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Sexual dimorphism in hybrids rats

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ABSTRACT

Laboratory rat strains descend from Wistar rats as a consequence of artificial selection. Previously we reported that the medial posterior division of the bed nucleus of the stria terminalis (BSTMP) was sexually dimorphic in Wistar and Long–Evans strains while the medial anterior division (BSTMA) and the locus coeruleus (LC) only showed sex differences in the ancestor Wistar strain. The lateral posterior division (BSTLP) was isomorphic in both strains. The present work studies the number of neurons in the BSTMP, BSTMA, BSTLP and LC of male and female Wistar and Long–Evans rats (F_0) and their hybrid F_1 and F_2 generations. The BSTMP is sexually dimorphic in the F_0, F_1 and F_2 generations while sex differences in the LC are only seen in F_0 Wistar rats but not in the F_0 Long–Evans or the F_1 and F_2 hybrid generations. Sex differences in the BSTMA are seen in F_0 Wistar but not in F_0 Long–Evans rats and completely disappear in the F_2 generations. The number of neurons in the LC of both males and females decreased in heterozygotic individuals (F_1) but increased in homozygotic (F_2). However, the number of neurons in the BSTMP changes significantly over the generations, although the ratio of neurons (female/male) is stable and unaffected in homo- or heterozygosis. Thus, the mechanism that regulates the neuronal female/male ratio would be different from the one that controls the number of neurons. The facts that sex differences in the BSTMP are not affected by homo- or heterozygosis and that they are seen in several mammalian orders suggest the existence of a "fixed" type of brain sex differences in the Mammalia Class.

1. Introduction

Sex differences in the mammalian brain have two main characteristics: First, they appear in neural networks, such as the vomeronasal pathway, which controls physiological and behavioral aspects of reproduction; and second, they take one of two opposite morphological patterns (Guillamon and Segovia, 1996; Segovia and Guillamon, 1993; Segovia et al., 1999). In some CNS structures males have larger morphological measurements (number of neurons, volume, etc.) than females, while in others the opposite is true. In the rat, the male>female pattern is characteristic of rat brain structures that receive vomeronasal input such as the accessory olfactory bulb, bed nucleus of the accessory olfactory tract, medial amygdala, posteromedial cortical amygdaloid nucleus, medial posterior division of the bed nucleus of the stria terminalis and the medial preoptic area (for a review, see Arnold and Gorski, 1984; Guillamon and Segovia, 1996; Segovia and Guillamon, 1993; Simerly, 2002), while the female>male pattern is seen in the anteroventral periventricular nucleus

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(Orikasa and Sakuma, 2003; Simerly et al., 1998), parastrial nucleus (Del Abril et al., 1990) and in the brain stem (i.e., locus coeruleus [LC]; Guillamon et al., 1988b).

Brain sex differences have also been reported in mice. The male>female pattern has been described in a cell group medial to the medial extension of the bed nucleus of the stria terminalis in two mice strains (Brown et al., 1999) and in the principal nucleus of the bed nucleus of the stria terminalis (Forger et al., 2004). The female>male pattern was found in cells located beneath the anterior commissure (Brown et al., 1999) and in the anteroventral periventricular nucleus (Forger et al., 2004; Simerly et al., 1998). Furthermore, these two patterns of sexual dimorphism have also been seen in rabbits (Bisenius et al., 2006; Segovia et al., 2006).

The bed nucleus of the stria terminalis (BST) is a useful model for the study of brain sex differences because it has dimorphic and isomorphic subdivisions. For instance, in Wistar rats the medial posterior division (BSTMP) presents the male>female pattern (Del Abril et al., 1987; Garcia-Falgueras et al., 2005; Guillamon et al., 1988a), while the medial anterior (BSTMA) and the lateral anterior (BSTLA) show a female>male pattern (Del Abril et al., 1987; Garcia-Falgueras et al., 2005; Guillamon et al., 1988a). The lateral posterior region (BSTLP) is isomorphic (Del Abril et al., 1987; Garcia-Falgueras et al., 2005). Moreover, BSTMP is one of the structures that receive vomeronasal input (Shipley et al., 2004) and, in the rat, has been related to the control of male copulatory behavior (Claro et al., 1995; Emery and Sachs, 1976). In Wistar rats, the LC, a structure that sends rich noradrenergic projections to olfactory structures (Shipley et al., 1985), is sexually dimorphic (Garcia-Falgueras et al., 2005; Guillamon et al., 1988b; Luque et al., 1992; Pinos et al., 2001), but not in the Sprague–Dawley (Babstock et al., 1997) and the Long–Evans (Garcia-Falgueras et al., 2005) strains.

In a recent paper we compared sex differences in the number of neurons in the BST and LC in Wistar and Long–Evans rats (Garcia-Falgueras et al., 2005). In that work we found that the BSTMP is sexually dimorphic (male>female) in both strains while the BSTMA, BSTLA and LC only show differences (female>male, in all cases) in the Wistar strain. The lateral juxtacapsular (BSTLjx) and the lateral posterior (BSTLP) BST subdivisions are isomorphic in the Wistar and the Long–Evans strains (Garcia-Falgueras et al., 2005).

It seems that selection has induced brain sexual dimorphism in some brain nuclei, like the BSTMP, that present a constant pattern of sexual dimorphism independently of strain and species (Allen and Gorski, 1990; Chung et al., 2000; Del Abril et al., 1987; Forger et al., 2004; Garcia-Falgueras et al., 2005; Guillamon et al., 1988a; Hines et al., 1985; Kruijver et al., 2000; Segovia et al., 2006; Stefanova and Ovtscharoff, 2000), while other nuclei, like the LC, show less stability and the sex differences may disappear not only in different species but also between strains of the same species (Garcia-Falgueras et al., 2005; Segovia et al., 2006).

Some studies have related sexual brain differences with genes. Forger et al. (2004) have related sex differences in the BSTMP with the *bax* gene and Brown et al. (1999) also related sex differences in the BST of SF-1 gene-disrupted mice. These works suggest a role of genes in sexual differentiation of the brain.

The production of hybrids, and the consequent increment of genetic variability, is another strategy to study the effect of genetic background on brain sex differences. We have seen a stable male>female pattern in the number of neurons in the BSTMP that appears in several species and strains. However, the female>male pattern for the number of neurons in the LC and the BSTMA seems to be unstable because it is seen in Wistar but not in Long–Evans rats. In the present work, we study the effect of heterozygosis of the two sexually dimorphic patterns over two successive generations. This was achieved by counting the number of neurons in the BSTMP, BSTMA, BSTLP and LC of male and female hybrids obtained by crossing individuals of the Wistar and the Long–Evans strains. The individuals are classified according to two criteria: matrilineal ascendance and molecular characteristics identified by the study of specific highly polymorphic DNA regions known as simple sequence length polymorphisms (SSLPs) or microsatellite DNA. These 1–6 simple nucleotide repeat sequences are often used as genetic markers for genotyping different strains.

2. Results

2.1. *The characterization of Long–Evans and Wistar strains*

With respect to the four molecular markers that characterized the Wistar and the Long–Evans strains, the transitions from F_0 to F_1 and from F_1 to F_2 had values of 100% homozygosis (F_0), 100% heterozygosis (F_1) and a partial recuperation of homozygosis (F_2) for the cited markers (Table 1).

The use of molecular markers restricts the number of subjects per group because only the homozygotic individuals are selected for analysis in F_2. Thus, it was not possible to apply both criteria (matrilineal and molecular) in all the experimental groups, but in those in which it was possible, the results obtained reinforce each other. In particular, we were able to apply both criteria to subjects whose brain structures did not show sex differences in F_2. In LC, BSTMA and BSTLP, there were no statistically significant differences in the number of neurons between the subjects grouped by the matrilineal criterion and those grouped by the molecular marker criterion: LC (WWLE, $t(19)=1.14$, n.s.; LELEW; $t(21)=0.04$, n.s.); BSTMA (WWLE: $t(14)=0.97$, n.s.; LELEW: $t(21)=0.80$, n.s.); BSTLP (WWLE: $t(12)=1.44$, n.s.; LELEW: $t(21)=0.98$, n.s.).

2.2. *Sex differences and comparison of the number of neurons between Wistar and Long–Evans strains*

2.2.1. *Bed nucleus of the stria terminalis*

There was a statistically significant main effect of sex ($F_{1,18}=24.02$; $p<0.0001$) in the BSTMP showing that males have more neurons than females in this structure in both Wistar and Long–Evans strains (Tables 2 and 3). There were statistically significant main effects of strain in the BSTMA ($F_{1,18}=6.77$; $p<0.02$) and the BSTLP ($F_{1,18}=11.01$; $p<0.005$) showing that Wistar rats have more neurons than Long–Evans rats in the BSTMA, while inspection of the means showed that female Wistar rats have more neurons in the BSTMA than the other groups (at least $p<0.05$) (Table 2). Thus, the Wistar strain showed sex differences (female>male) in the BSTMA (Table 2). With respect to the BSTLP, the Long–Evans females have more neurons than male and female Wistar rats ($p<0.05$ in both

Table 1 – Molecular analysis of the four SSLPs: D1Rat221 (248–336 bp), D4Rat2 (147–133 bp), D9Rat30 (172–158 bp) and D20Rat43 (218–261 bp) (columns) in F_0, F_1 and F_2 animals (rows) and experimental design of the investigation

Generation	Classification criteria of the subjects	Wistar strain, size of fragments obtained (bp) (D1Rat221, D4Rat2, D9Rat30 and D20Rat43)	Long–Evans strain, size of fragments obtained (bp) (D1Rat221, D4Rat2, D9Rat30, D20Rat43)
	Matrilineal	W	LE
F_0	SSLPs	100% of the subjects were homozygotic for the alleles characteristic of the Wistar strain 248-147-172-218 bp, 248-147-172-218 bp	100% of the subjects were homozygotic for the alleles characteristic of the Long–Evans strain 336-133-158-261 bp, 336-133-158-261 bp
	Matrilineal	WLE	LEW
F_1	SSLPs	100% of the offspring were heterozygotic for the alleles characteristic of the two strains 248-147-172-218 bp, 336-133-158-261 bp	100% of the offspring were heterozygotic for the alleles characteristic of the two strains 248-147-172-218 bp, 336-133-158-261 bp
	Matrilineal	WWLE	LELEW
F_2	SSLPs	Multiple genotypes. We selected the offspring with four alleles in homozygosis, characteristics of the Wistar strain 248-147-172-218 bp, 248-147-172-218 bp	Multiple genotypes. We selected the offspring with four alleles in homozygosis, characteristics of the Long–Evans strain 336-133-158-261 bp, 336-133-158-261 bp

cases) but are not different from the Long–Evans males (Tables 2 and 3). The BSTLP is isomorphic in both strains.

2.2.2. *Locus coeruleus*

There was a main effect of strain ($F_{1,17}=7.56$; $p<0.01$) showing that Wistar rats have a greater number of neurons in the LC than the Long–Evans rats. Sex interacted with strain significantly ($F_{1,17}=5.76$; $p<0.03$) since female Wistar rats have more neurons than the other groups (at least $p<0.05$). The LC showed sex differences in the Wistar (female > male) but not in the Long–Evans strain. The strain differences reflect the larger number of neurons in the female Wistar rats than in the Long–Evans rats or male Wistar (Tables 2 and 3).

2.3. *Study of the number of neurons in the bed nucleus of the stria terminalis through the F_0, F_1 and F_2 generations*

2.3.1. *Medial posterior division (BSTMP)*

The first step was to test the existence of sex differences in the number in neurons of the BSTMP. In this structure, males of both strains present more neurons than their corresponding females in all three generations (F_0, F_1, F_2) (at least $p<0.03$, in all cases) (Table 3).

In both male and female rats there was a statistically significant effect of group (males: $F_{5,32}=8.00$, $p<0.0001$; females: $F_{5,35}=8.93$, $p<0.0001$). Tables 4 (males) and 5 (females) display the means of all groups and, as can be seen, heterozygosis ($F_0 \rightarrow F_1$) does not affect males. With respect to females, heterozygosis does not affect females with a Wistar origin (W vs. WLE, n.s.) but does affect females with Long–Evans mothers (LE vs. LEW, $p<0.05$). In contrast, the increase of homozygosis ($F_1 \rightarrow F_2$) accompanied a decreased in the number of neurons in all groups of F_2 male and female rats ($p<0.05$ in all comparisons). Thus, in the BSTMP, a structure related to male copulatory behavior, the number of neurons in both male and female rats is decreased in the homozygotic F_2 animals.

Table 2 – Sex differences and comparison of the number of neurons in male and female Wistar and Long–Evans (F_0) rats (means ± SEM)

		BSTMP	BSTMA	BSTLP	LC
W	♂	23,179 ± 2487.4*	10,604 ± 1239.4*+	4061 ± 502.2	1604 ± 62.7*
	♀	15,105 ± 1957.9	17,264 ± 1807+	3597 ± 364.9	2090 ± 81.4+
LE	♂	25,053 ± 12,28.1*	8323 ± 1757	5506 ± 700	1571 ± 167.7
	♀	12,014 ± 2841.8	10,975 ± 1669	6776 ± 942++	1594 ± 48

W: Wistar; LE: Long–Evans; BSTMP: medial posterior division of the bed nucleus of the stria terminalis (BST); BSTMA: medial anterior division of the BST; BSTLP: lateral posterior division of the BST; LC: locus coeruleus. *At least $p<0.05$ with respect to female of the same strain; +at least $p<0.05$ with respect to the Long–Evans strain; +at least $p<0.05$ with respect to the Wistar strain.

Table 3 – Patterns of sex differences in the number of neurons in F_0, F_1 and F_2

		BSTMP	BSTMA	BSTLP	LC	BSTPM ♂/♀ ratio
F_0	W	♂ > ♀*	♀ > ♂**		♀ > ♂***	0.65
	LE	♂ > ♀*				0.48
F_1	WLE	♂ > ♀*	♀ > ♂**			0.68
	LEW	♂ > ♀*		♀ > ♂**		0.69
F_2	WWLE	♂ > ♀*				0.64
	LELEW	♂ > ♀*				0.59

W: Wistar; LE: Long–Evans; WLE: Wistar Long–Evans; LEW: Long–Evans Wistar; WWLE: Wistar Wistar Long–Evans; LELEW: Long–Evans Long–Evans Wistar; BSTMP: medial posterior division of the bed nucleus of the stria terminalis (BST); BSTMA: medial anterior division of the BST; BSTLP: lateral posterior division of the BST; LC: locus coeruleus. *At least $p<0.03$ in all cases; **$p<$ at least 0.01 in all cases; ***$p<0.0001$.

Table 4 – Variation of the number of neurons in males through F_0, F_1 and F_2 (means ± SEM)

	F_0		F_1		F_2
BSTMP	W, 23179 ± 2487.4	=	WLE, 27435 ± 1884.4	>	WWLE, 17230 ± 952.8
	LE, 25053 ± 1228.1	=	LEW, 27795 ± 2174.6	>	LELEW, 17046 ± 1588.7
BSTMA	W, 10604 ± 1239.4	=	WLE, 7828 ± 997.9	=	WWLE, 10175 ± 674.3
	LE, 8323 ± 1757	=	LEW, 14417 ± 2691.9	=	LELEW, 10285 ± 924.9
BSTLP	W, 3597 ± 364.9	=	WLE, 4318 ± 906.8	=	WWLE, 2794 ± 109.4
	LE, 5506 ± 700.9	=	LEW, 5042 ± 773.6	=	LELEW, 3705 ± 673.6
LC	W, 1604 ± 62.7	>	WLE, 1248 ± 37.2	<	WWLE, 1617 ± 76.6
	LE, 1571 ± 167.7	>	LEW, 1136 ± 91	<	LELEW, 1548 ± 54.1

W: Wistar; LE: Long–Evans; BSTMP: medial posterior division of the bed nucleus of the stria terminalis (BST); BSTMA medial anterior division of the BST; BSTLP lateral posterior subdivision of the BST; LC locus coeruleus. Symbols: < (lesser than) or > (greater than), statistical significance (at least $p<0.05$); = n.s.

2.3.2. *Medial anterior division (BSTMA)*

Female Wistar (F_0) and WLE (F_1) rats showed larger number of neurons than their counterpart males (at least $p<0.01$ in both cases) (Table 3). Sex differences were not seen in the remaining groups.

No effect of group was seen in males, indicating that they maintain a similar number of neurons through the generations (see Table 4). However, females presented a statistically significant group effect ($F_{5,32}=7.21$; $p<0.0001$); as shown in Table 5 heterozygosis only affected females of the Long–Evans strain (LE vs. LEW, $p<0.05$). However, homozygosis significantly decreased the number of BSTMP neurons in females of both strains (WLE vs. WWLE, $p<0.05$; LEW vs. LELEW, $p<0.05$).

2.3.3. *Lateral posterior division (BSTLP)*

In F_1, sex differences were detected between male and female LEW hybrids ($t=3.12$, $df=7$; $p<0.01$); females had a greater number of neurons than males (Table 3). There was not a main effect of group in the males, indicating that the number of neurons did not change significantly over the generations (Table 4). However, the group variable was statistically significant in females ($F_{5,32}=10.88$; $p<0.0001$) and there were a statistically significant increase in the number of neurons in the WLE generation (W vs. WLE, $p<0.05$) with a significant decrease in the number of neurons when homozygosis returned in F_2 generation (WLE vs. WWLE; LEW vs. LELEW; $p<0.05$ in both cases) (Table 5).

2.3.4. *Study of the locus coeruleus through the F_0, F_1 and F_2 generations*

Sex differences were only seen in the Wistar strain (F_0) ($t=9,42$; $df=8$; $p<0.0001$); as shown in Tables 2 and 3, females show a larger number of neurons than males. Thus, sex differences in the number of neurons in the LC are a characteristic of the Wistar strain that is lost after they are crossed with LE rats.

Both male and female rats showed a statistically significant group effect (males: $F_{5,38}=7.39$; $p<0.0001$; females: $F_{5,35}=12.98$; $p<0.0001$). As can be seen in Tables 4 and 5, the number of neurons was lower in heterozygotic individuals but higher in the LC of both male and female homozygotic rats.

2.3.5. *The fate of sex differences through generations*

As can be observed in Table 3, sex differences are maintained through generations only in the structure (male > female) that is implicated in the control of the male copulatory behavior: the BSTMP (Fig. 1). With respect to structures in which females have more neurons than males, which is a characteristic of the Wistar strain, sex differences are lost in the BSTMA in F_2 and in the LC from F_1 (Table 3). The BSTLP, an isomorphic structure in both strains, showed a transient sexual dimorphism in the LEW F_1 generation (Table 3).

3. Discussion

The main aim of the study was to observe the fortune of sex differences in the number of neurons in the BSTMP and the LC

Table 5 – Variation of the number of neurons in females through F_0, F_1 and F_2 (means ± SEM)

	F_0		F_1		F_2
BSTMP	W, 15105 ± 1957.9	=	WLE, 18772 ± 870.2	>	WWLE, 11063 ± 1507.7
	LE, 12014 ± 2841.8	<	LEW, 19194 ± 1178.7	>	LELEW, 10056 ± 670.5
BSTMA	W, 17264 ± 1807	=	WLE, 15961 ± 2071.9	>	WWLE, 10059 ± 750.7
	LE, 10975 ± 1669	<	LEW, 15541 ± 747.6	>	LELEW, 9963 ± 596.4
BSTLP	W, 4061 ± 502.2	<	WLE, 6810 ± 1049.2	>	WWLE, 3216 ± 363.6
	LE, 6776.2 ± 1179.7	=	LEW, 8131 ± 603	>	LELEW, 3233 ± 387.5
LC	W, 2090 ± 81.4	>	WLE, 1322 ± 76.5	<	WWLE, 1754 ± 134.2
	LE, 1594 ± 48	>	LEW, 1102 ± 45.5	<	LELEW, 1549 ± 85.64

W: Wistar; LE: Long–Evans; BSTMP: medial posterior division of the bed nucleus of the stria terminalis (BST); BSTMA medial anterior division of the BST; BSTLP lateral posterior subdivision of the of the BST; LC: locus coeruleus. Symbols: < (lesser than) or > (greater than), statistical significance (at least $p<0.05$); = n.s.

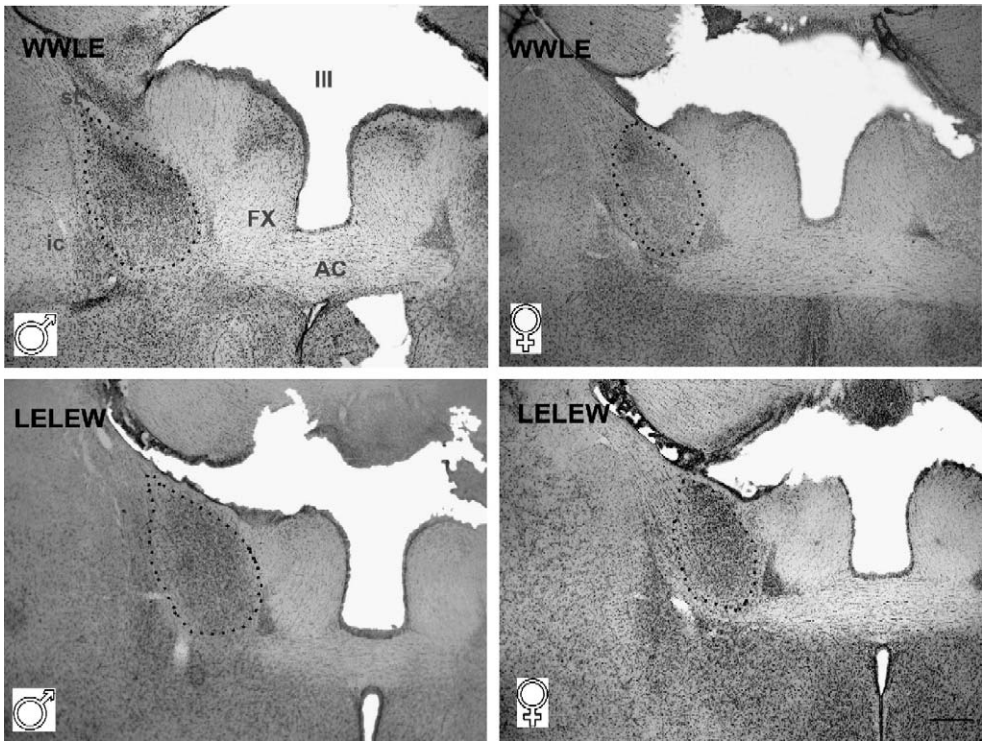

Fig. 1 – Coronal sections illustrating the medial posterior division of the bed nucleus of the stria terminalis (BSTMP) in the experimental groups of the F_2 generation. AC: anterior commissure; FX: fornix; ic: internal capsule; III: third ventricle; st: stria terminalis. Scale bar: 300 μm.

in Wistar and Long–Evans rats (F_0) and, after their cross breeding, in their descendants of the F_1 and F_2 generations.

Our findings show that males have more neurons than females in the BSTMP of the Wistar and Long–Evans strains, concurring with previous data in the literature (Del Abril et al., 1987; Garcia-Falgueras et al., 2005; Guillamon et al., 1988a) and this pattern is preserved in the F_1 and F_2 generations. A sexually dimorphic male > female pattern has been reported in the BSTMP in Wistar (Del Abril et al., 1987; Guillamon et al., 1988a), Long–Evans (Garcia-Falgueras et al., 2005) and Sprague–Dawley rat strains (Chung et al., 2000) as well as in mice (Forger et al., 2004), guinea pigs (Hines et al., 1985), rabbits (Segovia et al., 2006) and in the darkly stained posteromedial component of the human BST, a division that could be considered homologous to the rat BSTMP (Allen and Gorski, 1990). The medial posterior division of the BST belongs to the vomeronasal system, an olfactory pathway that controls reproductive physiology and behavior and that itself is sexually dimorphic (Guillamon and Segovia, 1996; Segovia and Guillamon, 1993; Segovia et al., 1999). The functional interest of the BSTMP is based on its participation in the control of male copulatory behavior. It was reported that lesions of the whole BST in Long–Evans rats (Emery and Sachs, 1976) or even limited to only the BSTMP in Wistar rats (Claro et al., 1995) disrupted copulatory behavior in males. If males have more neurons than females in the BSTMP in several species of mammals (Allen and Gorski, 1990; Chung et al., 2000; Del Abril et al., 1987; Forger et al., 2004; Guillamon et al., 1988a; Hines et al., 1985; Kruijver et al., 2000; Stefanova and Ovtscharoff, 2000) including rat strains (Garcia-Falgueras et al., 2005), and this phenotypic characteristic is not lost by cross breeding, which increases heterozygosis (step F_0 to F_1 in our experiment) and homozygosis (step F_1 to F_2), it could be suggested that this phenotypic trait in the male BSTMP might have a major evolutionary adaptive value and could be vital for species survival.

Taking into account lesion and stimulation studies, it has been suggested (Segovia and Guillamon, 1993) that the male > female pattern of brain sex differences may have a two-fold functional significance: first, it would enable males to display masculine sexual behavior, and second, it would inhibit lordotic and maternal behavior in males. Male copulatory behavior, in which the BSTMP is implicated, is crucial for mammalian species survival. It seems that the male > female pattern in the number neurons in the BSTMP is a fixed and highly conserved pattern that is maintained constant through mammalian species.

However, the results for the BSTMA and LC, two structures that present a pattern of female > male sexual dimorphism in respect to neuron number, suggest instability of this pattern. Sex differences in the LC are only seen in Wistar (F_0) but not in Long–Evans (F_0) rats. After crossing the strains sex differences are no longer seen in the LC in F_1 and F_2 . BSTMA sex differences are only seen in F_0 Wistar and F_1 WLE but not in F_0 Long–Evans, F_1 LEW or any F_2 individuals. The results for BSTMA and the LC in F_0 confirm previous findings (Garcia-Falgueras et al., 2005). The lack of consistency in this pattern of sex differences through the crossings and the fact that the BSTMA and the LC are isomorphic in the rabbit (Segovia et al., 2006) suggest that this Wistar strain

characteristic is of minor importance in terms of its adaptive value for the species. Probably, the female>male pattern seen in the BSTMA and the LC might be an evolutionarily less conserved pattern.

The loss of sexual dimorphism in the Wistar LC after the crossing with Long–Evans rats suggests that functions controlled by this structure are not apparently affected by sex differences. In F_1, heterozygosis is higher than in F_0 and is associated to the loss of sex differences in the LC. Thus, we could say that sexual dimorphism is not stable and, in the absence of an important selective pressure, it is lost.

However, the same degree of heterozygosis does not affect sexual dimorphism in the BSTMP, perhaps because the underlying functions in this structure have a greater adaptive value, and thus the selective pressure would maintain this dimorphism constant. It should be remembered that laboratory rats, whether albino or pigmented descend from Wistar as a consequence of artificial selection (Pass and Freeth, 1993; Zucker, 1960), a process that helped Darwin to define sexual selection. If artificial selection is a model for sexual selection, it could be that wild females would select males that would contributed to the stability of the male>female pattern in the BSTMP. In contrast, both males and females contributed equally to LC variability.

The BSTLP is isomorphic in the Wistar (F_0) and the Long–Evans (F_0) strains, as previously reported for Wistar rats (Garcia-Falgueras et al., 2005) and, except for F_1 WLE, it is also isomorphic in F_1 and F_2.

The fluctuation in the number of neurons between male and female rats of the Long–Evans and the Wistar strains through the F_0, F_1 and F_2 generations deserves some comment. The pattern of changes in neuronal number over generations differs between the LC (a brain stem structure) and the BST (a forebrain structure). With respect to the LC, males and females of both strains show an identical pattern of change: a decrease in the number of neurons in the F_1 with respect to the F_0 generation followed by an increase in neuron number in the F_2 generation with respect to the F_1 generation.

In spite of the stability of the sexual dimorphism in BSTMP over generations, the number of neurons changes significantly in this structure in both sexes between generations F_1 and F_2. However, the female/male neuron ratios in the BSTMP of Wistar and Long–Evans strains show a great deal of stability over the generations and this stability appears to be unaffected by homozygosis and heterozygosis. This seems to suggest the existence of mechanism(s) regulating the neuronal ratio between the sexes and that these mechanisms are different from those that control the number of neurons.

With respect to the number of neurons, testosterone is known to prevent neuronal death in the spinal nucleus of the bulbocavernosus (Nordeen et al., 1985). In the BSTMP, testosterone is responsible for the greater number of neurons found in the male Wistar rat (Guillamon et al., 1988a). Moreover, females have a greater number of apoptotic nuclei than males in the principal nucleus of the BST and that testosterone controls the incidence of apoptosis (Chung et al., 2000). Studies in mice have shown that Bax-dependent cell death is required for the sexual differentiation of cell number since a null mutation of the *Bax* gene completely eliminated sex differences in the overall cell number in the principal nucleus of the BST and the anteroventral periventricular nucleus (Forger et al., 2004; Forger, 2006). Nevertheless, the control of the number of neurons in the sexual differentiation of the brain is a very complex process. A developmental study in the LC has shown that cell death is greater in males on the day of birth but that adult sex differences are due to the greater postpuberal proliferation of neurons in females than in males (Pinos et al., 2001).

Our data suggest that the mechanism(s) that regulate the proportionality in the number of neurons between sexes might be different from those that control the number of neurons. We do not yet know what that mechanism is. However, if it is true that the *Bax* gene controls the number of neurons independently of testosterone in sexually dimorphic structures, as suggested by Forger et al. (2004, 2006), then gene *Bax* might be a candidate to explain the regulation of the stable ratio for the number of neurons between sexes observed by us.

In the present work, we have seen that there are structures like the BSTMP that maintain sex differences within a species after cross-breeding, which introduces variability. However, in other structures like the LC and the BSTMA, the same variability makes the sex differences disappear. This suggests two types of brain sexual dimorphism. First, a kind that could be call "fixed", when the same structure shows sex differences within a species and in different species and orders. This "fixed" sexual dimorphism is exhibited by the BSTMP. Something similar can be said for the sexually dimorphic nucleus of the preoptic area of the rat (Gorski et al., 1978) and similar nuclei of other species (Byne, 1998; Commins and Yahr, 1984; Hines et al., 1985; Hofman and Swaab, 1989; Roselli et al., 2004; Shapiro et al., 1991; Tobet et al., 1986).

This "fixed" sexual dimorphism appears to be constant with respect to Order species, strains within a species and generations. Taking into account our data and the revised literature on the BSTMP and the sexually dimorphic nucleus of the preoptic area, this "fixed" sexual dimorphism in these two structures might be a characteristic of the mammalian class.

Sex differences seen in the LC, BSTMA and BSTLP would belong to a second kind of brain sexual dimorphism that could be called "unstable", it would appear transiently and seems to be dependent on strains and species. This distinction could help to direct further research into brain and behavioral sexual dimorphism and focus attention on structures that have the same functional value in different species.

4. Experimental procedures

4.1. *Subjects and experimental design*

Ten adult male (5) and female (5) Wistar rats (Charles River, Criffa; Saint-Aubin-Les-Elbeuf, France) and ten adult male (5) and female (5) Long–Evans rats (Janvier, Madrid, Spain) constituted the F_0 generation (male Wistar: ♂W; female Wistar: ♀W; male Long–Evans: ♂LE, female Long–Evans: ♀LE) (Fig. 2). F_1 was obtained by crossing each female with a male of the other strain (Fig. 2); the hybrids were grouped according to their matrilineal origin and sex in the following

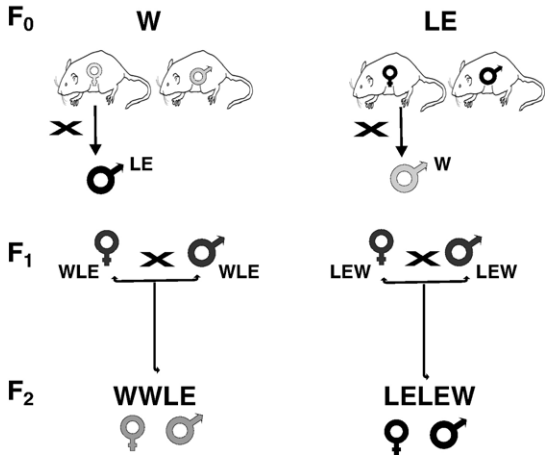

Fig. 2 – The experimental design: male and female Wistar (W) and the Long–Evans (LE) rats (F_0) were mated to obtain a F_1 generation (WLE and LEW). The F_2 (WWLE and LELEW) generation was obtained by crossing siblings of the F_1 generation.

groups: ♂WLE (6); ♀WLE (6); ♂LEW (6); and ♀LEW (6). The crossings to obtain F_2 were as follows: ♂WLE × ♀WLE: ♂WWLE (9) and ♀WWLE (8); and ♂LEW × ♀LEW: ♂LELEW (8) and ♀LELEW (7). The F_2 individuals were selected in two phases: (i) by their matrilineal ascendance and (ii) by molecular testing, to identify homozygotic animals by the molecular markers shown in Table 1.

During the experiment several animals were excluded because they died or the staining of their samples did not achieve sufficient quality for the stereological procedure. F_0: one LE female died and one LE male was not included in the LC study. F_1: one WLE male was lost for the BST. F_2: one LELEW female for the LC study and three WWLE males, two WWLE females and one LELEW male were lost for the BST study.

All subjects were housed in standard cages in same-sex and strain groups and maintained on a 12:12-h light–dark cycle (lights off at 8:00 PM). Food and water were provided *ad libitum*. The stock room was kept at 22±2 °C. Animal care throughout the experimental procedures was in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC).

4.2. *Molecular characterization criteria*

All the animals were analyzed using 37 molecular markers (SSLPs). The markers that were homozygotic in F_0 and F_2 (different for Wistar and Long–Evans; see Table 1) were chosen; these alleles were also easily distinguished on the agarose gels due to their different sizes. The four molecular markers chosen were located in chromosomes 1, 4, 9 and 20 (D1Rat221, D4Rat2, D9Rat30 and D20Rat43) (Fig. 3).

The subjects from F_0 were homozygotic for D1Rat221, D4Rat2, D9Rat30 and D20Rat43; the F_1 subjects were heterozygotic and in F_2 we selected the homozygotic individuals by the alleles that were characteristic for each strain (Table 1).

4.3. *SSLPs analysis*

DNA was obtained from 200 μl whole blood using the method described by Beyer et al. (1997). Primers were designed using DNA sequences from The Rat Genome Database http://rgd.mcw.edu/genomescanner (Table 6). PCR conditions were 50 ng DNA, 250 μM each dNTP, 2.5 mM $MgCl_2$, 0.4 μM each primer and 0.25 U Taq DNA Polymerase. Standard cycling conditions were carried out at 94 °C for 60 s, 57 °C for 60 s and 72 °C for 60 s (40 cycles) with a final extension at 72 °C for 10 min. Modified PCR programs were used to optimize some primer pairs when necessary (Table 6). PCR products were resolved either on 2% agarose gels stained with ethidium bromide or on a polyacrylamide gel (GeneGel Excel 12.5/24 Kit from Amersham Pharmacia Biotech) at 600 V for 40 min.

4.4. *Histology*

When subjects were 90 days old, they were deeply anesthetized with an intraperitoneal injection (250 mg/kg) of tribromoethanol (Sigma Aldrich), and 1 ml of blood was extracted from the left ventricle for the molecular analyses; then the animals were perfused intracardially with saline (0.9%) followed by 4% paraformaldehyde (Panreac) in PBS (Panreac). The brains were removed and stored in paraformaldehyde for 2 days followed by 3–5 days in 30% sucrose (Sigma Aldrich) at 4 °C. The brains were then frozen and coronally sectioned at a thickness of 50 μm (forebrain and midbrain) or of 40 μm (pons and cerebellum). All sections were stained with a 0.1% solution of cresyl violet (Merck) brought to pH 4 with glacial acetic acid (Panreac).

4.5. *Stereology*

Stereological methods were used to determine the number of neurons in the BST subdivisions and the LC. To measure each morphological parameter, we used a Diaplan Leitz

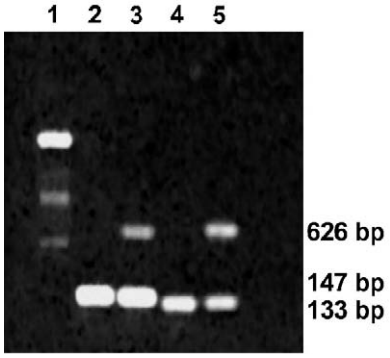

Fig. 3 – Amplification by PCR of the D4Rat2 marker and the *Sry* gene in four rats from F_0: (1) molecular weight marker. (2) A band of 147 bp from the D4Rat2 marker in a female Wistar rat. (3) A band of 147 bp from the D4Rat2 marker and another of 626 bp from the *Sry* gene, in a male Wistar rat from F_0. (4) A band of 133 bp from the D4Rat2 marker in a Long–Evans female rat. (5) A band of 133 bp from the D4Rat2 marker and another of 626 bp from the *Sry* gene, in a Long–Evans male.

Table 6 – Microsatellite markers employed to select the F_0 individuals which were crossed to generate F_1 and F_2 hybrid populations

SSLPs symbol	Primer sequences (5′-3′)	Chromosome location	Wistar (bp)	Long–Evans (bp)	Annealing temperatures
D1Rat221	GATGAGGAGTCTGGCTGAGG AGCCATGTGGAATGAGTTCC	1	248	336	64°
D4Rat2	CAAGGCTCAAATGTGTTCCA TAGGATGAGAATGCCCAAGG	4	147	133	62°
D9Rat30	ATGGTCTTCCTTCAAACACG GCTCCCACTCTGCAACAAAC	9	172	158	61°
D20Rat43	AGGAAGTTGAGATCAGGGCA GATTTGGCTTCTGTCCTCCA	20	218	261	61°
Sry gene	GGGAGGAGGGATGAATAT CATTGCAGCAGGTTGTACAGT	Yp	626	626	57°

The second column contains the primer sequences, the fourth and the fifth columns indicate the size of the amplified fragments and finally, the sixth column indicates the specific annealing temperatures for each SSLP and the Sry gene. The primer sequences were obtained from the available rat linkage maps http://rgd.mcw.edu/genomescanner.

microscope with a stage controlled by a computer software system (MultiControl 2000; Mörzhäuser Wetzlar, Germany). This system of motors is attached to the stage, enabling predetermined but randomly chosen steps to be generated on the *x* and *y* axes. Moreover, there is a specially fitted rotating stage so that the slices can be shifted by 360°, independently of their *x*–*y* movements. The stereological software package (GRID; Interactivision, Denmark) allows the patterns required (grids) to be superpositioned over the microscope image. Finally, an electronic microcator (Heidenhain, Germany) with a resolution of 0.5 μm is attached to the microscope so the *z* axis measurements of the stage can be taken.

The number of neurons in BSTMP, BSTMA, BSTLP and LC was estimated using the dissector and fractionator techniques (Sterio, 1984; Gundersen et al., 1988a,b. The sections were cut with a cryostat to a thickness of 50 μm (BST) and 40 μm (LC), but their actual width after using the microcator was approximately 20–25 μm. The optical dissector was used as follows: frames were generated over the area with a horizontal step of 90 μm and a vertical step of 90 μm for the BST and a horizontal step of 90 μm and vertical step of 50 μm for the LC. All of the frames that included the nuclear profile were considered. The profiles (neuronal nuclei) completely enclosed in the test frame (A[f]=842 μm^2 for all subdivisions of the BST and for the LC) and those intersected by the inclusion edges at a ×4323 magnification were counted. The height of the dissector was 12 μm. Finally, the total number of neurons in both structures was obtained applying the fractionator formula:

$$\Sigma Q = 1/\mathrm{ssf} \times 1/\mathrm{asf} \times 1/\mathrm{hsf}$$

In this formula, ΣQ is the total number of neurons counted; ssf is the section sampling fraction; asf is the area sampling fraction; and hsf is the height sampling fraction. To avoid overestimating the number of neurons the counting unit was the nucleolus. An observer, blinded to the group membership of each specimen, counted the number of neurons in each slide. For the BST and the LC, Ling et al. (1973) criteria were used to discriminate between neurons and glial cells.

4.6. *Statistical analyses*

The data showed homogeneity of variance for each structure and were submitted to different analyses: (a) to compare F_0 groups a two-way analysis of variance of sex (male and female) by strain (Wistar and Long–Evans); (b) to detect possible sex differences, two-tailed *t* tests were performed for each experimental group; (c) males and females of the F_0, F_1 and F_2 generations were analyzed separately using a one way analysis of variance, post hoc comparisons between groups were implemented using the Student–Newman–Keuls test, taking into account the appropriate error of variance.

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