1) than for cell bodies (P4). For radial processes the peak period of AB-2 immunoreactivity was earlier for males (E19) than females (P1). Thus, AB-2 immunoreactivity provides a sex-dependent marker for the timing during development of the expression of antigen(s) contained in specific elements in the developing hypothalamus.

The results of the present study indicate that the timing of certain developmental events differs between males and females. We also have shown that prenatal treatment with androgen can account for some aspects of these sex differences in development. Thus, in a specific portion of the hypothalamus an event such as the decrease of a particular marker occurs earlier in males than in females. Sex differences in the time course of specific molecular events may alter the potential for subsequent developmental steps (27) and in a cascading fashion result in stable sexual dimorphisms (28). If even a single early event is under hormonal control, sexual dimorphisms can result.

The immunocytochemical distribution obtained with a given antibody depends upon a number of factors, including the actual amount of antigen (combination of steady-state levels and turnover), accessibility of epitope/antigen (i.e., in a cryptic state in a membrane), and stability of antigen following fixation (both antigenicity and physical retention). The patterns of AB-2 immunoreactivity in the developing

hypothalamus reflect these factors. In addition, a recent report indicates that the penetration of intracardially injected fluorescein into midline brain structures is sex-dependent (29). The undetermined mechanism(s) for this difference also may affect other measures such as those reported here. We have yet to determine whether the amount of AB-2 antigen differs by sex and brain side or whether the accessibility or stability of antigen differs by sex and brain side.

An unexpected result in the present study is the large degree of asymmetry in AB-2 immunoreactivity. Transient morphological asymmetries have been described in other brain regions, including parts of rat cortex (30, 31) and chick medial habenula (32). As in our study, in the case of the medial habenula the greater degree of asymmetry in male chicks was also demonstrated in developing females that received exogenous testosterone (32). Asymmetric functional responses to exogenous androgen have also been reported. Unilateral implantation of gonadal steroids in the hypothalamus and preoptic area of neonatal (33) and adult (34) rats caused differential behavioral effects, dependent on the brain side of the implant. The results in our study may indicate an anatomical and biochemical locus for the generation of functional differences.

Two separate models could account for the observed asymmetry in immunoreactivity. (*i*) The two sides of the hypothalamus might be developing at different rates (33, 35). They might undergo qualitatively similar changes but with different temporal patterns. (*ii*) The two sides might be intrinsically different if one side contains elements not present in the other.

The region of hypothalamus in which AB-2 immunoreactivity is sex-dependent becomes sexually dimorphic in several ways by adulthood. AB-2 immunoreactivity is seen in radial glia during the period when neuronal migration occurs in the hypothalamus/preoptic area (36, 37). Hormonally influenced radial glia could play a developmental role (38) by directing the migration of neurons or their processes (23, 39). Sex dependence has been noted in the cell density of the anterior hypothalamic area (40) and in the volume of the bed nucleus of the stria terminalis [BST (41, 42)] and the ventromedial nucleus of the hypothalamus (43). Sex differences in connectivity of cells in the hypothalamus or BST have been suggested by electrophysiological studies (44), Golgi studies (45, 46), and the examination of other fiber systems for known neurotransmitters [e.g., serotonin (47), substance P (48)].

It has been suggested that certain developmental markers appear earlier in human females than in males because of growth-suppressive effects of testosterone (49). Though the results in our study of rats do not test this hypothesis, it raises some additional considerations. Androgens in normal male fetuses could alter rates of postnatal development either by accelerating or retarding particular embryonic events. For example, a hormonal response early in development may alter the molecular and cellular substrate for potential genetic and experimental effects later in development. Retardation at the later ages could be caused by lower numbers of available targets as a result of early hormone exposure. These targets could be less available either because an earlier event ceased more quickly in response to hormone or because an earlier event was delayed, thus leading to later maturation of the target. In extrapolating from knowledge of putative sex differences in cortical function (50), accurate predictions about hormonal influences will benefit from further definition of the early molecular events in sexual differentiation.

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