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Alpha-fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens

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Two clearly opposing views exist on the function of alpha-fetoprotein (AFP), a fetal plasma protein that binds estrogens with high affinity, in the sexual differentiation of the rodent brain. AFP has been proposed to either prevent the entry of estrogens or to actively transport estrogens into the developing female brain. The availability of *Afp* mutant mice (*Afp*$^{-/-}$) now finally allows us to resolve this longstanding controversy concerning the role of AFP in brain sexual differentiation, and thus to determine whether prenatal estrogens contribute to the development of the female brain. Here we show that the brain and behavior of female *Afp*$^{-/-}$ mice were masculinized and defeminized. However, when estrogen production was blocked by embryonic treatment with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione, the feminine phenotype of these mice was rescued. These results clearly demonstrate that prenatal estrogens masculinize and defeminize the brain and that AFP protects the female brain from these effects of estrogens.

Alpha-fetoprotein (AFP) is a plasma glycoprotein produced in great quantities during fetal life by the endodermal cells of the visceral yolk sac, by the hepatocytes and, in lesser amounts, by the gastrointestinal tract[1,2]. The synthesis of AFP decreases markedly soon after birth and only trace amounts are detected in adults[1]. At present, the physiological function of AFP during embryonic development remains largely unidentified. The observation that AFP is able to bind estrogens with high affinity in rats and mice has led to the suggestion that AFP may have a role in brain sexual differentiation[3–5]. However, two clearly opposing views exist on the role of AFP during brain sexual differentiation and thus on how the presence or absence of estrogens may induce the brain to develop as male or female[4,5]. The classic view holds that sex differences in the brain and behavior develop under the influence of estrogens derived from the neural aromatization of testosterone: the brain develops as male in the presence of estrogens and as female in their absence[6–8]. In agreement with this view, it has been proposed that the female brain needs to be protected from estrogens produced by the placenta and that AFP is the most likely candidate to achieve this protection because of its estrogen-binding capacity[4].

However, the idea that the female brain develops in the absence of estrogens and the role of AFP in protecting the brain against the differentiating action of estrogens have been challenged. First, the normal development of the female brain might actually require the presence of estrogens[5,9–11]. Second, the presence of AFP within neurons in the absence of any evidence for local AFP synthesis suggests that AFP is transported from the periphery into the brain[12,13]. It has thus been proposed that AFP acts as a carrier, which actively transports estrogens into target brain cells and, by doing so, has an active role in the development of the female brain[5].

At present, these two clearly opposing views on the function of AFP in brain sexual differentiation have not been experimentally tested owing to the absence of a suitable animal model and technical difficulties associated with measuring brain estrogen concentrations. The recent introduction of an *Afp* mutant (*Afp*$^{-/-}$) mouse model[14] has now made it possible to test these hypotheses and thus to determine the function of AFP in brain sexual differentiation. Male *Afp*$^{-/-}$ mice are fertile, but female *Afp*$^{-/-}$ mice are not, owing to a complete absence of ovulation[14]. Ovarian transplantations clearly demonstrate that anovulation in *Afp*$^{-/-}$ females is caused by an extragonadal factor probably localized at the level of the hypothalamus. Here we assessed whether the sexual differentiation of the brain and behavior is affected in female *Afp*$^{-/-}$ mice and whether a normal female phenotype can be induced by blocking estrogen production with an aromatase inhibitor during embryonic development. Our results show that the principal action of prenatal estrogen exposure, regardless of whether it occurs in female or male mice, is to defeminize and, to some extent, masculinize brain and behavior; further, we show that AFP, which binds estradiol circulating in the female fetus with high affinity and capacity, protects the developing brain from a male-typical organization by this steroid.

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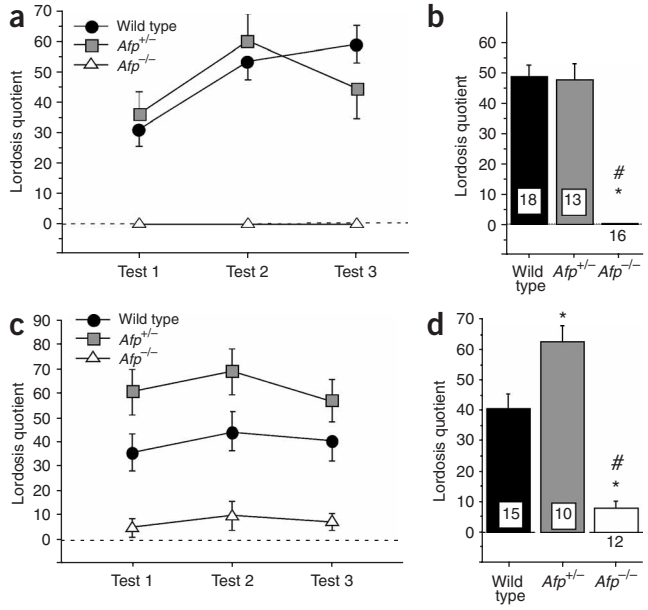

Figure 1 Complete absence of female sexual behavior in female mice lacking AFP. (**a**,**b**) Lordosis quotients in three consecutive tests of *Afp1*$^{-/-}$ females of the CD1 strain (**a**) and their average (**b**). (**c**,**d**) Lordosis quotients in three consecutive tests of *Afp1*$^{-/-}$ females of the C57Bl6/j strain (**c**) and their average (**d**). Results of *post-hoc* comparisons by the Fisher PLSD test are indicated as follows: *$P < 0.05$ compared to the wild type; #$P > 0.05$ compared to *Afp*$^{+/-}$. The numbers of subjects are indicated in the bars in **b** and **d**. Data are expressed as mean ± s.e.m.

RESULTS

Absence of female sexual behavior in *Afp*$^{-/-}$ mice

We generated two different *Afp*$^{-/-}$ mouse models[14]: *Afp1*$^{-/-}$ (in which a portion of exon 1 and both exons 2 and 3 were deleted) and *Afp2*$^{-/-}$ (in which a portion of exon 2 and all of exon 3 were deleted). The *Afp1*$^{-/-}$ alleles were generated in two different genetic backgrounds, the outbred CD-1 (*Afp1*$^{-/-}$*-CD1*) and the inbred C57Bl/6j strain (*Afp1*$^{-/-}$*-C57Bl/6j*), whereas the *Afp2*$^{-/-}$ allele was generated in only the CD-1 background (*Afp2*$^{-/-}$*-CD1*). All *Afp*$^{-/-}$ mouse models generated were tested for their ability to show female sexual behavior, and their behavior was always compared with that of their wild-type and heterozygous (*Afp*$^{+/-}$) littermates. Female mice were tested for female-typical sexual behavior over three weekly trials. To induce sexual receptivity, females were ovariectomized in adulthood and implanted with estradiol-releasing Silastic capsules. After 2 weeks, each female received a subcutaneous injection of 500-μg progesterone 2–4 h before she was placed in an observational cage with a sexually active male, and lordosis responses of the female to the male's mounting were quantified for 10 min. At the end of each test, a lordosis quotient was calculated by dividing the number of lordosis responses by the number of mounts received.

Afp1$^{-/-}$*-CD1* females never showed any female sexual behavior in any of the three trials, whereas wild-type and *Afp1*$^{+/-}$*-CD1* females showed substantial levels of female sexual behavior (**Fig. 1a**,**b**). Very similar results were obtained in *Afp* mutant females carrying the *Afp2*$^{-/-}$ allele (*Afp2*$^{-/-}$*-CD1* mice; data not shown). Repeated-measures analysis of variance (ANOVA) showed a significant effect of genotype on lordosis quotients ($F_{2,44} = 65.26$, $P < 0.001$), as well as a significant effect of repeated testing ($F_{2,88} = 5.83$, $P = 0.004$) and a significant interaction between these factors ($F_{4,88} = 2.56$, $P = 0.044$). Lordosis quotients increased in the second test as compared to the first in wild-type and *Afp1*$^{+/-}$*-CD1* females, but not in *Afp1*$^{-/-}$*-CD1* females.

The effects of estrogen receptor disruption are modified by the background strain used for the experiments[15]. Therefore, we also generated the *Afp1*-null allele in a *C57Bl/6j* background. However, the results were very similar to those obtained in *Afp1*$^{-/-}$ and *Afp2*$^{-/-}$ females of the *CD1* strain: *Afp1*$^{-/-}$*-C57Bl/6j* females showed much lower levels of sexual receptivity than did the wild-type and *Afp1*$^{+/-}$*-C57Bl/6j* females (**Fig. 1c**,**d**). Repeated-measures ANOVA of lordosis quotients only showed a significant effect of genotype ($F_{2,34} = 26.48$, $P < 0.001$). *Post-hoc* analysis showed that lordosis quotients of wild-type and *Afp1*$^{+/-}$*-C57Bl/6j* females were higher than those of *Afp1*$^{-/-}$*-C57Bl/6j* females. Furthermore, lordosis quotients were higher in *Afp1*$^{+/-}$*-C57Bl/6j* females than in wild-type females. The latter difference is most probably due to the below-normal lordosis quotients observed in the wild-type females of the present study, given that we previously observed lordosis quotients of about 60% (the same as those observed in the *Afp1*$^{+/-}$*-C57Bl/6j* females in the present study) in wild-type females of the *C57Bl/6j* strain[11].

Taken together, all the *Afp*$^{-/-}$ mouse models generated showed little or no female-typical sexual behavior even after being primed with estradiol and progesterone, suggesting that they had been defeminized.

Increased male-typical sexual behavior in Afp1$^{-/-}$ mice

Because perinatal estrogens have both defeminizing (that is, decrease in female-typical characteristics) and masculinizing (that is, increase in male-typical characteristics) effects on the brain[6–8], we tested whether *Afp* mutant females were behaviorally masculinized. Females of three genotypes—the wild type, *Afp1*$^{+/-}$ and *Afp1*$^{-/-}$ of the CD1-strain—were tested for male-typical coital behaviors over three weekly trials. Female mice were ovariectomized in adulthood and implanted with an estradiol-releasing Silastic capsule. Female mice show mounting and intromission-like behaviors when exposed to this hormone treatment[11,16]. Females were placed with a sexually receptive, hormonally primed female 2 weeks after the onset of hormone treatment, and male-typical coital behaviors were quantified for 30 min. For each test, we recorded the latency (in seconds) to initial onset and the frequency of mounts with pelvic thrusting and intromission-like movements.

Repeated-measures ANOVA of mount frequencies demonstrated a significant effect of genotype ($F_{2,18} = 4.55$, $P = 0.026$). The number of intromission-like movements and latencies to first mount did not differ among groups (data not shown). *Post-hoc* tests on mount frequencies indicated that *Afp1*$^{-/-}$*-CD1* females showed more mounting behavior

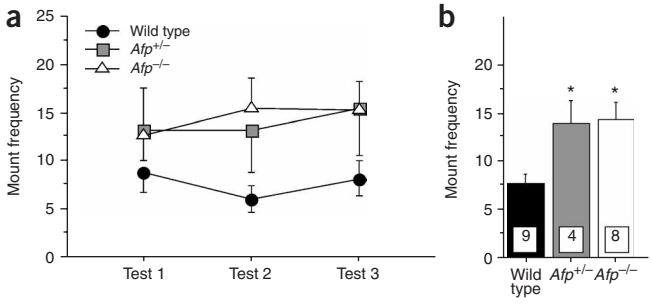

Figure 2 Increased male-typical sexual behavior in female mice lacking AFP. (**a**,**b**) Frequencies of mounts plus pelvic thrusting in three consecutive tests (**a**) and their average (**b**). Results of *post-hoc* comparisons by the Fisher PLSD test are indicated as follows: *$P < 0.05$ compared to the wild type. The numbers of subjects are indicated in the bars in **a** and **b**. Data are expressed as mean ± s.e.m.

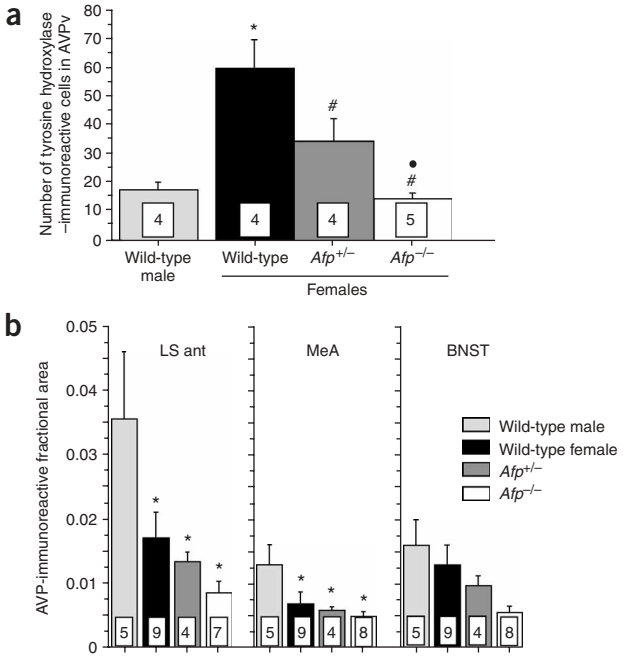

Figure 3 Neurochemical changes in female mice lacking AFP. (**a**) Numbers of tyrosine hydroxylase–immunoreactive neurons in the AVPv of $Afp^{-/-}$ females. (**b**) Brain AVP expression assessed by the fractional areas covered by AVP-immunoreactive structures in the lateral septum anterior (LS ant), medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST). Results of *post-hoc* comparisons by the Fisher PLSD test are indicated as follows: $*P < 0.05$ compared to wild-type males; $\#P < 0.05$ compared to wild-type females; $\bullet P < 0.05$ compared to $Afp^{+/-}$ females. The numbers of subjects are indicated in the bars. Data are expressed as mean ± s.e.m.

with pelvic thrusting than did the wild-type females, indicating that they were behaviorally masculinized (**Fig. 2a**,**b**). $Afp1^{+/-}$-*CD1* females also showed increased mounting behavior compared to the wild type, suggesting that these females were masculinized as well, probably because their brain was exposed to increased concentrations of estrogen owing to a decrease in the amount of AFP[14]. The binding affinity of estradiol for AFP (K_d 10^{-8} M) is lower than for its own receptor (K_d 10^{-11} M), stressing the importance of having excess levels of AFP during fetal development[17]. Lordosis behavior in $Afp^{+/-}$ females was not affected, which may indicate that masculinization is more sensitive than defeminization to the decrease in AFP.

Decreased tyrosine hydroxylase expression in $Afp1^{-/-}$ mice

To determine whether $Afp^{-/-}$ females were also affected at the neurochemical level, we determined the expression of tyrosine hydroxylase in the anteroventricular nucleus of the preoptic region (AVPv). The AVPv plays a critical role in female reproductive function by transducing hormonal feedback on luteinizing hormone secretion, and it is needed for hormonally induced ovulation[18]. The number of tyrosine hydroxylase–expressing neurons is sexually dimorphic in the AVPv: females have greater numbers than males[19–21]. We used immunocytochemical procedures to detect tyrosine hydroxylase–expressing neurons. We collected brains from new groups of females of three genotypes—wild-type, $Afp1^{+/-}$ and $Afp1^{-/-}$-*CD1*—as well as a group of wild-type males. All mice (male and female) had been gonadectomized in adulthood and implanted with an estradiol-releasing Silastic capsule 1 week before the experiment.

One-way ANOVA identified significant differences in the numbers of tyrosine hydroxylase–immunoreactive neurons between these groups ($F_{3,13} = 12.08$, $P < 0.001$). Wild-type females had greater numbers of tyrosine hydroxylase–immunoreactive cells in the AVPv than the wild-type males did, confirming the previously observed sex difference (**Fig. 3a**; refs. 19–21). Notably, $Afp1^{-/-}$-*CD1* females had decreased (that is, male-like) numbers of tyrosine hydroxylase–immunoreactive cells, whereas the numbers in $Afp1^{+/-}$ females were intermediate between those of the wild-type and $Afp1^{-/-}$ females. The decrease in tyrosine hydroxylase–immunoreactive neurons in the AVPv of $Afp1^{-/-}$ females may be related to their infertility. The intermediate numbers of tyrosine hydroxylase–immunoreactive neurons in the AVPv of $Afp1^{+/-}$ females may again indicate that they were exposed to increased levels of estrogens as a result of a partial decrease in the amount of AFP.

Unchanged vasopressin expression in $Afp1^{-/-}$ mice

To determine whether the brains of $Afp1^{-/-}$ females were masculinized, we determined the expression of arginine-vasopressin (AVP) in the lateral septum and a few other brain areas because AVP expression has been found to be sexually dimorphic in the lateral septum of a number of species, including mice[22], rats[23] and prairie voles[24]. Generally, males show a higher expression of AVP, particularly in the lateral septum, than do females. We used immunocytochemical procedures to detect AVP-expressing neurons. Brains were collected from new groups of females of three genotypes—wild-type, $Afp1^{+/-}$ and $Afp1^{-/-}$-*CD1*—as well as from a group of wild-type males that had been gonadectomized in adulthood and implanted with an estradiol-releasing Silastic capsule 1 week before the experiment.

We observed clear sex differences in measures of the fractional areas covered by AVP-immunoreactive structures: males showed higher immunoreactivity than females in the lateral septum, both at the anterior and posterior level, in the medial amygdala and possibly in the bed nucleus of the stria terminalis (the effect did not reach statistical significance in this nucleus), confirming previously observed sex differences in terms of AVP expression in these brain regions (**Fig. 3b**; refs. 22–24). However, for each brain region analyzed, the areas of AVP-immunoreactive structures in $Afp1^{-/-}$-*CD1* females were very similar to those in wild-type and $Afp1^{+/-}$ females (**Fig. 3b**). This was confirmed by a one-way ANOVA, which showed an overall significant group effect for the lateral septum in its anterior ($F_{3,21} = 4.33$, $P = 0.016$) and posterior parts ($F_{3,21} = 10.92$, $P < 0.001$; data not shown) and in the medial amygdala ($F_{3,22} = 4.04$, $P = 0.020$) and an almost significant group effect in the bed nucleus of the stria terminalis ($F_{3,22} = 2.88$, $P = 0.059$). *Post-hoc* analysis showed that males had higher densities of AVP-immunoreactive structures in the lateral septum and medial amygdala than did the wild-type, $Afp1^{+/-}$ and $Afp1^{-/-}$ females. The latter three groups did not differ among each other.

Prenatal ATD restores female phenotype of $Afp^{-/-}$ mice

Our initial observations—of a complete absence of female sexual behavior in $Afp^{-/-}$ females and of male-like numbers of tyrosine hydroxylase–immunoreactive cells in their AVPv—did not allow us to discriminate between the two competing theories about the role of AFP in brain sexual differentiation. It could be argued that the brains of $Afp^{-/-}$ females were defeminized because they were no longer protected from the estrogens produced by their mother or male siblings. This argument would support the hypothesis that AFP serves primarily to protect the female brain from excessive exposure to estrogens[4]. By contrast, it could be argued that the brains of $Afp^{-/-}$ females were not feminized because they were lacking the AFP to transport small

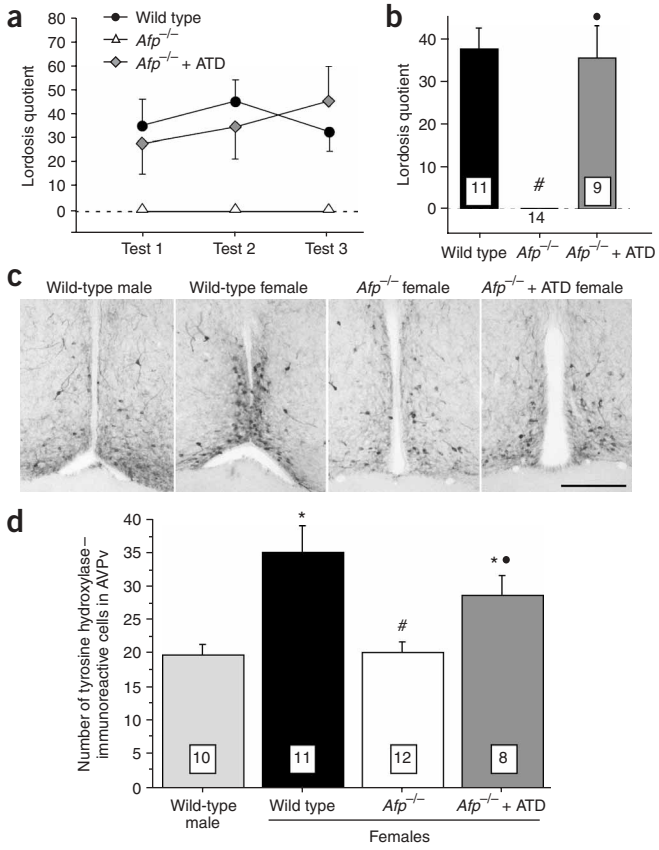

Figure 4 Prenatal treatment with the aromatase inhibitor ATD rescued the female phenotype of *Afp2*$^{-/-}$ females. (**a**,**b**) Lordosis quotients in three consecutive tests (**a**) and their average (**b**). (**c**) Photomicrographs of sections in AVPv stained by immunohistochemistry for tyrosine hydroxylase in males and females of the three genotypes. Scale bar, 200 μm. (**d**) Number of tyrosine hydroxylase–immunoreactive neurons in these sections. Results of *post-hoc* comparisons by the Fisher PLSD test are indicated as follows: *$P < 0.05$ compared to wild-type males; #$P < 0.05$ compared to wild-type females; ●$P < 0.05$ compared to *Afp2*$^{-/-}$ females. The numbers of subjects are indicated in the bars. Data are expressed as mean ± s.e.m.

amounts of estrogens to target brain areas and thus to promote the differentiation of these structures in a female direction. This argument would favor the hypothesis that AFP is needed to transport estrogens into the brain and thus has an active role in female brain differentiation[5]. To discriminate between these competing hypotheses, we blocked estrogen production during prenatal development by treating pregnant female mice heterozygous for the *Afp* mutation with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD). If AFP protects the female brain from being exposed to estrogens, *Afp*$^{-/-}$ offspring of ATD-treated mothers should not be defeminized, as the ATD treatment would prevent the formation, during prenatal development, of high defeminizing levels of estradiol. However, if AFP actually acts as an estrogen carrier and is thus necessary for feminizing the female brain, then the *Afp*$^{-/-}$ offspring of ATD-treated mothers should not show normal female sexual behavior because of a lack of feminization by estrogens (absence of the steroid and of its carrier).

Pregnant female mice heterozygous for the *Afp* mutation (*Afp2*$^{+/-}$-*CD1*) received daily injections with the aromatase inhibitor ATD during the last 10 d of pregnancy. Female progeny were ovariectomized in adulthood and implanted with an estradiol-releasing Silastic capsule and, 2 weeks later, tested for female sexual behavior over three weekly trials (after receiving a subcutaneous injection with progesterone 2–4 h before each test).

Prenatal treatment with ATD clearly rescued female sexual behavior in *Afp2*$^{-/-}$-*CD1* females (**Fig. 4a**,**b**). Such females showed levels of lordosis behavior similar to those in wild-type females, whereas—as expected—*Afp2*$^{-/-}$-*CD1* females treated prenatally with propylene glycol as a control did not show female sexual behavior. Repeated-measures ANOVA confirmed a significant group effect of lordosis quotients ($F_{2,31} = 10.55$, $P < 0.001$).

At the end of behavioral testing, we collected the brains for immunocytochemical detection of tyrosine hydroxylase–expressing neurons in the AVPv. Prenatal treatment with ATD also prevented the previously observed decrease in the number of tyrosine hydroxylase–immunoreactive cells in the AVPv of *Afp*$^{-/-}$ females (**Fig. 4c**,**d**). The numbers of tyrosine hydroxylase–immunoreactive cells in ATD-treated *Afp2*$^{-/-}$ females were similar to those in wild-type females, whereas *Afp2*$^{-/-}$ females treated with propylene glycol as a control again had male-like numbers. ANOVA on the number of tyrosine hydroxylase–immunoreactive cells showed a significant group effect ($F_{3,37} = 7.99$, $P < 0.001$). *Post-hoc* analysis showed that wild-type females and ATD-treated *Afp*$^{-/-}$ females had significantly higher numbers of tyrosine hydroxylase–immunoreactive cells than did the *Afp*$^{-/-}$ females and wild-type males. The last two groups did not differ from one another.

Taken together, our results indicate that prenatal treatment with an aromatase inhibitor rescued the female phenotype in *Afp*$^{-/-}$ females, suggesting that prenatal estrogens masculinize and defeminize the brain and that AFP protects the female brain from these estrogens.

DISCUSSION

The classic view of sexual differentiation in mammalian species is that testosterone secreted by the testes causes masculinization (increase in male-typical characteristics) and defeminization (decrease in female-typical characteristics) of the brain and behavior. Many of these actions of testosterone on the developing brain are mediated by estradiol, which is formed locally in the brain through the transformation of testosterone by the enzyme aromatase (the 'aromatization hypothesis'; refs. 6–8). The aromatization hypothesis further implies that estrogens produced by the placenta or fetal gonads and subsequently secreted into circulation do not have a role in the sexual differentiation of the rodent brain because they are bound with high affinity to AFP—a major fetal plasma protein present in many developing vertebrate species and produced transiently in great quantities by the endodermal cells of the visceral yolk sac, by the hepatocytes of the fetal liver and in the gastrointestinal tract during gestation[1–3]. Thus, it has been proposed that AFP serves to protect the developing female brain from excessive exposure to estrogens and thus from becoming masculinized and defeminized[4]. If estradiol were not bound to AFP, it would be free to enter the brain, and because significant concentrations of estradiol circulate in newborn females[25], female rats would be masculinized and defeminized. Indirect evidence for a protective role of AFP comes from a study[26] showing that the addition of neonatal serum to [^{3}H]estradiol strongly reduces its uptake into the brain of adult female rats.

The presence of AFP within neurons[12] and the absence of any evidence of local AFP synthesis[13] suggest, however, that AFP could be transported from the periphery into the brain. It has thus been proposed that AFP can act as a carrier that actively transports estrogen into target brain cells and actually controls the level of estradiol available to interact with its receptor in neurons, thereby modulating brain sexual differentiation[5].

Our findings that *Afp*$^{-/-}$ females showed no female sexual behavior at all, and that normal levels of this behavior could be induced by blocking estrogen action during embryonic development, demonstrate that the principal action of prenatal estrogen exposure is to defeminize individuals (that is, decrease their capacity to display female sexual behavior later in life) and that AFP normally binds estradiol circulating in the female fetus and thereby protects the developing brain from defeminization. Thus our findings corroborate the hypothesis originally proposed[4] and argue against the model that considers AFP a carrier delivering estrogen to the brain[5]. Why AFP is present in brain cells in some brain regions but not in others remains unclear, however. Little or no intraneuronal AFP is found in limbic, hypothalamic and amygdaloid areas, whereas large amounts are present in adjacent regions[27]. This could indicate that AFP protects from estrogens those brain regions that are involved in reproductive functions, such as the hypothalamus, but may deliver estrogens to other brain regions and thereby influence the sexual differentiation of various functions, including nonreproductive behaviors such as learning and memory[28].

It is unclear whether, in other species, AFP similarly protects the brain from estrogens during sexual differentiation. In contrast to AFP in mice and rats, human AFP does not bind estrogens, although, notably, AFP has been reported to have an antiestrogenic activity in estrogen-sensitive breast cancers[29], probably by a mechanism other than the sequestration of estrogen[30]. In primates, the main high-affinity steroid hormone–binding protein (androgens and to a lesser extent estrogens) is the sex hormone–binding globulin (SHBG), which has little structural homology with AFP[31]. The primate SHBG may have a function in embryonic development similar to that of AFP in rodent species. It should be noted that androgen is the main hormone causing brain masculinization in primate species[32]. SHBG may thus protect the developing female human brain from being masculinized by androgens.

AFP also binds other ligands[33] such as fatty acids (for example, arachidonic and docosahexaenoic acids) or bilirubin but, for the following reasons, it is likely that the effects observed in the present study relate only to estrogen action: (i) the phenotype of *Afp*$^{-/-}$ mice is strictly related to reproductive functions (anovulation as shown in ref. 14 and the absence of lordosis as shown in the present study) and (ii) only female *Afp*$^{-/-}$are affected, which is in line with the proposed role of AFP in protecting the female brain from estrogens. If AFP were acting by another mechanism such as the binding of fatty acids, a broader phenotype affecting both sexes would be expected.

The demonstration that AFP protects the embryonic female brain from estrogens does not, however, exclude a role for estrogens in female development. The classic concept of the female brain developing in the absence of any hormonal secretions was based on early studies showing that male-typical sexual behaviors can be induced in female mammals by treating them perinatally with testosterone, whereas female-typical sexual behaviors can be induced in male mammals by castrating them at birth[34–36]. However, a possible role of sex steroids in the differentiation of the female brain was suggested by several behavioral studies showing that perinatal exposure of female rats to small amounts of estrogens facilitates their capacity to display female sexual behavior in adulthood[9,37]. More recent studies also demonstrated that female aromatase knockout (*Cyp19*$^{-/-}$) mice show reduced levels of lordosis behavior[11] and anovulation[38] even after adult treatment with estradiol and progesterone. Together these data thus suggest that the female brain does not only need to be protected from prenatal estrogens by AFP, but that, in addition, exposure to small amounts of estrogens may also be necessary for a normal development of the female phenotype. If true, then estrogens most probably act during the postnatal period when the amount of AFP has decreased substantially and no longer has a protective role. AFP levels are the highest at birth, after which they decrease by about 50% during the first 24 h. Only trace levels (about 0.01% of fetal levels) are detected at 3 weeks of age[3]. Accordingly, several studies in which estrogen exposure was manipulated in both sexes during the perinatal developmental period have suggested that different critical periods exist for male- and female-typical organization of the brain and behavior[39–41].

One notable observation in the present study is that AVP expression remained female-like in *Afp*$^{-/-}$ females in spite of their exposure to large amounts of estradiol during embryonic development. This finding is particularly unexpected in light of earlier observations showing an important role for perinatal estrogens in the sexual differentiation of the AVP system in rats[42]. It is possible, however, that the development of sex differences in AVP expression in the mouse brain depends more on the perinatal action of testosterone than on that of estradiol, an idea supported by recent findings in male *Cyp19*$^{-/-}$ mice (ref. 43 and F. Allieri *et al.*, *Soc. Behav. Neuroendocrinology Abstr.* **46**, 2004).

Compared to wild-type males, gonadally intact male *Ark*$^{-/-}$ mice indeed show reduced AVP immunoreactivity in the lateral septum, medial amygdala and bed nucleus of the stria terminalis, suggesting that the sexually dimorphic AVP system of the mouse forebrain is under the control of estradiol[43]. However, when treated with estradiol in adulthood, male *Cyp19*$^{-/-}$ mice show normal, male-like AVP expression in these brain structures, suggesting that these effects of estradiol are mainly activational (in adulthood) and that the organization, during development, of the sexually dimorphic AVP system is not under the control of estrogens but may require the presence of testosterone (F. Allieri *et al.*, *Soc. Behav. Neuroendocrinology Abstr.* **46**, 2004).

Alternatively, sex chromosomes may also contribute directly to the development of the sexually dimorphic AVP system in mice[21]. Indeed, XY males and XY female mice (that is, females with a deletion of the *Sry* gene, which encodes the testes-determining factor) are more masculine than XX mice with regard to the density of AVP-expressing fibers in the lateral septum[22]. Future studies using both *Afp*$^{-/-}$ and *Cyp19*$^{-/-}$ mouse models should determine whether the AVP system in mice depends on the action of perinatal testosterone or whether genetic factors are actually involved.

METHODS

Mice and experimental manipulations. All breeding and genotyping was performed at the Laboratoire de Biologie du Développement, Université Libre de Bruxelles, Gosselies, Belgium. The *Afp1*$^{-/-}$*-CD1* mice used in the present study had been backcrossed for a minimum of 18 generations, whereas the *Afp1*$^{-/-}$*-C57Bl/6j* and the *Afp2*$^{-/-}$*-CD1* had been backcrossed for a minimum of 12 and 8 generations, respectively. All mice were housed in groups of the same sex (genotypes, however, were mixed) under a reversed light-dark cycle (12 h: 12 h light/dark). Food and water were always available *ad libitum*. All behavioral experiments were conducted in accordance with the guidelines set forth by the National Institutes of Health Guiding Principles for the Care and Use of Research Animals and were approved by the Ethical Committee for Animal Use of the University of Liège.

Stimulus males were derived from a CD-1 breeding colony at the Laboratoire de Biologie du Développement, Université Libre de Bruxelles, and were left gonadally intact. All stimulus-treated females were derived from a C57Bl/6j breeding colony at the Center for Cellular and Molecular Neurobiology, University of Liège. In adulthood, all experimental and stimulus females were ovariectomized in adulthood under general anesthesia using a mixture of ketamine (80 mg kg^{-1} intraperitoneally (i.p.)) and medetomidine (Domitor from Pfizer, 1 mg kg^{-1} i.p.). Mice received atipamezole (Antisedan from Pfizer, 4 mg kg^{-1}, subcutaneously) at the end of the surgery to antagonize medetomidine-induced effects, thereby accelerating their recovery. At the time of

ovariectomy, all females received a 5-mm-long Silastic capsule subcutaneously (inner diameter: 1.57 mm; outer diameter: 2.41 mm) containing crystalline 17β-estradiol (diluted 1:1 with cholesterol).

To block estradiol synthesis during embryonic development, pregnant female *Afp2*$^{+/-}$*-CD1* mice received daily subcutaneous injections of 100 µl of a solution containing 40 mg ml^{-1} ATD dissolved in propylene glycol starting on day 12 of pregnancy. An additional group of pregnant *Afp2*$^{+/-}$*-CD1* females received injections with the solvent only to serve as controls. As ATD treatment could potentially interfere with maternal behavior and lactation, pups were delivered by cesarean section at day 21 of pregnancy and subsequently placed with a foster mother. At the same time, blood was taken from the ATD-treated females and estradiol plasma concentrations were determined by radioimmunoassay as previously described[14]. All ATD-treated females had estradiol levels that were below the detection limit of the assay (10 pg ml^{-1}). On some occasions, the mother gave birth naturally and also started to lactate her pups spontaneously. In this case, pups were left with their natural mother and no blood was taken from the mother so as to not disrupt lactation. A total of three out of nine prenatally ATD-treated *Afp*$^{-/-}$ females were born naturally. Initially, this factor was included in the statistical analysis of the behavioral data. As no effect was found on their later sexual behavior, the results were combined. At 21 d of age, pups were weaned and the tip of their tail cut for later analysis of their genotype[14]. It should be noted that embryonal treatment with the aromatase inhibitor ATD restores fertility in *Afp*$^{-/-}$ females[44].

Behavioral analyses. (i) Female sexual behavior. All lordosis tests were conducted in a Plexiglas aquarium (35 cm long × 25 cm high × 19 cm wide) whose floor was covered with fresh sawdust. At the beginning of each test, a sexually experienced male of the CD1 strain was placed alone in the aquarium and allowed to adapt for 15 min. Subsequently, an experimental female was placed in the aquarium and we recorded the lordosis responses of the female to the mounts of the stimulus male. The test lasted until the female had received 10 mounts or 10 min had elapsed. We tested both the *Afp1*$^{-/-}$ and *Afp2*$^{-/-}$ mouse models generated in the CD-1 strain for female sexual behavior, and we observed a similar phenotype for both alleles. We also obtained similar behavioral results in *Afp1*$^{-/-}$ females of the CD-1 and C57Bl/6j strains, showing no effect of background strain. Also, all female sexual behavioral experiments were performed in duplicate: that is, for each knockout model, two independent groups of female mice of three genotypes (the wild type, *Afp*$^{+/-}$ and *Afp*$^{-/-}$) were tested for receptivity. As the second replicate always confirmed the results from the first, data were combined in this presentation.

(ii) Male sexual behavior. All male sexual behavior tests were conducted in a Plexiglas aquarium (35 cm long × 25 cm high × 19 cm wide) whose floor was covered with fresh sawdust. At the beginning of each test, the female subject was placed alone in the aquarium to adapt for 15 min. Subsequently, an estrous female of the C57Bl/6j strain was introduced, and the number of mounts and intromission-like behaviors shown by the female subject were scored for 30 min. In addition, we recorded latencies to mounts and intromission-like behaviors. The female subject was never mounted by the stimulus female. We compared the capacity to display male sexual behavior in one group of *Afp1*$^{-/-}$*-CD1* females and one group of *Afp1*$^{-/-}$*-C57Bl/6j* females to their wild-type and *Afp*$^{+/-}$ littermates. However, only a few female subjects of the *Afp1*$^{-/-}$*-C57Bl/6j* strain mounted the stimulus female (both the wild-type and *Afp*$^{-/-}$ subjects; data not shown). It should also be noted that in contrast with the female sexual behavior assessment, this experiment was not performed in duplicate.

Immunohistochemistry. To determine tyrosine hydroxylase expression, brains of male and female wild-type mice, female *Afp1*$^{+/-}$ mice and *Afp1*$^{-/-}$ (CD-1) mice were fixed by perfusion with 4% paraformaldehyde and further processed by immunocytochemistry using standard procedures. To determine AVP expression, the brains were fixed with 5% acrolein (method adapted from ref. 21). In the ATD experiment, tyrosine hydroxylase expression was determined in wild-type males and females and in *Afp2*$^{-/-}$ and ATD-treated *Afp2*$^{-/-}$ (CD-1) females. Mice (males and females) were always gonadectomized and implanted with a Silastic capsule containing estradiol[11]. Immunohistochemistry was carried out using avidin-biotin-peroxidase–diaminobenzidine (ABC-DAB) colorimetric detection. Primary antibodies were specific to AVP (ICN Biomedicals; 1:1,000) and tyrosine hydroxylase (ICN Biomedicals; 1:5,000). Numbers of tyrosine hydroxylase–expressing neurons in the AVPv were directly counted on drawings prepared with a camera lucida. AVP expression was analyzed by computer-assisted densitometry as described previously[38].

Statistical analysis. Data were compared by ANOVA including eventually one repeated factor, followed when appropriate by *post-hoc* comparisons with the Fisher protected least significant difference test (Fisher PLSD). Level of significance was set at 0.05, and only significant results are mentioned in detail.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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