Androgen receptor deficiency alters the arginine-vasopressin sexually dimorphic system in Tfm rats.

ANDROGEN RECEPTOR DEFICIENCY ALTERS THE ARGININE-VASOPRESSIN SEXUALLY DIMORPHIC SYSTEM IN TFM RATS.

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LIST OF ABBREVIATIONS

AIS	**androgen insensitivity syndrome**
AR	**androgen receptor**
AVP	**arginine vasopressin**
AVP-ir	**arginine vasopressin immunoreactive**
BST	**bed nucleus of the stria terminalis**
DHT	**dihydrotestosterone**
E_2	**estradiol**
ER	**estrogen receptor**
LS	**lateral septum**
MeA	**medial amygdaloid nucleus**
MPOA	**medial preoptic area**
PVN	**paraventricular nucleus**
SON	**supraoptic nucleus**
T	**testosterone**
Tfm	**testicular feminilization mutation**

ABSTRACT

In rodents as well as in many other mammalian and non-mammalian species, the arginine-vasopressin (AVP) system includes a parvocellular sexually dimorphic portion located within the bed nucleus of the stria terminalis (BST), the medial amygdaloid nucleus (MeA) and the lateral septum. In this system, males have more cells and denser projections than females, neurons show androgen and estrogen receptors, and gonadal hormones are required for the activation. However, the role of these hormones for the differentiation of the system is not clear. Previous studies performed on aromatase knock out mice suggested that estradiol is not necessary for the differentiation of the system, but it is important for its activation in adulthood.

To elucidate the role of androgens on differentiation and functioning of AVP parvocellular system, we compared male and female rats with a non-functional mutation of androgen receptor (*Tfm*, testicular feminization mutation) to their control littermates. Our data show that the lack of a functional androgen receptor significantly decreases the expression of AVP immunoreactivity within the BST and MeA of male *Tfm*. Thus supporting the hypothesis that androgens, through the action of their receptor, should have a relevant role in the organization and modulation of the AVP parvocellular sexually dimorphic system.

INTRODUCTION

Gonadal steroids, through their interaction with intracellular receptors, induce several effects in target cells; these receptors are members of ligand-activated transcription factors family that bind responsive elements located on the genome and therefore are able to modulate the transcription of a wide variety of genes (McCarthy, 2008). Specific nuclear receptors for estrogens and androgens are present in the developing brain of rodents (DonCarlos and Handa, 1994, DonCarlos, 1996, McAbee and DonCarlos, 1998). Therefore, gonadal steroids are likely to play a crucial role in brain plasticity and development; in particular, they may induce the differentiation of sexually dimorphic structures and behaviors (Panzica et al., 1995, McCarthy and Konkle, 2005, McCarthy and Arnold, 2011, Lenz et al., 2012).

Sex difference in testosterone (T) levels during development has been recognized as the driving force of sexual differentiation of the mammalian central nervous system. In male rats, T secretion reaches the peak from prenatal days 18-20 (Perakis and Stylianopoulou, 1986). This early elevation in T levels coincides with the period (*critical perinatal period*) when T can permanently influence the development of behavior and neural structures (Cooke et al., 1998). Within the brain, T is metabolized into estradiol (E_2) by aromatase or into 5α-dihydrotestosterone (5α-DHT) by 5α-reductase. These two steroids may bind their receptors inducing the differentiation of neural circuits. In some cases both androgen (AR) and estrogen receptors (ER) may contribute to structural plasticity of nervous circuits, as for the posterior part of the amygdala (MeP) (Cooke et al., 2003).

The AR mediates age- and tissue-specific actions of androgens and is responsible for the development, maintenance and regulation of the male reproductive system (Raskin et al., 2009, Juntti et al., 2010). AR is encoded by a X-linked gene, which consists of 8 exons, and it is essential for androgenic action, of both T and 5α-DHT (Gobinet et al., 2002).

In humans, mutations of the AR gene represent the molecular basis of androgen insensivity syndrome (AIS), a rare X-linked disorder characterized by a defective virilization in genetic males ranging from partial forms, in which 46, XY phenotypic males are infertile, to the complete syndrome showing a female phenotype (Brinkmann, 2001, McPhaul, 2002, Hughes et al., 2012)

In mammals, as well as in most vertebrates, part of the arginine-vasopressin (AVP) neuronal system is sexually dimorphic and gonadal steroids sensitive in adulthood (for a review see De Vries and Panzica, 2006). The bed nucleus of the stria terminalis (BST) and the amygdala contain more AVP-immunoreactive (AVP-ir) cells in male than in female and projections from these nuclei to the lateral septum (LS) are denser in male than in female; in addition, several other brain regions show sexually dimorphic AVP innervation (De Vries et al., 1984b, Rood et al., 2013).

Histological investigations demonstrated several sexually dimorphic features (volume, cell number and cell size) in BST and amygdala. The BST shows a consistent pattern of sex differences in several mammalian species [guinea pig (Hines et al., 1992); rat (Del Abril et al., 1987); rabbit (Segovia et al., 2006)] and even in humans (Zhou et al., 1995). Both nuclei have a crucial role in the regulation of rodent male and female sexual and aggressive behaviors (Veening and Coolen, 1998, Veening et al., 2005). In particular, the rodent parvocellular AVP system is involved in the control of social behaviors, such as pair bonding, social recognition, aggressivity, depression and anxiety (see Compaan et al., 1993, Veenema and Neumann, 2008, Choleris et al., 2009, Rotzinger et al., 2010).

Several studies have investigated the development of sexually dimorphic AVP system in mammals and other vertebrates and the role played by gonadal hormones for its regulation (see for a review De Vries and Panzica, 2006).

Gonadectomy and early T replacement therapy affect the organization of limbic AVP system and AR expression, suggesting that both T and AR are involved in the differentiation of this system (Bingham and Viau, 2008). Also aromatase (the enzyme converting T into E_2) expression is reduced by gonadectomy and restored by T replacement (Roselli et al., 1987). However, whereas the lack of a functional aromatase gene (ArKO) induces an apparent reduction of AVP cell number and fiber innervation in BST, amygdala and LS (Plumari et al., 2002), adult administration of E_2 in the same mice induced a full recovery of male AVP system (Pierman et al., 2008). Therefore, it seems that E_2 is not necessary for the differentiation of the sexually dimorphic AVP system, while it plays a major role in adulthood to stimulate the same system.

The *Tfm* rats (characterized by a testicular feminilization due to a spontaneous mutation of the AR gene) may help in elucidate the role of androgens and ARs in the activation/organization of the sexually dimorphic AVP system. These animals show a

low level of the aromatase protein itself, even in T-treated gonadectomized *Tfm* male rats (Roselli et al., 1987). In addition, *Tfm* rodents show alterations in some behaviors [i.e. adult playful attack and defense (Field et al., 2006), anabolic androgenic steroid-induced aggressive behavior (Wu et al., 2009, Robinson et al., 2012), anxiety-related behaviors (Zuloaga et al., 2008, Zuloaga et al., 2011) or social investigation (Tejada and Rissman, 2012)] that are partially dependent by the AVP system (Veenema and Neumann, 2008, Veenema et al., 2010). Also, some neuronal circuits are altered in these animals, as the nitric oxide producing system of BST and other hypothalamic nuclei (Martini et al., 2008).

As *Tfm* rodents have decreased AR binding but apparently normal ER binding (Attardi et al., 1976), these animals offer a way to examine the contribution of the AR to the sexual differentiation of rodent brain in gonadally intact individuals.

MATERIALS AND METHODS

Animals

Female *Tfm* rats from the colony of the Department of Psychobiology of UNED, Madrid, Spain, (for the origin of the strain see Garcia-Falgueras et al., 2005) were analyzed and identified by polymerase chain reaction (PCR, Fernandez et al., 2003) as females heterozygous for the *Tfm* mutation (X^{Tfm}/X). These rats were mated with Wistar male rats (Charles River; Criffa, Barcelona) to obtain the animals used in this experiment (N = 20). The animals obtained in the matings have the same genetic background (X^{Tfm}/X x Wistar male, X/Y) and therefore are comparable to each other. The experimental groups, classified after PCR analyses, were as follows: (a) *Tfm* males (X^{Tfm}/Y, N = 5); (b) *Tfm* carrier females (X^{Tfm}/X, N = 5); (c) control littermate males (X/Y, N = 5), and (d) control littermate females (X/X, N = 5).

At the age of 21 days, the animals were housed in monosexual groups in standard cages and maintained in 12:12 light/dark cycle (lights off at 8:00 PM). Food and water were provided *ad libitum*. The animal room was kept at 22 ± 2 °C. Animal care and handling throughout the experimental procedures were in accordance with the European Union Council Directive of 24 November 1986 (86/609/EEC) and the experiment was approved by the ethical committee of the UNED. The genotyping of the animals was performed at the Department of Pharmacological and Biomedical

Sciences (Milano, Italy) by using 200 µl of peripheral blood according to the protocol previously developed (Fernandez et al., 2003, Garcia-Falgueras et al., 2005).

Colchicine injection and sacrifice

At the age of 60 days, all the animals were subjected to an intracerebroventricular injection of colchicine to increase the AVP immunostaining (Compaan et al., 1993, Plumari et al., 2002, Pierman et al., 2008). Females were inspected for the stage of the cycle (examination of vaginal smears) and injected only in estrus. Briefly, the animals were deeply anaesthetized with a mixture of ketamine (80mg/Kg of body weight), valium (10mg/Kg of body weight), and atropine (0,05mg/Kg of body weight) and positioned in a stereotaxic apparatus (Kopf, Tujunga, CA, USA). They were then stereotaxically injected into the left lateral ventricle with 10µl of solution corresponding to 250µg/Kg of body weight of colchicine dissolved in distillated water. Accuracy of the position of the injection cannula was controlled by observing the increase in pressure in the cannula when it entered the ventricle. Later inspection of brain sections revealed no trace of damaged tissue around the lateral ventricle except for the penetration path of the needle. 48 hours after the colchicine injection, rats were deeply anesthetized with the same anesthetic mixture (ketamine-valium-atropine) and perfused through the heart with saline solution (0.9%) until vessels were completely blood-free, and then with fixative (4% paraformaldehyde in 0,1 M phosphate buffer, pH 7,3). Brains were dissected out of the skull, post-fixed for 2 hours in the same fixative and rinsed in 0.01 M phosphate buffer saline (PBS). They were then placed overnight in a 30% sucrose solution in PBS, frozen in precooled liquid isopentane at -35°C, and stored in a deep freezer at –80°C until sectioning.

Immunohistochemistry

Brains were serially cut in the coronal plane at 25 µm thickness using a cryostat and collected in a cryoprotectant solution (Watson et al., 1986) at -20°C. Every forth section one was stained for AVP by immunohistochemistry using the free floating technique. Sections were immunostained using a commercial AVP antibody (ICN Pharmaceuticals, CA; USA) diluted 1:20,000. The same antibody has been previously used to stain the AVP cells and fibers in rodents species (Ferris et al., 1995, Villalba et al., 1999, Plumari et al., 2002, Pierman et al., 2008). The specificity of this

antibody to vasopressin (and the absence of any cross-reactivity with oxytocin) has been validated previously (Ferris et al., 1995). In the present study, we have performed the following controls: a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); b) the secondary antibody was omitted. In these conditions, cells and fibers were totally unstained.

Briefly, the free-floating sections collected in the cryoprotectant solution were washed overnight in PBS at pH 7.3. The following day, sections were first washed in PBS containing 0.2 % Triton X-100 for 30 min and then treated to inhibit endogenous peroxidase activity with a solution of PBS containing methanol/hydrogen peroxide for 20 min (Streefkerk, 1972). Sections were incubated for 30 min with normal goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at room temperature with anti-AVP antibody diluted 1:20,000 in PBS, pH 7.3-7.4, containing 0.2% Triton X-100. A biotinilated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was then employed at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with avidin-peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). The peroxidase activity was visualized with a solution containing 0.400 mg/ml 3,3-diamino-benzidine (Sigma, Milan, Italy) and 0.004% hydrogen peroxide in 0.05M Tris-HCl buffer pH 7.6. Sections were mounted on chromallum coated slides, air dried, cleared in xylene and cover slipped with Entellan (Merck, Milan, Italy). Sections were collected on chromalum-coated slides, air-dried, washed in xylene, and cover slipped with Entellan (Merck, Milano, Italy).

An alternate set of sections was stained with toluidine blue to set up a series of Nissl-stained section that were used for anatomical orientation. Identification of all structures was based on the rat brain atlas of Paxinos and Watson (1998).

Quantitative analysis

For quantitative analysis, all slides were first coded so that the observer was blind to the experimental treatments. Sections were chosen to match similar levels of the regions to be investigated. Selected fields were acquired through a Zeiss Axioplan I microscope equipped with a Leica DFC 320 digital telecamera connected to an Apple G4 Macintosh. Digital images were later analyzed with Image J program (version

1.46, a public domain program written by W. Rasband at the U.S. National Institutes of Health, Bethesda, MD, USA).

The cells count and the fractional area, covered by AVP-ir structures, were evaluated according to the following protocols in the regions schematically reported in Fig. 1:
- in the BST and amygdala we counted the number of AVP-ir cells (identified for the presence of a clearly labeled cell body) using the cell counter plug-in of ImageJ. We counted all the positive neurons within a rectangle of fixed size (500,000 μm^2) placed over the nuclei (Fig. 1). The measures were performed in two separate rostro-caudal levels of the bed nucleus of the stria terminalis (BST: -0.40 and -0.80 relative to Bregma, (Paxinos and Watson, 1998), and medial amygdaloid nucleus (MeA: -2.12 and –2.56 relative to Bregma, (Paxinos and Watson, 1998) to control for a possible heterogeneity of AVP distribution in these structures.

- In the LS, as well as in the BST and amygdala, we measured the density of AVP-ir structures by calculating, in binary transformations of the images (threshold function of Image J), the fractional area (percentages of pixels) covered by positive structures in predetermined regions with a fixed extension (500,000 μm^2) as described in our previous studies in mice and quails (Viglietti-Panzica et al., 2001, Plumari et al., 2002, Viglietti-Panzica et al., 2007, Pierman et al., 2008). In particular we considered for BST, and MeA the same levels described above and for LS two additional rostro-caudal levels (coordinates 0.48 and -0.26 relative to Bregma in the rat brain atlas, see Fig. 1).

Statistical analysis

Statistical analyses were performed using the software Statview 5.0 (Abacus Concepts, Berkely, CA, USA).

The average from 2 coronal sections was used for data analysis, since a preliminary analyses revealed no significant difference between the anterior-posterior levels for each considered nucleus (BST, MeA, and LS). We have therefore performed a two-way analysis of variance (ANOVA, genotype and sex as independent factors) on the average cells count (BST and MeA) or on the average fractional area value (BST, MeA, and LS). When appropriate, we applied a post-hoc comparison test (Fisher PLSD). Differences were considered statistically significant for $p \leq 0.05$.

RESULTS

The qualitative observation of the immunostained sections obtained from colchicine-injected WT male rats (XY) showed a distribution of AVP-ir structures in line with what has been previously described in rodents (De Vries et al., 1985, Plumari et al., 2002, Grassi et al., 2010, Rood and De Vries, 2011). In particular, we observed a dense population of AVP-ir parvocellular neurons and fibers in the BST (Fig.2), and the MeA (Fig.3), whereas the presence of positive fibers within the LS (Fig.4) was rather weak. The comparison with WT females evidenced an obvious sex difference (females have less immunoreactivity than males) for BST, MeA, and LS.

When comparing AVP immunostained sections of XY and *Tfm* (X^{tfm}/Y) male rats we observed a sharp decrease of AVP-ir elements in the BST and in the MeA of *Tfm* males (Figs.2-3). A slight decrease of the vasopressinergic innervation was detectable in the LS (Fig.4). These qualitative observations were confirmed by quantitative analysis of AVP immunoreactivity, whose results are summarized in Fig.5.

Regarding the average fractional area value, for BST, and MeA the two-way ANOVA reported significant effects for all the considered parameters (sex, genotype, and interaction sex-genotype). For the BST: sex ($F_{1,1}$=32.76, $p<0.0001$), genotype ($F_{1,1}$=15.03, p=0.001), interaction between sex and genotype ($F_{1,1,1}$=10.32, p=0.005). For the MeA: sex ($F_{1,1}$=17.88, p=0.0006), genotype ($F_{1,1}$=9.61, p=0.007), interaction between sex and genotype ($F_{1,1,1}$=8.70, p=0.009). The post-hoc Fisher PLSD test reported highly significant differences ($p<0.0001$ for BST, and $p<0.001$ for MeA) between male XY and male X^{tfm}Y, male XY and female XX, and male XY and female X^{tfm}X.

For the LS, the two-way ANOVA reported a significant effect only for sex ($F_{1,1}$= 10.32, p=0.003). The post-hoc Fisher PLSD test reported significant differences (p=0.01) for comparisons of male XY against female XX or female X^{tfm}X.

For the average cell counts value in the BST and MeA the two-way ANOVA reported significant effects for the considered parameters (sex, genotype, and interaction sex-genotype). For the BST: sex ($F_{1,1}$=161.63, $p<0.0001$), genotype ($F_{1,1}$=19.34, p=0.0004), interaction between sex and genotype ($F_{1,1,1}$=17.00, p=0.0008). For the MeA: sex ($F_{1,1}$=6.40, p=0.0223), genotype ($F_{1,1}$=4.83, p=0.0430), interaction between

sex and genotype ($F_{1,1,1}$=4.547, p=0.0488). The post-hoc Fisher PLSD test reported highly significant differences (p<0.0001 for BST, and p<0.01 for MeA) between male XY and male $X^{tfm}Y$, male XY and female XX, and male XY and female $X^{tfm}X$.

DISCUSSION

Present results confirm the existence of significant sexual differences in the parvocellular part of the AVP system of rat brain (De Vries and Panzica, 2006). In particular, we found the presence of highly significant differences in the density of AVP-ir elements (higher in males than in females) in BST, MeA, and LS nuclei. In addition, we show that the absence of a fully functional AR, causing the testicular feminization syndrome, induces significant decrease of the expression of AVP in these nuclei. This decrease is so marked that in *Tfm* rats the sex dimorphism of AVP is no longer observed. In fact, male $X^{tfm}Y$ shows a significant decrease of AVP-ir in the BST, and the MeA nuclei in comparison to male XY, whereas the observed decrease of the immunoreactivity in the LS is not significant. The lack of significant effect of AR mutation on the AVP innervation of LS is intriguing, because these fibers derive from the BST/medial amygdala system (De Vries and Buijs, 1983, Caffé et al., 1987). However, the presence of a decrease not reaching the significance could be due to the large variation of experimental measurements (see the extent of standard error in the bar graphs), as well as to the use of colchicine injection to increase the immunoreactivity in cell bodies of BST and MeA. This treatment is, in fact, interfering with the axonal flux (Brat and Brimijoin, 1992) and we observed an overall decrease of the AVP immunoreactivity in extra-hypothalamic fibers' systems of our animals.

The masculinization of the rodent hypothalamus (i.e. volume of the structure, number of neurons, expression of neurotransmitters, and neuropeptides) is widely assumed to be due to estrogenic metabolites of testosterone interacting with ERs during perinatal critical period (aromatization hypothesis), while the α-fetoprotein (AFP) protects the developing female brain from masculinization and defeminization by estrogens (for a review see McCarthy et al., 2009). However, this is not the only mechanisms present in the vertebrate brain, for example, in the avian hypothalamus estradiol has exactly the opposite effect, inducing the demasculinization of those avian behaviors and

circuits that are sexually differentiated (for a review see Balthazart and Adkins-Regan, 2002), while, in non human primates masculinization of brain and behavior is an androgen dependent process (for a review see Wallen, 2005). Finally, recent studies hypothesized an important role of epigenetic changes to induce brain sexual differentiation (for a review see Lenz et al., 2012).

In adult rats, AVP projections from the BST and MeA are dependent by the levels of circulating gonadal hormones: gonadectomy eliminates AVP expression and replacement of hormones reinstates it (De Vries et al., 1984a). Many AVP-ir neurons of BST and medial amygdala express estrogen and androgen receptors (Axelson and Van Leeuwen, 1990, Zhou et al., 1994). However, the activational effect of gonadal hormones cannot explain all differences in AVP expression because males and females exposed to similar steroid levels still differ (De Vries and Al Shamma, 1990, Wang et al., 1993), so the residual differences should be due to organizational effects of hormones. Similar effects have been described for almost all vertebrates (Goodson and Bass, 2001, Panzica et al., 2001, De Vries and Panzica, 2006).

In rodents, the mechanism by which gonadal hormones may influence the differentiation of parvocellular AVP system has been studied in a variety of models. Neonatal treatment of male and female rats gonadectomized at postnatal day 0 (PND-0) with either T, E_2 or E_2-DHT increased the number of vasopressinergic cells in the BST over that of DHT or oil treatment. However, treatment with DHT also significantly increased the number of vasopressinergic cells over that of oil treatment. Hence, these data confirm the role of E_2 in causing sexual differentiation of the brain; in addition they provide a demonstration of a masculinizing effect of a nonaromatizable androgen on a sexually dimorphic neuropeptide system (Han and De Vries, 2003).

Scordalakes and Rissman (2004) performed a study of AVP expression in *Tfm*, ArKO and ArKO-Tfm (DKO) mice. They looked the LS, the BNST and the MePD and found a clear sex difference only in the LS. Moreover, they found also that the *Tfm* background had no effect on AVP expression (WT and *Tfm* males had the greatest amount of AVP-ir in the LS, and displayed the most aggression in resident–intruder tests). These data seem in contradiction with our present results, but the study was performed in mice and without colchicine treatment, so there are both methodological and species differences.

In the same experiment Scordalakes and Rissman (2004) found in the DKO mice less AVP immunoreactivity as compared to the mutants lacking either ERalpha or AR, suggesting again a contribution of both ERalpha and AR for the differentiation of this system. In partial contrast with these studies, in our previous experiments using aromatase knock out mice (ArKO) we demonstrated that early exposure to estrogens is not necessary for the full differentiation of the parvocellular AVP system of BST and MeA. In fact, ArKO adult males do not show AVP immunoreactivity in these regions (Plumari et al., 2002), but, if they are exposed in adulthood to E_2, AVP immunoreactivity is restored to levels observed in intact wild type males (Pierman et al., 2008). This suggests that the decrease in AVP expression in gonadally intact male ArKO mice was largely due to the absence of activational rather than to organizational effects of E_2 in these mice. These results are in line with previous studies on alpha-fetoprotein knockout mice (AFP-KO). Indeed, AFP-KO females, not protected by alpha-fetoprotein, are exposed to a large amount of maternal estrogens during development, but they did not show a male-typical AVP system suggesting that, in these conditions, estrogens have probably no masculinizing effect on this sexually dimorphic neuropeptide system (Bakker et al., 2006). Finally, XY, XY^{-Sry}, XX^{Sry}, that are all male mice (in which Y^- symbolize the deletion of the gene Sry and ^{Sry} stand for the insertion of a Sry transgene) are more masculine than XX and XY^- female mice with regard to the density of AVP-expressing fibers in the lateral septum, suggesting a direct contribution of sex chromosome for the development of AVP sexual dimorphism (De Vries et al., 2002).

Recent studies, performed in mice, demonstrate that ARs have a role in masculinization of some behaviors (Raskin et al., 2009, Juntti et al., 2010). Our data on *Tfm* rats suggest that androgens, mediated by the AR, may have an important role, also in the differentiation of some circuits of the rodent brain, in particular in the differentiation of AVP-ir elements in the parvocellular sexually dimorphic system located in rat BST and MeA. This effects is sex-specific, in fact, the *Tfm* mutation apparently had no effect in heterozygous carrier female, that express a wild type copy and a mutated copy of the AR gene and, thus, can compensate the effect with the wild type AR. Based on similar studies on *Tfm* mutants, an important role for AR to induce sex differences has been proposed, in rodents, also for other sexually dimorphic neural circuits, such as spine formation in the prefrontal cortex (Hajszan et al., 2007), the size of neuronal somata in the rat MeP (Morris et al., 2005) and VMHvl (Dugger

et al., 2007), the calbindin immunoreactivity in the mouse medial preoptic area (Edelmann et al., 2007), the expression of neuronal nitric oxide synthase (nNOS) in rat BST and medial preoptic area (Martini et al., 2008), or the astrocyte number in the rat medial amygdala (Johnson et al., 2008).

The expression of mRNA for AR in the rat BST and other forebrain regions has been investigated by in situ hybridization, demonstrating its presence from embryonic day 20 (ED-20). In addition, AR mRNA levels increase throughout the perinatal period. The developmental profile of AR mRNA in the forebrain is consistent with a hypothetical role for androgens acting via the AR in the organization of male typical neural circuits (McAbee and DonCarlos, 1998).

Although males and females both show increases in relative AR mRNA expression between ED-20 and PND-10, the progression of AR mRNA levels is sex and region specific. On ED-20, PND-0, and PND-4, the relative expression of AR mRNA is not different in males and females in any of forebrain areas analyzed. Between PND-4 and PND-10 (i.e. during the second half of the critical period for sexual differentiation), sex differences in AR mRNA expression arise in the BST and the MPOA, but not in the other analyzed regions (lateral septum, ventromedial hypothalamus, and arcuate nucleus). No data were provided for MeA in this study (McAbee and DonCarlos, 1998). These region-specific patterns in the profile of AR mRNA expression suggest a functional difference in the sensitivity of individual areas to the potential developmental actions of androgens. Experimental studies involving gonadectomy at PND-0 demonstrated that the onset of this sexual difference in the AR mRNA expression at PND-10 is due to circulating levels of T (McAbee and DonCarlos, 1999b), but males gonadectomized on PND-0 and treated daily with DHT, a nonaromatizable androgen, had low levels of AR mRNA that were not significantly different from AR mRNA levels in intact females. In contrast, males gonadectomized on PND-0 and treated daily with diethylstilbestrol (DES), a synthetic estrogen, maintained male-typical levels of AR mRNA in the BST and the MPOA. Treatment with the androgen receptor antagonist, flutamide had no effect on AR mRNA expression. The conclusion is that AR mRNA is up-regulated by estrogen but is not regulated by androgen during the early postnatal period (McAbee and DonCarlos, 1999a).

In summary, available data demonstrate that AR and ER are largely present in the AVP system of BST/MeA, that the expression of these receptors in BST is sexually

differentiated from early stage of postnatal development, and that the development of AR mRNA differences is due to the presence of estrogens. Therefore we can hypothesize that both ER and AR are necessary to develop the sexual dimorphism of AVP parvocellular system of BST and MeA, and that their action is sequential during the perinatal critical period. The lack of effect of aromatase deletion (Pierman et al., 2008) could be due to a compensatory effect of the DHT metabolite 3beta-diol that can stimulate the AVP promoter through ERbeta (Pak et al., 2007, 2009).

The present analysis cannot determine whether the morphological effects of a defective AR are due to the protein dysfunction during the perinatal, pubertal, or adult period because *Tfm* rats have defective AR throughout development. Whether AR stimulation acts on the developing and/or the adult parvocellular AVP system must await further study. The mechanism could also involve epigenetic changes. In fact, a recent study demonstrated that a transient reduction in the amygdala of the expression of methyl-CpG-binding protein 2 (MeCP2, a protein binding to methylated DNA) decreased AR mRNA in two-week old males (but not in the adult), whereas it permanently eliminated the sex difference in the AVP system (Forbes-Lorman et al., 2012). Thus, the reduction in AR expression during early postnatal life may play a role in the decrease in AVP expression in MeCP2-siRNA treated males.

In conclusion, present data indicate that androgens, through the action of AR and in cooperation with estrogens, may have a relevant role in the organization and modulation of gender-specific components of the neural circuits that mediates male social behaviors, in particular the AVP producing population of BST and MeA.

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Legends to the figures

Fig.1. Schematic diagrams showing the levels and the regions (grey squares) where the counts were performed. Letters are referred to the different level of Bregma: a) 0.48 for lateral septum; b) -0.26 for lateral septum; c) -0.40 for bed nucleus of the stria terminalis; d) -0.80 for bed nucleus of the stria terminalis; e) -2.12 for medial amygdaloid nucleus; f) -2.56 for medial amygdaloid nucleus. Drawings from the atlas of Paxinos and Watson (1998).

Fig.2. Representative photomicrographs showing AVP-immunoreactive staining in the bed nucleus of the stria terminalis (BST) of male (XY) and female (XX) wild type rats as well as in *Tfm* rats of both sexes ($X^{tfm}Y$ and $X^{tfm}X$). f – fornix; opt – optic tract. The scale bar represents 150 µm.

Fig.3. Representative photomicrographs showing AVP-immunoreactive staining in medial amygdaloid nucleus (MeA) of male (XY) and female (XX) wild type rats as well as in *Tfm* rats of both sexes ($X^{tfm}Y$ and $X^{tfm}X$). f – fornix; opt – optic tract. The scale bar represents 150 µm.

Fig.4. Representative photomicrographs showing AVP-immunoreactive staining in the lateral septum (LS) of male (XY) and female (XX) wild type rats as well as in *Tfm* rats of both sexes ($X^{tfm}Y$ and $X^{tfm}X$). * : third ventricle. The scale bar represents 100 µm.

Fig.5. Bar graphs illustrating the fractional area covered by AVP-ir elements (means+/- S.E.M.; top panel) and the cells mean (means+/- S.E.M.; bottom panel) in the bed nucleus of the stria terminalis (BST), medial amygdaloid nucleus (MeA), and lateral septum (LS), of male (XY) and female (XX) wild type rats as well as in *Tfm* rats of both sexes ($X^{tfm}Y$ and $X^{tfm}X$). ***$p<0.001$, **$p<0.01$, *$p<0.05$ different from XY males;

REFERENCES

Attardi B, Geller LN, Ohno S (1976) Androgen and estrogen receptors in brain cytosol from male, female, and testicular feminized (tfm/y hermaphrodite) mice. Endocrinology 98:864-874.

Axelson JF, Van Leeuwen FW (1990) Differential localization of estrogen receptors in various vasopressin synthetizing nuclei of the rat brain. J Neuroendocrinol 2:209-216.

Bakker J, De Mees C, Douhard Q, Balthazart J, Gabant P, Szpirer J, Szpirer C (2006) Alpha-fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens. Nat Neurosci 9:220-226.

Balthazart J, Adkins-Regan E (2002) Sexual differentiation of brain and behavior in birds. In: Hormones, Brain and Behavior, vol. 4 (Pfaff, D. W. et al., eds), pp 223-301 New York: Academic Press.

Bingham B, Viau V (2008) Neonatal gonadectomy and adult testosterone replacement suggest an involvement of limbic arginine vasopressin and androgen receptors in the organization of the hypothalamic-pituitary-adrenal axis. Endocrinology 149:3581-3591.

Brat DJ, Brimijoin S (1992) A paradigm for examining toxicant effects on viability, structure, and axonal transport of neurons in culture. Mol Neurobiol 6:125-135.

Brinkmann AO (2001) Molecular basis of androgen insensitivity. Mol Cell Endocrinol 179:105-109.

Caffé AR, Van Leeuwen FW, Luiten PGM (1987) Vasopressin cells in the medial amygdala of the rat project to the lateral septum and ventral hyppocampus. J Comp Neurol 261:237-252.

Choleris E, Clipperton-Allen AE, Phan A, Kavaliers M (2009) Neuroendocrinology of social information processing in rats and mice. Front Neuroendocrinol 30:442-459.

Compaan JC, Buijs RM, Pool CW, De Ruiter AJH, Koolhaas JM (1993) Differential lateral septal vasopressin innervation in aggressive and nonaggressive male mice. Brain Res Bull 30:1-6.

Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM (1998) Sexual differentiation of the vertebrate brain: principles and mechanisms. Front Neuroendocrinol 19:323-362.

Cooke BM, Breedlove SM, Jordan CL (2003) Both estrogen receptors and androgen receptors contribute to testosterone-induced changes in the morphology of the medial amygdala and sexual arousal in male rats. Horm Behav 43:336-346.

De Vries GJ, Al Shamma HA (1990) Sex differences in hormonal responses of vasopressin pathways in the rat brain. J Neurobiol 21:686-693.

De Vries GJ, Buijs RM (1983) The origin of the vasopressinergic and oxytocinergic innervation of the rat brain with special reference to the lateral septum. Brain Res 273:307-317.

De Vries GJ, Buijs RM, Sluiter AA (1984a) Gonadal hormone actions on the morphology of the vasopressinergic innervation of the adult rat brain. Brain Res 298:141-145.

De Vries GJ, Buijs RM, Van Leeuwen FW (1984b) Sex differences in vasopressin and other neurotransmitter systems in the brain. Prog Brain Research 61:185-203.

De Vries GJ, Buijs RM, Van Leeuwen FW, Caffé AR, Swaab DF (1985) The vasopressinergic innervation of the brain in normal and castrated rats. J Comp Neurol 233:236-254.

De Vries GJ, Panzica GC (2006) Sexual differentiation of central vasopressin and vasotocin systems in vertebrates: different mechanisms, similar endpoints. Neuroscience 138:947-955.

De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-Badge R, Burgoyne PS, Arnold AP (2002) A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. J Neurosci 22:9005-9014.

Del Abril A, Segovia S, Guillamon A (1987) The bed nucleus of the stria terminalis in the rat: regional sex differences controlled by gonadal steroids early after birth. Dev Brain Res 32:295-300.

DonCarlos LL (1996) Developmental Profile and Regulation of Estrogen Receptor (Er) mRNA Expression in the Preoptic Area of Prenatal Rats. Dev Brain Res 94:224-233.

DonCarlos LL, Handa RJ (1994) Developmental profile of estrogen receptor mRNA in the preoptic area of male and female neonatal rats. Dev Brain Res 79:283-289.

Dugger BN, Morris JA, Jordan CL, Breedlove SM (2007) Androgen receptors are required for full masculinization of the ventromedial hypothalamus (VMH) in rats. Horm Behav 51:195-201.

Edelmann M, Wolfe C, Scordalakes EM, Rissman EF, Tobet S (2007) Neuronal nitric oxide synthase and calbindin delineate sex differences in the developing hypothalamus and preoptic area. Dev Neurobiol 67:1371-1381.

Fernandez R, Collado P, Garcia Doval S, Garcia-Falgueras A, Guillamon A, Pasaro E (2003) A molecular method for classifying the genotypes obtained in a breeding colony from testicular feminized (Tfm) rats. Horm Metab Res 35:197-200.

Ferris CF, Delville Y, Miller MA, Dorsa DM, De Vries GJ (1995) Distribution of small vasopressinergic neurons in golden hamsters. J Comp Neurol 360:589-598.

Field EF, Whishaw IQ, Pellis SM, Watson NV (2006) Play fighting in androgen-insensitive tfm rats: evidence that androgen receptors are necessary for the development of adult playful attack and defense. Dev Psychobiol 48:111-120.

Forbes-Lorman RM, Rautio JJ, Kurian JR, Auger AP, Auger CJ (2012) Neonatal MeCP2 is important for the organization of sex differences in vasopressin expression. Epigenetics 7:230-238.

Garcia-Falgueras A, Pinos H, Collado P, Pasaro E, Fernandez R, Jordan CL, Segovia S, Guillamon A (2005) The role of the androgen receptor in CNS masculinization. Brain Res 1035:13-23.

Gobinet J, Poujol N, Sultan C (2002) Molecular action of androgens. Mol Cell Endocrinol 198:15-24.

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Res Rev 35:246-265.

Grassi D, Amorim MA, Garcia-Segura LM, Panzica G (2010) Estrogen receptor alpha is involved in the estrogenic regulation of arginine vasopressin immunoreactivity in the supraoptic and paraventricular nuclei of ovariectomized rats. Neurosci Lett 474:135-139.

Hajszan T, Maclusky NJ, Johansen JA, Jordan CL, Leranth C (2007) Effects of androgens and estradiol on spine synapse formation in the prefrontal cortex of normal and testicular feminization mutant male rats. Endocrinology 148:1963-1967.

Han TM, De Vries GJ (2003) Organizational Effects of Testosterone, Estradiol, and Dihydrotestosterone on Vasopressin mRNA Expression in the Bed Nucleus of the Stria Terminalis. J Neurobiol 54:502–510.

Hines M, Allen LS, Gorski RA (1992) Sex Differences in Subregions of the Medial Nucleus of the Amygdala and the Bed Nucleus of the Stria Terminalis of the Rat. Brain Res 579:321-326.

Hughes IA, Davies JD, Bunch TI, Pasterski V, Mastroyannopoulou K, MacDougall J (2012) Androgen insensitivity syndrome. Lancet 380:1419-1428.

Johnson RT, Breedlove SM, Jordan CL (2008) Sex differences and laterality in astrocyte number and complexity in the adult rat medial amygdala. J Comp Neurol 511:599-609.

Juntti SA, Tollkuhn J, Wu MV, Fraser EJ, Soderborg T, Tan S, Honda S, Harada N, Shah NM (2010) The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors. Neuron 66:260-272.

Lenz KM, Nugent BM, McCarthy MM (2012) Sexual differentiation of the rodent brain: dogma and beyond. Front Neurosci 6:26.

Martini M, Di Sante G, Collado P, Pinos H, Guillamon A, Panzica GC (2008) Androgen receptors are required for full masculinization of nitric oxide synthase system in rat limbic-hypothalamic region. Horm Behav 54:557-564.

McAbee MD, DonCarlos LL (1998) Ontogeny of region-specific sex differences in androgen receptor messenger ribonucleic acid expression in the rat forebrain. Endocrinology 139:1738-1745.

McAbee MD, DonCarlos LL (1999a) Estrogen, but not androgens, regulates androgen receptor messenger ribonucleic acid expression in the developing male rat forebrain. Endocrinology 140:3674-3681.

McAbee MD, DonCarlos LL (1999b) Regulation of Androgen Receptor Messenger Ribonucleic Acid Expression in the Developing Rat Forebrain. Endocrinology 140:1807-1814.

McCarthy MM (2008) Estradiol and the developing brain. Physiol Rev 88:91-134.

McCarthy MM, Arnold AP (2011) Reframing sexual differentiation of the brain. Nat Neurosci 14:677-683.

McCarthy MM, Konkle ATM (2005) When is a sex difference not a sex difference? Front Neuroendocrinol 26:85-102.

McCarthy MM, Wright CL, Schwarz JM (2009) New tricks by an old dogma: mechanisms of the Organizational/Activational Hypothesis of steroid-mediated sexual differentiation of brain and behavior. Horm Behav 55:655-665.

McPhaul MJ (2002) Androgen receptor mutations and androgen insensitivity. Mol Cell Endocrinol 198:61-67.

Morris JA, Jordan CL, Dugger BN, Breedlove SM (2005) Partial demasculinization of several brain regions in adult male (XY) rats with a dysfunctional androgen receptor gene. J Comp Neurol 487:217-226.

Pak TR, Chung WC, Hinds LR, Handa RJ (2007) Estrogen receptor-beta mediates dihydrotestosterone-induced stimulation of the arginine vasopressin promoter in neuronal cells. Endocrinology 148:3371-3382.

Pak TR, Chung WC, Hinds LR, Handa RJ (2009) Arginine vasopressin regulation in pre- and postpubertal male rats by the androgen metabolite 3beta-diol. Am J Physiol Endocrinol Metab 296:E1409-1413.

Panzica GC, Aste N, Castagna C, Viglietti-Panzica C, Balthazart J (2001) Steroid-induced plasticity in the sexually dimorphic vasotocinergic innervation of the avian brain: behavioral implications. Brain Res Rev 37:178-200.

Panzica GC, Aste N, Viglietti-Panzica C, Ottinger MA (1995) Structural sex differences in the brain: influence of gonadal steroids and behavioral correlates. J Endocrinol Invest 18:232-252.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. Fourth Edition. San Diego: Academic Press.

Perakis A, Stylianopoulou F (1986) Effects of a prenatal androgen peak on rat brain sexual differentiation. J Endocrinol 108:281-285.

Pierman S, Sica M, Allieri F, Viglietti-Panzica C, Panzica GC, Bakker J (2008) Activational effects of estradiol and dihydrotestosterone on social recognition and the arginine-vasopressin immunoreactive system in male mice lacking a functional aromatase gene. Horm Behav 54:98-106.

Plumari L, Viglietti Panzica C, Allieri F, Honda S, Harada N, Absil P, Balthazar J, Panzica GC (2002) Changes in the Arginine-Vasopressin Immunoreactive Systems in Male Mice Lacking a Functional Aromatase Gene. J Neuroendocrinol 14:971–978.

Raskin K, de Gendt K, Duittoz A, Liere P, Verhoeven G, Tronche F, Mhaouty-Kodja S (2009) Conditional inactivation of androgen receptor gene in the nervous system: effects on male behavioral and neuroendocrine responses. J Neurosci 29:4461-4470.

Robinson S, Penatti CA, Clark AS (2012) The role of the androgen receptor in anabolic androgenic steroid-induced aggressive behavior in C57BL/6J and Tfm mice. Horm Behav 61:67-75.

Rood BD, De Vries GJ (2011) Vasopressin innervation of the mouse (Mus musculus) brain and spinal cord. J Comp Neurol 519:2434-2474.

Rood BD, Stott RT, You S, Smith CJ, Woodbury ME, De Vries GJ (2013) Site of origin of and sex differences in the vasopressin innervation of the mouse (Mus musculus) brain. J Comp Neurol 521:2321-2358.

Roselli CE, Salisbury RL, Resko JA (1987) Genetic evidence for androgen-dependent and independent control of aromatase activity in the rat brain. Endocrinology 121:2205-2210.

Rotzinger S, Lovejoy DA, Tan LA (2010) Behavioral effects of neuropeptides in rodent models of depression and anxiety. Peptides 31:736-756.

Scordalakes EM, Rissman EF (2004) Aggression and arginine vasopressin immunoreactivity regulation by androgen receptor and estrogen receptor alpha. Genes Brain Behav 3:20-26.

Segovia S, Garcia-Falgueras A, Carrillo B, Collado P, Pinos H, Perez-Laso C, Vinader-Caerols C, Beyer C, Guillamon A (2006) Sexual dimorphism in the vomeronasal system of the rabbit. Brain Res 1102:52-62.

Streefkerk JG (1972) Inhibition of erythrocyte peroxidase activity by treatment with hydrogen peroxide following methanol. J Histochem Cytochem 20:829-831.

Tejada LD, Rissman EF (2012) Sex differences in social investigation: effects of androgen receptors, hormones and test partner. J Neuroendocrinol 24:1144-1153.

Veenema AH, Beiderbeck DI, Lukas M, Neumann ID (2010) Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. Horm Behav 58:273-281.

Veenema AH, Neumann ID (2008) Central vasopressin and oxytocin release: regulation of complex social behaviours. Prog Brain Res 170:261-276.

Veening JG, Coolen LM (1998) Neural activation following sexual behavior in the male and female rat brain. Behav Brain Res 92:181-193.

Veening JG, Coolen LM, de Jong TR, Joosten HW, de Boer SF, Koolhaas JM, Olivier B (2005) Do similar neural systems subserve aggressive and sexual behaviour in male rats? Insights from c-Fos and pharmacological studies. Eur J Pharmacol 526:226-239.

Viglietti-Panzica C, Balthazart J, Fratesi S, Plumari L, Absil P, Panzica GC (2001) Estradiol mediates effects of testosterone on vasotocin-immunoreactivity in the adult quail brain. Horm Behav 40:445-461.

Viglietti-Panzica C, Mura E, Panzica GC (2007) Effects of early embryonic exposure to genistein on male copulatory behavior and vasotocin system of Japanese quail. Horm Behav 51:355-363.

Villalba C, Auger CJ, de Vries GJ (1999) Androstenedione effects on the vasopressin innervation of the rat brain. Endocrinology 140:3383-3386.

Wallen K (2005) Hormonal influences on sexually differentiated behavior in nonhuman primates. Front Neuroendocrinol 26:7-26.

Wang Z, Bullock NA, De Vries GJ (1993) Sexual differentiation of vasopressin projections of the bed nucleus of the stria terminals and medial amygdaloid nucleus in rats. Endocrinology 132:2299-2306.

Watson RE, Wiegand SJ, Clough RW, Hoffman GE (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7:155-159.

Wu MV, Manoli DS, Fraser EJ, Coats JK, Tollkuhn J, Honda S, Harada N, Shah NM (2009) Estrogen masculinizes neural pathways and sex-specific behaviors. Cell 139:61-72.

Zhou JN, Hofman MA, Gooren LJG, Swaab DF (1995) A sex difference in the human brain and its relation to transsexuality. Nature 378:68-70.

Zhou L, Blaustein JD, De Vries GJ (1994) Distribution of androgen receptor immunoreactivity in vasopressin- and oxytocin-immunoreactive neurons in the male rat brain. Endocrinology 134:2622-2627.

Zuloaga DG, Morris JA, Jordan CL, Breedlove SM (2008) Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic-pituitary-adrenal axis. Horm Behav 54:758-766.

Zuloaga DG, Poort JE, Jordan CL, Breedlove SM (2011) Male rats with the testicular feminization mutation of the androgen receptor display elevated anxiety-related behavior and corticosterone response to mild stress. Horm Behav 60:380-388.

Figure 1

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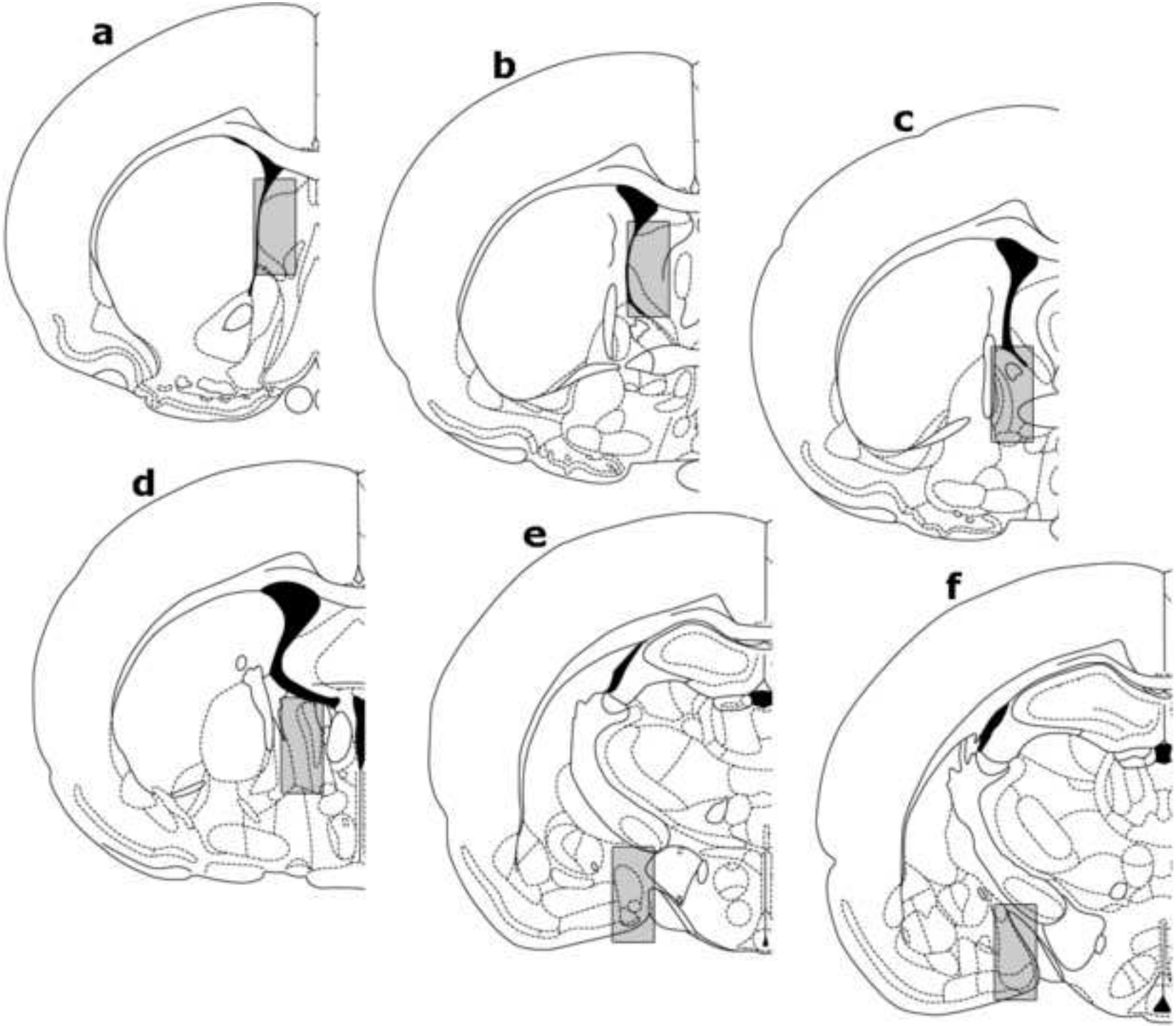

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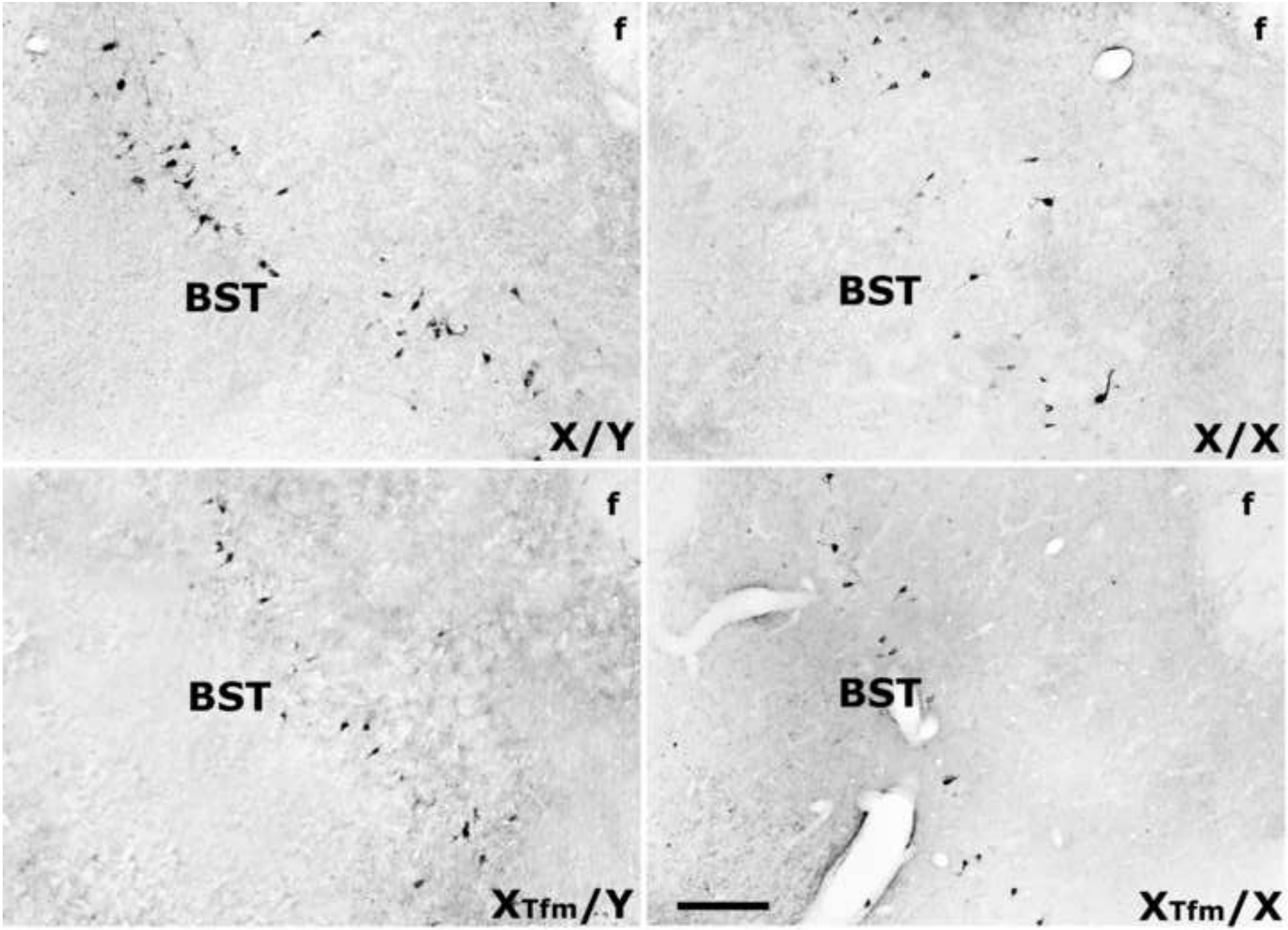

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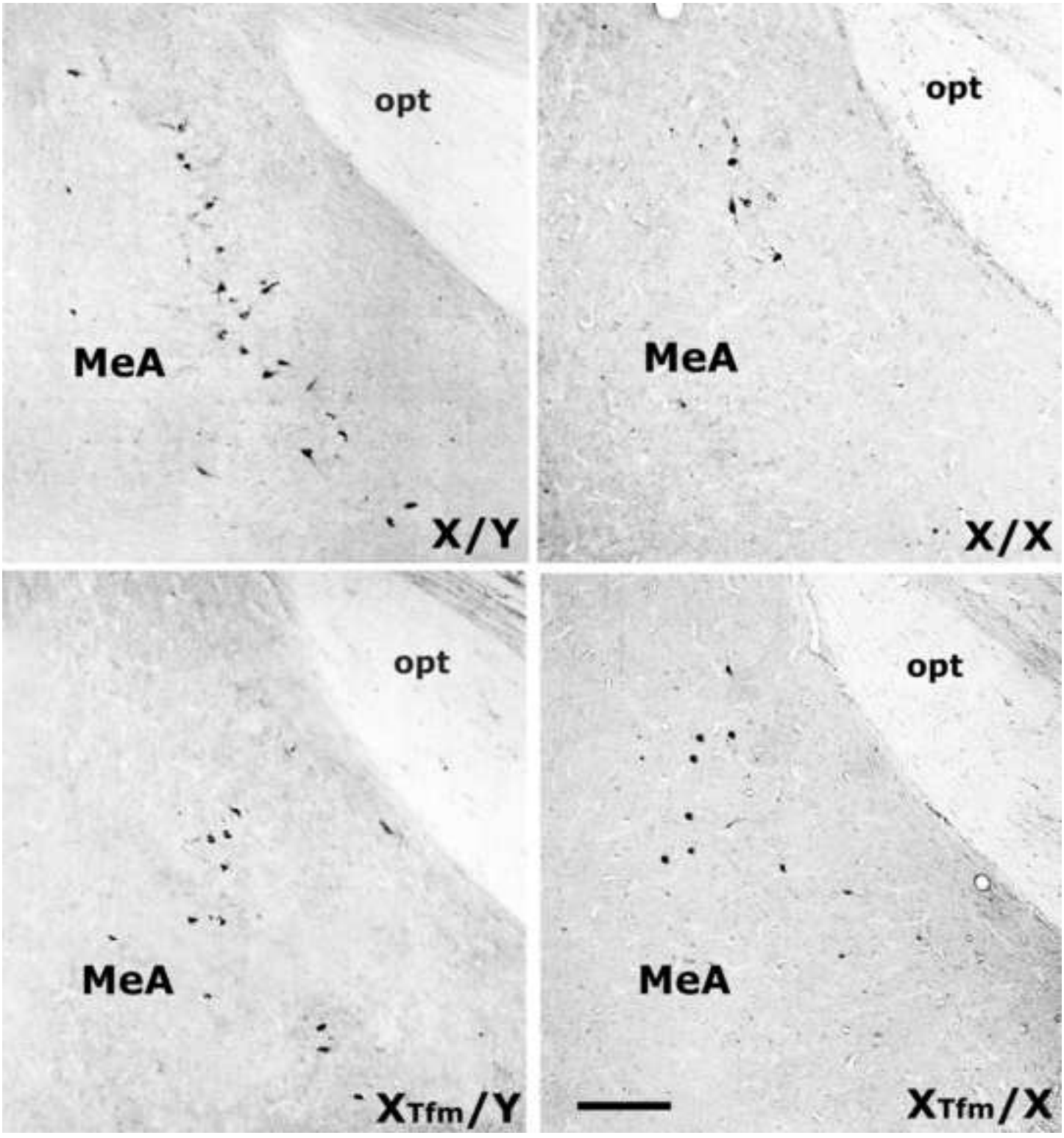

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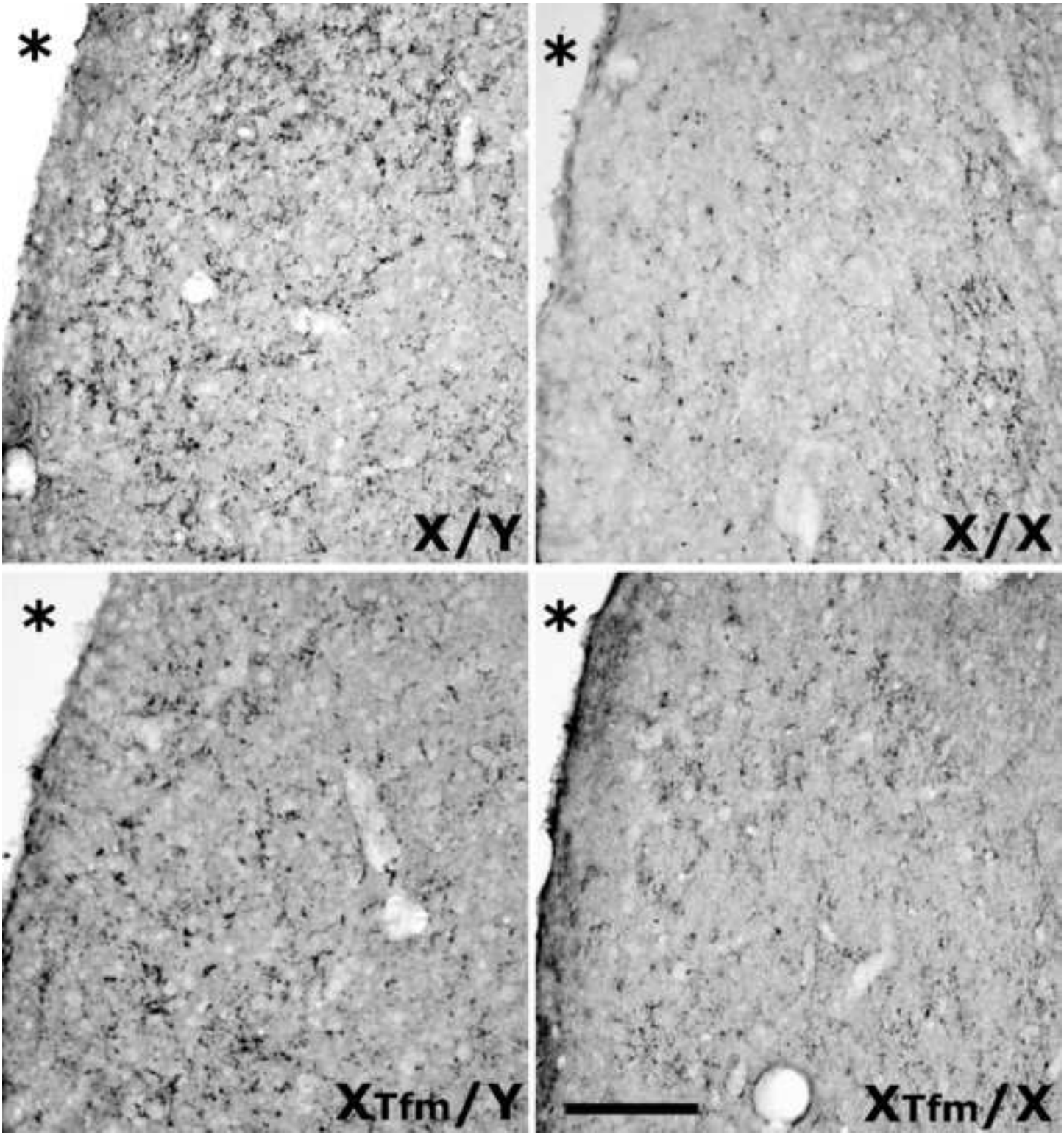

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