litter in which individuals are reared are often ignored. Previous research with estrogen receptor α knockout (ERKO) mice suggests that litter composition influences the sociosexual behaviors of these KO mice in adulthood (Crews et al., 2004). Namely, when Genotype is considered as a component of litter composition, behavioral differences between the Genotypes are more sharply defined and less variable than reported previously. The present study utilized the same paradigm as described in Crews et al. (2004). Thus, pups derived from mating males and females heterozygous (HTZ) for a null mutation of estrogen receptor α (ERα) were sexed and genotyped within 2 days after birth. With this information litters were reconstituted, forming either same-sex/same-genotype controls, same-sex/mixed-genotype litters of equal numbers of KO and wildtype (WT) individuals, mixed-sex/same-genotype, or mixed-sex/mixed-genotype litters of equal numbers of male or female young.

We hypothesized that behavioral variations caused by genetic and developmental factors within the litter environment correlate with differences in behavior and COX activity in specific brain areas later in adulthood. Using genetically-modified mice we demonstrate that the sex and genotype ratio of the litter has a powerful effect on the organization of the neural mechanisms controlling social and anxiety-related behaviors.

MATERIALS AND METHODS

MICE

Mice were produced by mating male and nulliparous female mice HTZ for a dysfunctional ERα gene, which were backcrossed to C57B/6J mice. These mice were obtained from the breeding colony maintained at Rockefeller University. Two to three females were housed with a male, and, when they were visibly pregnant (3–5 days before the day of delivery based on *post hoc* analyses), they were singly housed. Animal rooms were maintained on a 12:12 h Light:Dark cycle at constant temperature (22°C) with food (PicoLab Rodent Diet 20, Oakville, ON, Canada) and water available *ad libitum*. All procedures were approved by the Rockefeller University, University of Tsukuba, and University of Texas Animal Use and Care Committees.

All pups were sexed and genotyped within 2 days of birth and the litters re-constituted on PND 2. During this period, each pup received individual identification marks on the body by a Sharpie™ permanent marker, a procedure that was repeated each day. After genotyping, individuals were identified with individually specific toe clips for permanent identification. In all instances, HTZ females that had contributed young to the study served as foster mothers.

Littermates were separated in a systematic manner to insure that litter source was not overrepresented in each group. Pups were reared in litters of four; all individuals survived to weaning. As in previous studies with these animals, individuals continued to be group-housed with littermates of the same sex and genotype following weaning. Unlike previous studies, individuals were housed according to genotype (e.g., ♂WT with only ♂WT) after weaning. Two weeks before behavior testing, all animals were individually housed. Behavioral testing began at approximately 8 weeks of age for all mice and included tests of anxiety (Light:Dark transition test) and of social and aggressive behaviors (resident-intruder test) in this order. All animal husbandry, testing, and euthanasia followed approved institutional IACUC guidelines and NIH Guidelines for the Use of Animals in Research.

RECONSTITUTION OF LITTERS

Two separate studies were performed, one focusing on males and the other on females. Experimental litters were reconstituted from natal litters into one of 16 possible combinations: same-sex, same-genotype; same-sex, mixed-genotype; mixed-sex, same-genotype; mixed-sex, mixed-genotype totaling more than 450 pups. Only four litter types of the 16 possible combinations were impossible to construct in adequate sample sizes and so the present report is limited to the 12 litter types that were reconstituted in sufficient sample sizes for statistical analysis. **Table 1** details the distribution of a male or a female animals' genotype (experimental cohort) with respect to the sex and genotype of the other half of the animals in the litter (Sibling Type) along with the number of litters from each of these groups. The number of individuals and experimental litters reconstituted from natal litters totaled were as follows: for males, a total of 200 individuals divided into 39 same-sex, and 22 mixed-sex litters; for females, a total of 168 individuals divided into 42 same-sex, and 13 mixed-sex litters.

As adults an individual of each sex and genotype from the experimental cohort was chosen at random from each of the litters and tested for anxiety and affiliative behaviors. Another individual was selected at random for the COX histochemistry. Hence individuals used for assessment of brain metabolic activity were not tested before sacrifice, but were the same age as those individuals that were tested.

BEHAVIORAL TESTING

Light:Dark transition test

The test apparatus consisted of clear plastic box (50 cm × 50 cm × 35 cm) and a black (light opaque) covered-plastic box (50 cm × 25 cm × 25 cm) was inserted in one side (the dark side). The black box had an open doorway (2 cm × 5 cm) that led to the light side of the apparatus, which was illuminated by a 40-W white bulb. Mice were moved from the living room to the experimental room at least 1 h before the test. At the beginning of the tests, mice were removed from their cages, gently placed at the doorway, and released into the dark side of the apparatus (Ogawa et al., 2003, 2005). A Digiscan analyzer and software (Omnitech Electronics Inc., Dartmouth, NS, Canada) were used to collect and store horizontal-activity, which was monitored by infrared beams. For each mouse, total activity (total number of beam breaks) and cumulative time spent in each compartment were recorded during 10 min tests. Gonadally intact male and female mice were tested once at 8 weeks of age.

Table 1 | Sex and genotype combinations in reconstituted litters from mating mice heterozygous for estrogen receptor α (WT, wildtype; KO, knockout). The "individual" rows indicate the experimental cohort (mice in which dependent variables were measured) while the columns "Sibling Type" indicates the stimulus cohort (siblings used as independent variables). All litters consisted of four individuals. A single individual from each litter was used in the behavioral studies and another used for cytochrome oxidase histochemistry.

	Sibling type			
Individual	**♂WT**	**♂KO**	**♀WT**	**♀KO**
♂WT	11	7	7	6
♂KO	11	10	NA	NA
♀WT	11	NA	17	11
♀KO	11	NA	8	6

NA means not available as the number of litters created for these groups did not reach the minimum required of six.

Resident-intruder test

To determine if litter composition influences affiliative behavior in adulthood, mice were tested in a resident-intruder paradigm as in previous studies from this laboratory (Ogawa et al., 1996, 1997, 1998a,b). All individuals were gonadally intact. In females, the stage of estrous cycle at the time of testing was not determined for experimental females. Behavior tests lasted for 15 min during the dark phase (4–8 h after lights off) under red light. Experimental males were tested with olfactory bulbectomized C57BL/6J male intruders and females were tested with ovariectomized C57BL/6J female intruders. Cumulative duration of social interaction including sniffing, genital licking, and grooming was recorded for each experimental mouse. Experimental males and females were tested on two consecutive days at 9 weeks of age.

STATISTICS FOR BEHAVIORAL MEASURES

Behavioral data were analyzed after they were log-transformed, if necessary, so as to normalize the distributions and remove non-homogeneity. They were then analyzed for group differences of each sex by ANOVAs followed by *t*-test *post hoc* analyses.

TISSUE PREPARATION AND CYTOCHROME OXIDASE IMAGING

Brains were removed immediately following decapitation and prepared for COX imaging as per Gonzalez-Lima and Cada (1998). The same individual measured all of the areas after extensive training in neuroanatomy. All measures were done blind, the key being broken only after the completion of all measurements.

For the purposes of comparing COX activity between each batch, a two-way ANOVA was performed using whole brain (WB) measures that were partitioned into the factors of three anatomical regions and into the batches in which these brains were stained. The three anatomical regions (rostral, medial, and caudal) as well as the nuclei measured are depicted in **Figure 1**. Testing the arbitrary subdivisions of the mouse brain with a small representative random sample of the study population ($n = 12$) indicated that further subdivision of WB regions from the original subdivisions did not yield significant regional effects on individual nuclei activity after normalization (two-way ANOVA, $F = 0.845$, $df = 9, p = 0.575$). This finding suggests that subdivision into three regions is sufficient to capture the regional variation in WB activity. Normalization is used to correct for observed staining variability and allow comparisons between different groups of animals or nuclei. For normalization, the average COX activity of a given nucleus was divided by the respective averaged regional WB COX activity for each individual to generate this value for normalized nucleus activity. This correction was made for all nuclei in the entire population of experimental animals and was used in all subsequent analyses other than ANOVA comparisons; in the latter instance a two-way ANOVA was performed for the factors of Genotype and Sex for WB activity and for each of the 20 nuclei using Hochberg's GT2 *post hoc* analysis.

PRINCIPAL COMPONENTS ANALYSIS

Data reduction performed with principal component (PC) analysis was used to decompose the full set of all nuclei influences between one another into eigenvectors or PCs that selected a few nuclei that were contributing to most of the variance. In this study, PCs were

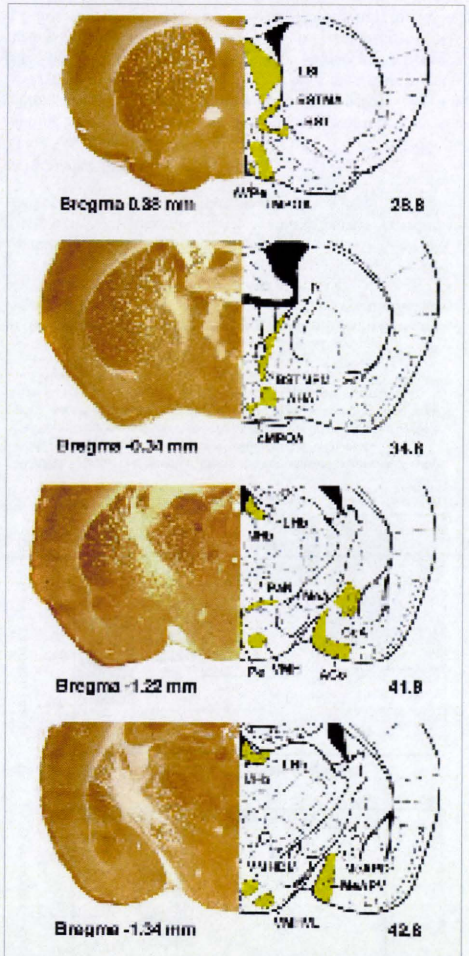

FIGURE 1 | Left column is a composite of sample mouse brain images histochemically stained for cytochrome oxidase with immediate right column delineating the boundaries of nuclei measured in yellow in the corresponding coronal sections in Paxinos and Franklin (2001); the bottom left number refers to Bregma and the bottom right number refers to the figure number.

derived in iteratively repeated attempts to account for as much of the variance in the original correlation matrix as possible, with each subsequent PC accounting for less variance than the PC before it. A plot of these ordered eigenvalues is called a SCREE plot, and can be used to help visually interpret the following criteria for the significance of a PC as outlined by Ecker et al. (2007) (**Figure 2**):

(1) Kaiser's rule, stated in the following way: $l^2 > 1$, was used to find PCs that account for non-trivial variance within the correlation matrix (Loehlin, 2004).
(2) If only a few PCs account for most of the variance, as would be expected in the case where there was some amount of shared variance between multiple nuclei, then a few PCs would have a high l^2 and the rest would have a low l^2. If one PC's eigenvalue l^2_n, where n is the index representing the ordered occurrence of each PC, accounts for more variance than the following PCs, then $(l^2_n - l^2_{n+1})$ – the difference between the large eigenvalue and the subsequent smaller eigenvalue – should be large in comparison to the difference between the next pair of eigenvalues $(l^2_{n+1} - l^2_{n+2})$. Following this reasoning, Ecker et al. (2007) used Equation 1 to determine the significance of the nth PC:

$$\frac{l^2_n - l^2_{n+1}}{l^2_{n+1} - l^2_{n+2}} \geq 3.0 \quad (1)$$

Each time that this equation is true, a significant drop in the eigenvalues has occurred, and should correspond to a large drop on the SCREE plot.
(3) Only eigenvalues occurring before the end of the first series of large drops in the SCREE plot are considered significant. This is also called the elbow criterion after Catell (1966) for reasons that become apparent when viewing **Figure 2**.

Principal components analysis (PCA) was used to derive PCs from a data set containing all males ($n = 37$) and later all females ($n = 34$) in their respective studies that had no missing values for a given set of nuclei used in their analyses. The analysis was also used on each sex for subsets of each of the genotypes used in the analysis. In all, PC analysis was performed three times for each sex: once for all animals in the parsed data set, again for the WT, and then KO within those data sets. After the significant PCs for each group were determined, the coefficients of each nucleus for the significant PCs were checked against the following criterion: if the value of the coefficient was found to be greater than 0.60, then the nucleus of that coefficient is placed into a set of nuclei for further analysis using Pearson's bivariate correlation along with the jackknifing procedure. All nuclei found significant in a single PC were considered independently of the nuclei considered in another PC unless the same nuclei occurred with a coefficient greater than 0.60 in both PCs; this rule had one exception during analysis of the male tissue that served as a control. The coefficient value used in this analysis was selected for its ability to separate nuclei into smaller groups that did not have a large number of shared nuclei and following the observation that nuclei values below this coefficient were often much lower (0.00–0.30) than the value of 0.6.

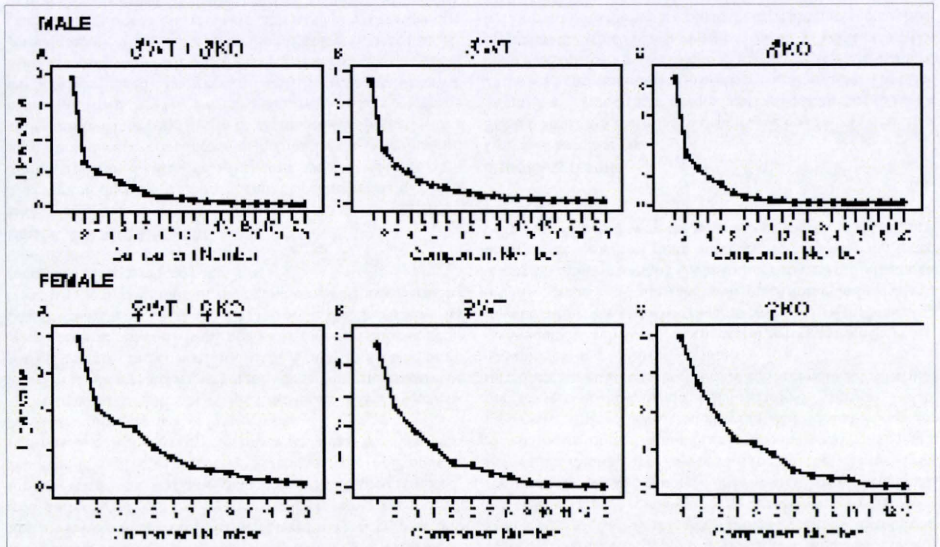

FIGURE 2 | SCREE plots corresponding to the amount of variance that each eigenvalue accounts for. The largest point on the left of each plot is the eigenvalue representing the first principal component derived, which is the first in an iterative process of attempting to account for as much variance as possible in the correlation matrix and its subsequent residual matrices. A precipitous slope, as seen in each SCREE plot, indicates that the amount of variance accounted for by the following component is not nearly as great as the previous. This observation is the basis of Eq. 1. For males each plot shows a main component and a few less significant components that quickly become trivial (triviality is $\lambda^2 < 1$, see **Table 4**), indicating that among the 20 nuclei considered there is an underlying group of nuclei that is primarily driving the strength of the correlation matrix. Females show a less steep slope. Eigenvalues shown in SCREE plots were derived using principal component analysis for males on the top row and females on the bottom. Top row: **(A)**: all males (♂WT + ♂KO; $n = 37$), **(B)**: ♂WT only ($n = 21$), and **(C)**: ♂KO only ($n = 16$). Bottom row: **(A)**: all females (♀WT + ♀KO; $n = 34$), **(B)**: ♀WT only ($n = 17$), and **(C)**: ♀KO only ($n = 17$).

INTER-REGIONAL CORRELATIONS IN METABOLIC ACTIVITY

Each nucleus selected from a given PC was subjected to a modified jackknifing procedure similar to that used by Sakata et al. (2000) to find correlations that were consistently significant between nuclei. To find group differences when appropriate, groups were segregated into subsets of the full data set depending upon an experimental factor (i.e., Genotype or Sibling Type) and then subjected to the jackknifing procedure. Correlations found to be significant after jackknifing in multiple groups were tested for a statistically significant difference in correlations between groups. This was done by taking the correlation coefficient from each $n-1$ step in the jackknifing procedure and comparing this set of correlations with the equivalent correlations in the comparison group using the t-test with assumed unequal variance.

LIMBIC LANDSCAPES

For visualization of how discrete nuclei are modified by the litter environment, the limbic landscape method described in Crews et al. (2006) was utilized.

RESULTS

BEHAVIOR: MALES

Resident-intruder test

Aggression in male mice was significantly influenced by both the Sex and Genotype of the littermates (**Figure 3A**). When raised in litters having either ♂KO or ♀KO littermates, ♂WT were more aggressive than when raised in litters containing either ♂WT or ♀WT littermates. KO males were not aggressive when raised in litters containing either ♂KO or ♂WT littermates.

Light:Dark transition test

The cumulative duration spent in the light compartment was significantly increased in ♂WT mice raised with ♀WT littermates, but not when raised with ♀KO littermates (**Figure 4**). The latency to move to the light compartment was increased by the presence of ♂KO or ♀KO pups in the litter, but not when the littermates were ♀WT or ♂WT.

BEHAVIOR: FEMALES

Resident-intruder test

Overall, ♀KO reared only with ♀KO sisters showed significantly less social contact in the resident-intruder test compared to ♀WT raised with ♀WT sisters or when raised with ♀KO or ♂WT littermates (**Figure 3B**). Social contact time in ♀KO mice was increased by the presence of ♂WT brothers or ♀WT sisters in the litter; although in the latter instance ♀KO were not significantly from those raised with ♀KO sisters. In contrast, ♀KO raised with ♂WT brothers were not different from ♀WT regardless of litter type.

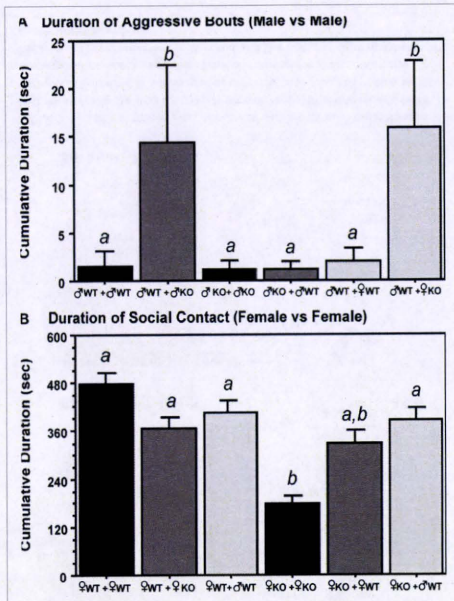

FIGURE 3 | Litter composition affects the sociosexual behaviors of adult male and female estrogen receptor α knockout (ERKO) mice. **(A)** Represents the duration (seconds) of aggressive bouts exhibited by male mice in resident-intruder tests. **(B)** Represents the duration seconds) of social contact exhibited by females in resident-intruder tests. Mean and SE is indicated; letters above bars indicate significant differences.

Light:Dark transition test

There were no significant differences among females according to the Sex or Genotype of their littermates.

CYTOCHROME OXIDASE ACTIVITY: MALES

There was a significant effect ($p < 0.01$) on WB activity due to Sibling Type but not to Genotype or an interaction between the Sibling Type and Genotype (**Table 2**). Further analysis (two-way ANOVA with WB region and Sibling Type as main factors) indicated no significant effect of Sibling Type on interaction between regional WB activity and Sibling Type ($F = 0.112$, df $= 6$, $p = 0.995$). A Bonferroni corrected *post hoc* analysis of these results for Sibling Type revealed that male mice raised with KO brothers had an increased level of WB activity with respect to males raised with WT brothers ($p = 0.016$).

Subsequent analysis on individual non-normalized male nuclei using a two-way ANOVA for Genotype and Sibling type with Hochberg's GT2 *post hoc* indicated that significant differences

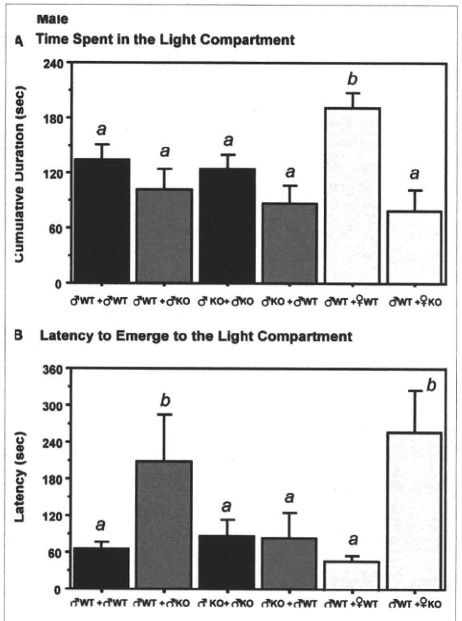

FIGURE 4 | The presence of different genotype of littermates influence later adult behavior in gonadally intact adult male estrogen receptor α knockout (ERKO) mice in Light:Dark transition tests. (A) Represents the time (s) spent in the light compartment. **(B)** Indicates the latency to emerge to the light compartment. Mean and SE is indicated; letters above bars indicate significant differences.

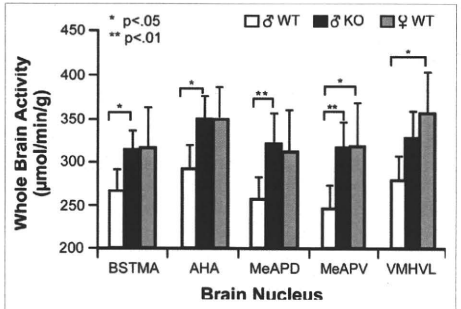

FIGURE 5 | Sibling Type affects metabolic activity in limbic nuclei of male mice in this study. Hochberg's GT2 *post hoc* analysis for a two-way ANOVA comparing sibling type and genotype effects per nucleus showed only significant sibling type effects on metabolic activity in 5 of the 20 limbic nuclei studied in the male mice. Mean and 95% confidence intervals depicted with one asterisk indicating a significance of $p < 0.05$ and two asterisks a significance of $p < 0.01$. Abbreviations: BSTMA, bed nucleus of the stria terminalis medial division, anterior; AHA, anterior hypothalamic area, anterior; MeAPD, medial amygdaloid (MeA) nucleus, posterodorsal; MeAPV, medial amygdaloid (MeA) nucleus, posteroventral; VMHVL, ventromedial hypothalamic nucleus, ventrolateral.

Table 2 | Two-way ANOVA based on the effect an individual's Genotype and Sibling Type has on cytochrome oxidase activity in whole brain measures of limbic areas in male and female mice raised in reconstituted litters.

Variable	df	*F*-value	Significance
MALES			
Genotype	1	7.435	0.518
Sibling Type	3	7.436	0.005
Genotype X Sibling type	1	0.095	0.540
FEMALES			
Genotype	1	72.319	0.000
Sibling Type	2	20.511	0.000
Genotype X Sibling type	2	2.683	0.069

were attributable only to Sibling type and not to Genotype type, conferring with the results seen in the WB analysis. In five of the 20 nuclei measured (BSTMA, AHA, MeAPD, MeAPV, and VMHVL) (**Figure 5**), males raised with WT brothers had significantly lower mean COX activity ($p < 0.05$) compared to males raised with KO brothers. For example, ♂WT raised with WT brothers had significantly lower average metabolic activity in the AHA, BSTMA, MeAPD, and MeAPV when compared to ♂WT raised with KO brothers, and lower activity in the MeAPV and VMHVL relative to ♂WT raised with WT sisters.

CYTOCHROME OXIDASE ACTIVITY: FEMALES

Analysis of female WB activity showed that both the experimental factors of Genotype and Sibling Type significantly affected the mean WB activity (**Table 2**). Comparison of the means for Genotype shows that ♀WT show a greater overall WB activity when compared to ♀KO ($p < 0.001$). A Bonferroni *post hoc* analysis showed female mice raised with ♀WT siblings had a significantly greater ($p < 0.01$) mean WB activity when compared to females raised with ♂WT or ♀KO littermates. These results for females differ from results for males as males had no significant effect of Genotype on WB, and the effect of Sibling Type on each sex appears to differ reciprocally.

A two-way ANOVA on each nucleus using the factors of Sibling Type and Genotype with Hochberg's GT2 *post hoc* analysis indicate that both Sibling Type and Genotype caused significant ($p < 0.05$) mean differences in certain nuclei activities for the females. In 10 nuclei the mean COX activity differed significantly for Genotype: cMPOA ($p = 0.013$), AHA ($p = 0.009$), PaN ($p = 0.004$), MeAA ($p = 0.005$), CeA ($p = 0.033$), VMH ($p = 0.001$), MeAPV ($p = 0.049$), VMHDM ($p = 0.027$), and VMHVL ($p = 0.004$). In each, the ♀KO had consistently lower mean nuclei activity, which agrees with the lower activity seen for the same comparison with mean WB activity in females. The analysis also showed a less extensive affect on mean nuclei activity due to Sibling Type, with significant effects found only in the cMPOA ($p = 0.016$), AHA ($p = 0.043$), and VMHVL ($p = 0.017$).

CORRELATIONS IN CYTOCHROME OXIDASE ACTIVITY IN MALES AND FEMALES

After assessing the effects on overall mean activity of the WB and individual nuclei, interactions between the nuclei were assessed for significance. An initial analysis using Pearson's bivariate correlations on normalized nuclei measures with a two-tailed test of significance showed that males had 83 out of 190 possible pair-wise correlations significant at $p < 0.05$ and 60 out of 190 pair-wise correlations significant at $p < 0.01$ without jackknifing. Females showed 87 out of 210 possible interactions that were significant at $p < 0.05$, and 37 out of 210 at $p < 0.01$ (the increased number of possible interactions due to the inclusion of extra nuclei). When females are compared to the males, the fraction of significant interactions and number of reliably significant interactions is lower in females. This indicates that female nuclei interactions, as shown by correlated COX activity between nuclei tested, are probably not as strong as that shown in males.

PRINCIPAL COMPONENTS ANALYSIS, INTER-REGIONAL CORRELATIONS IN METABOLIC ACTIVITY, AND LIMBIC LANDSCAPES

To find functionally interactive networks, PCs were extracted for three different groups in each sex, making a total of six groups overall. These groups were all ♂(both WT and KO), all ♀ (both WT and KO), ♂WT males only, ♀WT only, ♂KO only, and ♀KO only. Finally, the Limbic Landscape method described in Crews et al. (2006) to illustrate how activity in a defined network of limbic nuclei was modified by the litter environment.

Males

Principal components analysis. Principal components for males were extracted for three different groups: all males (♂WT and ♂KO), ♂WT only, and ♂KO only. The plots of sequentially derived eigenvalues are given as SCREE plots for each group subjected to PCA, shown in **Figure 2**. Using the eigenvalues derived from PCA, the three criteria for determining significant PCs were applied to the eigenvalues of each PC in the three groups. Those eigenvalues that passed the first criterion of $l^2 > 1$ are given in **Tables 3A,B** along with the test for significance based on Eq. 1 from the second criterion; there was no case where the third criterion was invalid and all other criteria were found significant.

When both ♂WT and ♂KO were combined there were two significant PCs (significance ≥ 3.0) and when ♂WT and ♂KO males were considered separately, there was only one significant PC in each. The significant PCs in the individual ♂WT and ♂KO groups had several nuclei with coefficients greater than 0.60 (**Table 3A**) that shared coefficients of similar strength to those in the PCA of combined males. Furthermore, in the ♂WT and ♂KO groups the second, non-significant PC (included for comparison in **Table 3A**) differed from the first significant PC in its respective group for the values of its coefficients, but shared many similarities in coefficient values with the significant PC of the other male group as well as a significant PC of the combined males group. For example, the second PC for ♂WT was dissimilar to the first PC in that group, but similar to the first significant PC in ♂KO and the second significant PC in the combined males group (**Table 3A**).

After consideration, the similarity between PCs in each group of males suggests only two significant PCs that are consistent across genotype. Consequently, nuclei with a coefficient greater than 0.60 in significant PCs believed to be similar were placed together to represent one of the two putatively conserved clusters of nuclei (**Table 3B**). The first significant cluster contained several rostral amygdalar and hypothalamic nuclei. This cluster was labeled as the male rostral limbic cluster (mRLC) and contained the following nuclei: AVPe, cMPOA, AHA, PaN, VMH, MeAA, CeA, and ACo. The second cluster was labeled the male caudal limbic cluster (mCLC) and contained hypothalamic and amygdalar nuclei that were caudal to those used in the mRLC. The nuclei in the mCLC were VMH, PaN, MeAPD, MeAPV, VMHDM, and VMHVL. The inclusion of the PaN, which was not significant in any PCs of the mCLC, served as a control of between cluster correlations as the VMH showed strong correlations in both clusters. It also served as a control for demonstrating that the choice of 0.6 as the coefficient of significance was reasonable.

Inter-regional correlations in metabolic activity. Before the jackknifing procedure could be conducted, the combined male data set was subdivided into groups of shared experimental traits. These groups consisted of all animals that had the same treatment with regard to a given experimental variable. For example, ♂WT mice raised with either ♂WT or ♂KO brothers shared a similar Genotype but not Sibling Type, and were grouped together to form a ♂WT group for Genotype comparison. When the groups had been appropriately partitioned, only the nuclei within a certain cluster were used during the jackknifing procedure for that group. At the completion of jackknifing all groups had been jackknifed for both the mRLC and the mCLC (Table 1A in Supplementary Material). In many groups there were still several between nuclei correlations that maintained significance at $\alpha = 0.05$ throughout the jackknifing procedure. Further analysis also used a values of $\alpha = 0.02$ and 0.01. Within each colored cell of Table 1A in Supplementary Material is the averaged pair-wise correlation coefficient following jackknifing for the two nuclei that are denoted above and to the right of the cell. The significance of each correlation, significant and insignificant, is shown in Table 1B in Supplementary Material. Only half of each table is shown due to the symmetric nature of a pair-wise correlations table. Those correlations that were not significantly different from $r = 0$ at $\alpha = 0.05$ were deemed insignificant and were not included in Table 1B in Supplementary Material. If after jackknifing two nuclei maintain a significant correlation, then the two nuclei may be considered functionally associated, coupled, or connected. If functional connections were found between comparable groups, then a *t*-test with an assumption of unequal variance was performed on the correlation coefficients for the connection in each group. This analysis yielded several significant differences for functional connections between different Genotypes and Sibling Types.

Several nuclei showed functional associations in both ♂WT and ♂KO mice (**Figure 6**, top panel), while other functional associations were unique to ♂WT and ♂KO mice (**Figure 6**, bottom panel). Common functional associations, at $\alpha = 0.01$, were found among the amygdalar nuclei in the mRLC are: the ACo with the CeA and MeAA, and the MeAA with the CeA, PaN and VMH. In the mCLC

Table 3 | (A) Principal component analysis (PCA) of three groups of males [all males, wildtype (WT) males only, and estrogen receptor α knockout (ERKO) males only]. For each group there were 20 principal components (PCs), but only those given below satisfied the first criterion of $l^2 > 1$. Significant eigenvalues, marked as bolded numbers with asterisks, satisfied the second and third criterion. Other eigenvalues that shared similarities to significant eigenvalues in other groups are marked with a tilde (~). Both marked eigenvalues correspond to PCs that were considered in later analyses. **(B)** Rotated component matrix with significant PCs derived from the principal component analysis displayed in **Table 3A**. The columns are the significant PCs and PCs of shared similarity to significant PCs in other groups. The coefficient value for each PC is given after VARIMAX rotation.

	All ♂			♂WT only			♂ERKO only		
	Eigenvalue	**% Variance**	**Sign.**	**Eigenvalue**	**% Variance**	**Sign.**	**Eigenvalue**	**% Variance**	**Sign.**
A. PRINCIPAL COMPONENT ANALYSIS									
1	7.687	38.436	***8.247**	7.363	36.814	***4.306**	8.638	43.192	***5.973**
2	2.582	12.910	***3.729**	3.165	15.827	~2.196	3.340	16.698	~1.358
3	1.963	9.817	0.359	2.190	10.951	0.807	2.453	12.266	1.355
4	1.797	8.985	1.715	1.746	8.728	2.381	1.800	9.000	0.828
5	1.334	6.670	0.787	1.196	5.978	0.239	1.318	6.590	0.791
6	1.064	5.321	0.476						

	All ♂		♂WT only		♂ERKO only	
	***1**	***2**	***1**	**2**	***1**	**2**
B. MATRIX COMPONENT						
rMPOA	0.04	–0.05	0.22	0.01	0.11	–0.18
BST	–0.07	0.07	–0.05	–0.10	0.06	–0.05
BSTMA	0.40	0.25	0.13	0.25	0.29	0.45
AVPe	0.52	–0.15	***0.70**	0.04	–0.13	0.52
Pe	0.15	0.06	0.41	0.09	0.20	0.06
MPOA	0.19	0.21	0.41	0.36	0.23	0.11
BSTMPM	0.08	–0.07	0.01	–0.26	0.12	0.07
cMPOA	0.53	0.31	***0.67**	0.44	0.35	0.43
ACo	***0.84**	0.25	***0.92**	0.07	0.57	0.66
AHA	***0.78**	0.14	***0.69**	0.22	0.21	0.87
PaN	***0.84**	0.19	***0.66**	0.47	0.14	0.95
CeA	***0.84**	0.19	***0.89**	0.04	0.45	0.71
MeAA	***0.84**	0.22	***0.84**	0.04	0.46	0.73
MHb	0.25	0.00	0.36	0.07	0.05	0.20
LHb	–0.13	0.06	–0.05	0.14	–0.05	–0.23
VMH	0.56	0.45	***0.65**	0.28	***0.78**	0.36
MeAPD	0.38	***0.81**	0.15	0.71	***0.84**	0.35
MeAPV	0.26	***0.90**	–0.05	0.90	***0.86**	0.36
VMHDM	0.22	***0.80**	0.28	0.83	***0.87**	0.12
VMHDL	0.09	***0.86**	0.12	0.90	***0.90**	0.05

Asterisk indicates nuclei with a coefficient greater than 0.60 in significant PCs. These significant nuclei were placed in a cluster of nuclei representative of one of the two putatively conserved PCs. These probable conserved clusters can be noted by comparing coefficient values between PCs thought to conform to the same cluster such as PC 1 in all ♂ mice, PC 1 in ♂WT only, and PC 2 in ♂KO only. Significant values and PCs are indicated by bold numbers with asterisks.

common associations were between proximal subnuclei – the MeAPD with the MeAPV and the VMHVL with the VMHDM. It should be noted that there were no significant associations between these two clusters that are shared across genotypes.

Given the large number of functional associations shared by either genotype, there were still many functional associations that were unique to ♂WT mice. In the mRLC these include associations by the AHA with the cMPOA, CeA, PaN, VMH, and MeAA; by the VMH with the PaN, AVPe; and by the CeA with the cMPOA. In the mCLC of the ♂WT mice, unique functional associations are limited to the MeAPV with the VMHDM and VMHVL. In the ♂KO mice there are fewer functional associations: in the mRLC these are made by the cMPOA with the VMH and the MeAA and by the CeA with the PaN. In the mCLC, ♂KO mice had a functional association of the VMH with the MeAPD and the MeAPV.

Similarly, there were common functional associations among males that are housed with either ♂WT or ♂KO brothers (**Figure 7**, top panel), but other functional associations that were unique to males raised with ♂WT or ♂KO brothers (**Figure 7**, bottom panel).

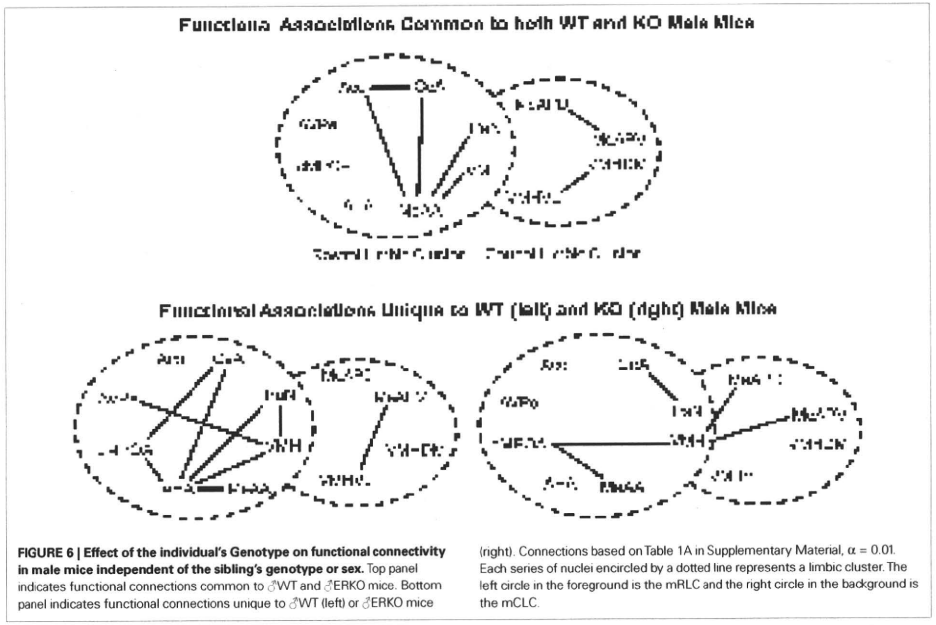

FIGURE 6 | Effect of the individual's Genotype on functional connectivity in male mice independent of the sibling's genotype or sex. Top panel indicates functional connections common to ♂WT and ♂ERKO mice. Bottom panel indicates functional connections unique to ♂WT (left) or ♂ERKO mice (right). Connections based on Table 1A in Supplementary Material, α = 0.01. Each series of nuclei encircled by a dotted line represents a limbic cluster. The left circle in the foreground is the mRLC and the right circle in the background is the mCLC.

Common associations were found among the amygdalar nuclei: the ACo with CeA, the CeA with MeAA, and the MeAA with the VMH in the mRLC. Functional associations also occurred between the MeAPD and MeAPV and between the VMHDM and VMHVL in the mCLC. Again, there were no significant associations between nuclei of the mRLC and mCLC suite of nuclei shared between Sibling Type groups. As with the male mice in this study, having a WT brother resulted in a number of unique functional associations, including significant associations between nuclei in the mRLC and mCLC suite of nuclei (**Figure 7**, top panel). These included associations of the PaN with the CeA, AHA, and VMH; the AHA with the VMH and cMPOA; and the cMPOA with the VMH in the mRLC. In the mCLC, the MeAPV associated with the PaN, VMH, and VMHVL. There were only two functional associations unique to males having KO brothers, and these were between the ACo and the MeAA in the mRLC and between the MeAPV and the VMHVL in the mCLC. The paucity of interactions for males raised with ♂KO may be due to the small sample size.

Limbic landscapes. To illustrate changes in the metabolic activity in selected nuclei (BNSTma; AVPe; MPOA; AHA; MeAPD; MeAPV; VMHVL) with defined neural connections, it is clear that the effect of having KO brothers or sisters in the litter have different consequences on WT males (**Figure 8**, bottom row). Further, in the mixed-sex, mixed-genotype litters, the effect of the genotype of the brothers or sisters had different effects on the limbic landscape of WT males (**Figure 8**, right column). The effect of Genotype in all-male litters indicated an overall decrease in activity, but no differences in activity pattern among the nuclei (**Figure 9**).

Females

Principal components analysis. In order to further compare the functional connectivity between males and females PCA was performed as before on three different groups generated from a subset of the female study population. Because of a large number of missing nuclei measures for individual animals, the full set of animals and nuclei had to be parsed to avoid the problem of missing values when performing PCA. The data set was then optimized for the maximum number of animals and nuclei. The best result featured 34 animals and 13 nuclei. Those nuclei were the LSI, MPOA, Pe, ACo, MeAA, CeAA, VMH, LHb, MHb, MeAPD, MeAPV, VMHDM, and VMHVL. It should be noted that the number of female animals used for PCA is nearly the same size as that used in the PCA of males, but with seven fewer nuclei considered. The types of groups used for PCA were discussed above.

The results of PCA for the three female groups can be visualized using the SCREE plots (**Figure 2**), and the analysis for significant PCs (**Tables 4A,B**). The number of significant PCs between groups and significant nuclei in each of those PCs are dissimilar to the results found in males. When all females were considered in the

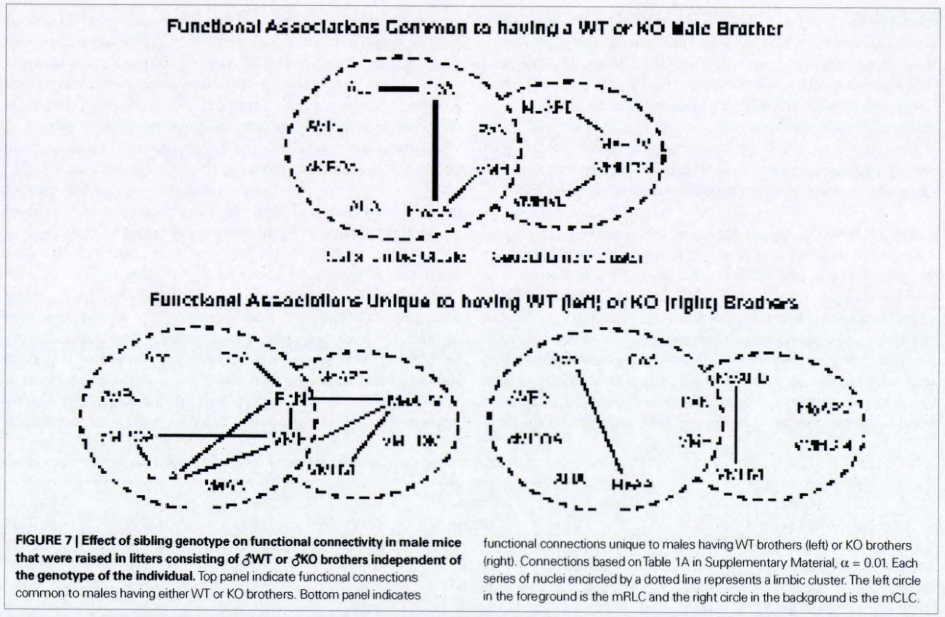

FIGURE 7 | Effect of sibling genotype on functional connectivity in male mice that were raised in litters consisting of ♂WT or ♂KO brothers independent of the genotype of the individual. Top panel indicate functional connections common to males having either WT or KO brothers. Bottom panel indicates functional connections unique to males having WT brothers (left) or KO brothers (right). Connections based on Table 1A in Supplementary Material, $\alpha = 0.01$. Each series of nuclei encircled by a dotted line represents a limbic cluster. The left circle in the foreground is the mRLC and the right circle in the background is the mCLC.

PCA there was only one significant PC, and the nuclei that showed significance were all amygdalar: ACo, MeAA, CeA, MeAPD, MeAPV. Although the subdivided groups of ♀WT and ♀KO females were evenly numbered at 17, PCA performed on each group resulted in PCs with significant nuclei that were not represented in both genotypes. The significant PCs and the significant nuclei within each of those PCs for all three groups (**Table 4A**) indicated one significant PC for all females, four significant PCs for ♀WT, and three significant PCs for ♀KO females (**Figures 10 and 11**).

These results differ from males due to two reasons: (i) There was a significant PC (PC2) in WT females with no analog in ♀KO females. (ii) Unlike males, where almost all significant nuclei in similar PCs were at or near significance between genotypes, similar PCs in females only contained a majority of shared significant nuclei between genotypes. Also, when comparing similar PCs between genotypes many nuclei showing significance in one genotype were not even near significance in the other.

Inter-regional correlations in metabolic activity. Although these significant clusters were less conserved than in the males, a jackknifing analysis was still conducted by genotype to distinguish the nature of the connectivity between different genotypes and to indicate qualitatively the difference between sexes. From the three possible similar PCs, only two showed an indication of multinuclei connections or clusters (>2 shared nuclei, with a majority of the nuclei in each PC shared) among female mice (**Table 4B**). These clusters shared some similarities to males in the nuclei considered, but were otherwise quite distinct (**Figures 10 and 11**). The more rostral of the two clusters, the female rostral limbic cluster contained the Pe, ACo, MeAA, CeA, MeAPD, and MeAPV, while the more caudal cluster, female caudal limbic cluster contained the LHb, MeAPV, VMH, VMHDM, and VMHVL. Like the males, the females maintained a cluster of nuclei associated with the amygdala and a cluster associated with the VMH and subnuclei, however this appeared to be the extent of the similarity. As before, nuclei were selected to enter into the cluster if significance was shown in either of the putative clusters. Results of the jackknifing can be seen in **Table 4B** for a comparison of genotype, and further demonstrated the differing nature of male and female connectivity.

Limbic landscapes. Mapping the limbic landscape in selected nuclei with defined neural connections, it is clear that the effect of having KO brothers or sisters in the litter have different consequences on WT females (**Figure 12**, bottom row). Further, in the mixed-sex, mixed-genotype litters, the effect of the genotype of the brothers or sisters had different effects on the limbic landscape of WT males (**Figure 8**, right column). The effect of Genotype in all male litters indicted an overall decrease in activity, but no differences in the pattern of activity among the nuclei (**Figure 9**).

DISCUSSION

An individual's genotype interacts with its environment throughout life to continuously shape the phenotype. In mammals, the formative environment for social and anxiety-related behaviors

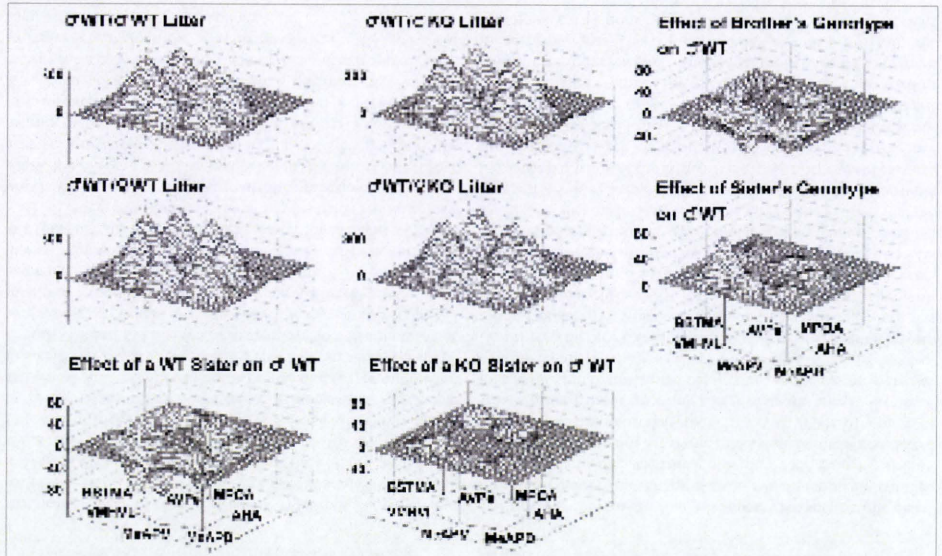

FIGURE 8 | The Sex and Genotype of siblings in the litter influences metabolic activity in the limbic landscape of adult male mice wildtype (WT) for the estrogen receptor α gene. Limbic landscapes of ♂WT raised with brothers or sisters of the same or different genotype are depicted. Illustrated is the mean cytochrome oxidase (COX) activity in each nucleus of each ♂WT. Groups are presented according to the type of sibling with which the ♂WT individual was raised; ♂WT, ♂KO; ♀WT; ♀KO. The limbic landscape maps on the bottom row give the difference between the ♂WT/♂WT and ♂WT/♀WT (left) and the ♂WT/♂WT and ♂WT/♀KO maps (right), indicating the effect of the sister's genotype on ♂WT. The far right column indicates the effect of sibling's genotype; on the right top is the effect of the brother's genotype and in the middle row is the effect of sister's genotype; the far right column is the difference between the ♂WT/♂WT and ♂WT/♂KO (top right) and the ♂WT/♀WT and ♂WT/♀KO maps (middle right). The nuclei are presented in a clockwise fashion reflecting a rostral-caudal dimension: main bed nucleus of the stria terminalis (BNSTma); anteroventral periventricular nucleus (AVPe); medial preoptic area (MPOA); anterior hypothalamus, anterior (AHA); medial amygdaloid nucleus, posterodorsal (MeAPD); medial amygdaloid nucleus, posteroventral (MeAPV); ventromedial hypothalamic nucleus, ventrolateral (VMHVL).

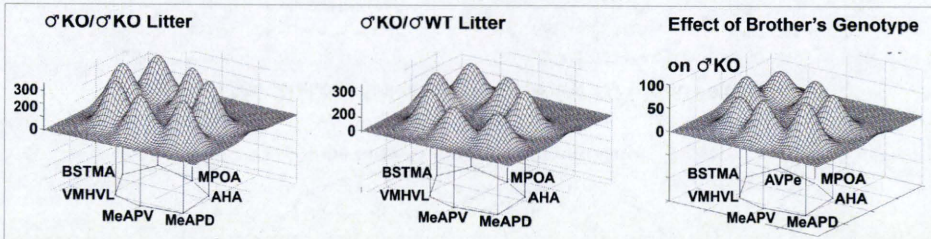

FIGURE 9 | The genotype of brothers in the litter influences metabolic activity in the limbic landscape of adult male estrogen receptor α knockout mice (♂KO). Groups presented according to the type of sibling with which the male was raised (with ♂KO or ♂WT brothers). The limbic landscape map on the right represents the difference between ♂KO/♂KO litter and ♂KO/♂WT litter, or the Effect of the Brother's Genotype on ♂KO mice. Other details as in **Figure 8**.

is the family unit; in the case of rodents, this is the litter and the mother-young bond. A deciding factor in this environment is the sex ratio of the litter and, in the case of mice lacking functional copies of gene(s), the ratio of the various genotypes in the litter. A summary of the behavioral data obtained with males indicate that when raised in litters having either ♂KO or ♀KO littermates, ♂WT are more aggressive than when raised in litters containing either ♂WT or ♀WT littermates. KO males are not aggressive

Table 4 | (A) Principal component analysis of three groups of female mice (all females, wildtype (WT) females only, and knockout (KO) females only). For each group there were 21 principal components (PCs), but only those given below satisfied the first criterion of $\lambda^2 > 1$. Significant eigenvalues, marked as bolded numbers with asterisks, satisfied the second and third criterion. Other eigenvalues that shared similarities to significant eigenvalues in other groups are marked with a tilde (~). Both marked eigenvalues correspond to PCs that were considered in later analyses. **(B)** Rotated component matrix for groups of female mice with significant PCs derived from the principal component analysis displayed in **Table 4.** The columns below are the significant PCs; there were no insignificant PCs of shared similarity. The coefficient value for each PC is given after VARIMAX rotation.

	All ♀			♀WT only			♀ERKO only		
	Eigenvalue	**% Variance**	**Sign.**	**Eigenvalue**	**% Variance**	**Sign.**	**Eigenvalue**	**% Variance**	**Sign.**
A. PRINCIPAL COMPONENT ANALYSIS									
1	3.935	30.268	***4.786**	4.629	35.604	***2.179**	3.950	30.383	***1.529**
2	2.081	16.006	2.879	2.690	20.689	***1.901**	2.691	20.700	***1.323**
3	1.693	13.026	0.250	1.800	13.844	***0.793**	1.868	14.369	***5.974**
4	1.559	11.991	2.408	1.332	10.244	***10.342**	1.246	9.585	0.376
5	1.021	7.851	0.796	0.742	5.706	0.083	1.142	8.784	0.637
6	3.935	30.268	4.786	4.629	35.604	2.179	3.950	30.383	1.529

	All ♀	♀WT only				♀ERKO only		
	***1**	***1**	***2**	***3**	***4**	***1**	***2**	***3**
B. MATRIX NUCLEI								
LSI	−0.002	0.240	***0.771**	0.207	−0.246	−0.183	0.033	−0.036
MPOA	−0.023	−0.195	***0.875**	−0.029	−0.277	0.100	0.074	−0.007
PE	0.137	−0.139	***0.839**	−0.080	0.237	***0.721**	−0.164	0.148
ACO	***0.755**	***0.874**	0.150	0.130	−0.212	***0.775**	0.364	−0.152
MEAA	***0.885**	***0.946**	0.083	0.073	−0.006	***0.786**	0.069	−0.393
CEA	***0.858**	***0.904**	−0.138	−0.185	0.085	***0.835**	0.178	0.006
VMH	0.258	−0.113	0.229	***0.697**	−0.472	0.386	***0.831**	−0.112
LHb	−0.254	−0.146	0.311	***−0.796**	0.048	−0.290	−0.103	***0.899**
MHb	0.117	0.041	−0.109	0.060	***0.865**	0.121	0.037	***0.864**
MEAPD	***0.648**	***0.820**	−0.204	0.261	0.160	0.280	0.192	0.338
MEAPV	***0.726**	***0.873**	−0.125	0.236	0.112	0.387	***0.650**	0.246
VMHDM	−0.017	0.067	0.374	***0.727**	0.414	−0.131	***0.736**	−0.007

Asterisk bolded numbers indicate nuclei with a coefficient greater than 0.60 in significant PCs.

when raised in litters containing either ♂KO or ♂WT littermates. Anxiety-like behaviors, reflected in behavior in the Light:Dark box, were modified in WT males if they had WT sisters in the litter, an effect that was absent if the sisters were KO mice. A summary of the behavioral data obtained with females indicate that the social behavioral profile of ♀KO mice is most clearly distinguished from that of ♀WT mice when ♀KO mice are raised in litters containing only ♀KO mice; ♀KO mice raised with ♂WT mice behave similarly. ♀KO mice are more similar to ♂WT mice than they are to ♀WT mice in their behavior, metabolic activity, and functional connectivity, suggesting that ♀KO mice play a male-type role in the preweaning sibling environment.

Regarding the effect of Genotype and Sex ratios of the litter on brain metabolic activity later in adulthood, it is noteworthy that ♂WT and ♂KO do not differ in their WB metabolic activity, but when male mice were raised in litters having ♂KO, or females of either Genotype, overall brain activity was higher than if raised in litters having only WT males. In females, those having ♀WT siblings had higher WB activity, and the presence of ♀KO or ♂WT siblings had the effect of lowering WB activity. Whether this and the other observed effects were due to direct interactions of the pups, or indirectly a consequence of differences in maternal behavior with different types of litters is not known. It appears there may be a neural mechanism affected by the sex of the individual that reinforces the complete activation or depression of COX activity when a WT sibling of the same sex is present. This can be taken to mean that a WT sibling of the same sex induces a physiological or neurological response that alters nuclei and WB activity. Since KO and opposite sex siblings do not induce this response, there is likely some differentiation by the individual (or possibly mother) of what is a normal sibling.

Most social information is gained through olfaction in rodents and, after transduction of chemosensory signals, transmitted through well-defined neural circuits (Blaustein, 2008; De Vries and Simerly, 2002; Hull and Rodriguez-Manzo, 2008; Newman, 1999). Prominent nuclei in this system are the medial amygdala (including the MEAA, MeAPD, and MeAPV), which project to the bed nucleus of the stria terminalis (including the BST, BSTMA, and BSTMPM) and, in turn, to the medial preoptic area and anterior hypothalamus. In the present study COX activity in the MeAPD, MeAPV, BSTMA, and AHA, was

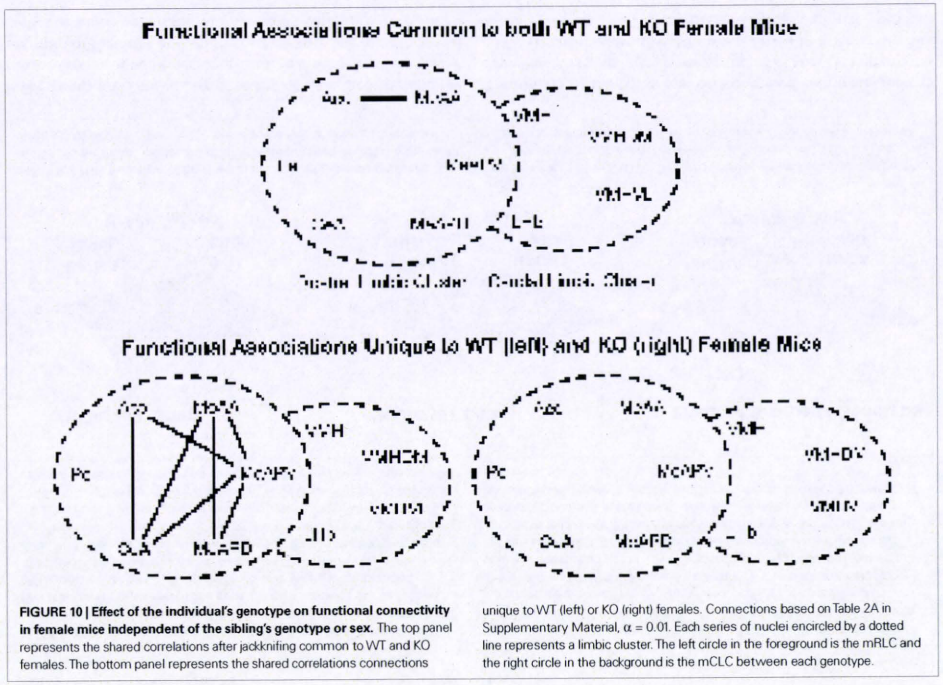

FIGURE 10 | Effect of the individual's genotype on functional connectivity in female mice independent of the sibling's genotype or sex. The top panel represents the shared correlations after jackknifing common to WT and KO females. The bottom panel represents the shared correlations connections unique to WT (left) or KO (right) females. Connections based on Table 2A in Supplementary Material, $\alpha = 0.01$. Each series of nuclei encircled by a dotted line represents a limbic cluster. The left circle in the foreground is the mRLC and the right circle in the background is the mCLC between each genotype.

significantly lower in males raised with WT brothers compared to males raised with KO brothers. Further, males raised with WT sisters had higher COX activity in the MeAPV and the VMHVL compared to males raised with WT brothers. The functional associations of metabolic activity in the network of nuclei was also significantly modified by the litter composition. The differences in limbic landscapes of male and female mice of different genotype and sibling type captured in **Figures 8, 9, 12, and 13** illustrate the effects of Sex versus Genotype in both WT and KO male and female mice. Note that the effect of Sex (male versus female) is substantially different among WT and KO mice, just as the effect of Sibling Genotype is different between male and female mice. It is of interest that ♂KO and ♀WT share similar means, although the female is not statistically different from male WT (possibly due to decreased sample size) for all nuclei except the VMHVL, suggesting this nucleus may be affected by female siblings preferentially. Further, the behavioral differences observed are likely a function of some combination of altered WB, nuclei, or network activity. For example, males raised with ♂WT siblings behave differently than do males raised with ♂KO siblings. This may result from lowered WB activity or from one or more specific nuclei being significantly lowered in activity. The AHA is significantly lowered in activity for males raised with ♂WT siblings and also appears to be strongly correlated with several other nuclei in the RLC and not at all in males raised with ♂KO siblings.

Principal Component Analysis appeared to successfully determine portions of underlying networks of functionally coupled nuclei. Applying the jackknifing procedure to these networks can be thought of as looking for strong correlations in nuclei preselected for probable strong correlations (Sakata et al., 2000). In support of what appears to be an arbitrary choice of significance in the use of PCs, the resulting functional connections in the cluster were not completely interconnected (which would imply too high of a selection criteria for the PC coefficient), nor too large to begin interpretation (too low of a selection criteria for the coefficient), nor was there an excessively large list of nuclei to even begin interpretation and comparison (also suggesting too low of a selection criteria). Using PCA revealed males to have a network of functional associations more conserved between genotypes than females, possibly due to a smaller nuclei sample in females. The observation that the first and second PCs shared similar coefficients between Genotypes led to the reduction of the 20 nuclei into two independent clusters, the RLC and the CLC. These clusters shared two nuclei throughout jackknifing, the VMH and the PaN. While the VMH met the selection criterion in one of the PCs for both clusters, the PaN did not meet selection criteria in the mCLC. Thus, the PaN was used to determine if lower selection criterion for the PCs would yield more meaningful results. This comparison showed only four significant functional correlations between the PaN and some other nucleus

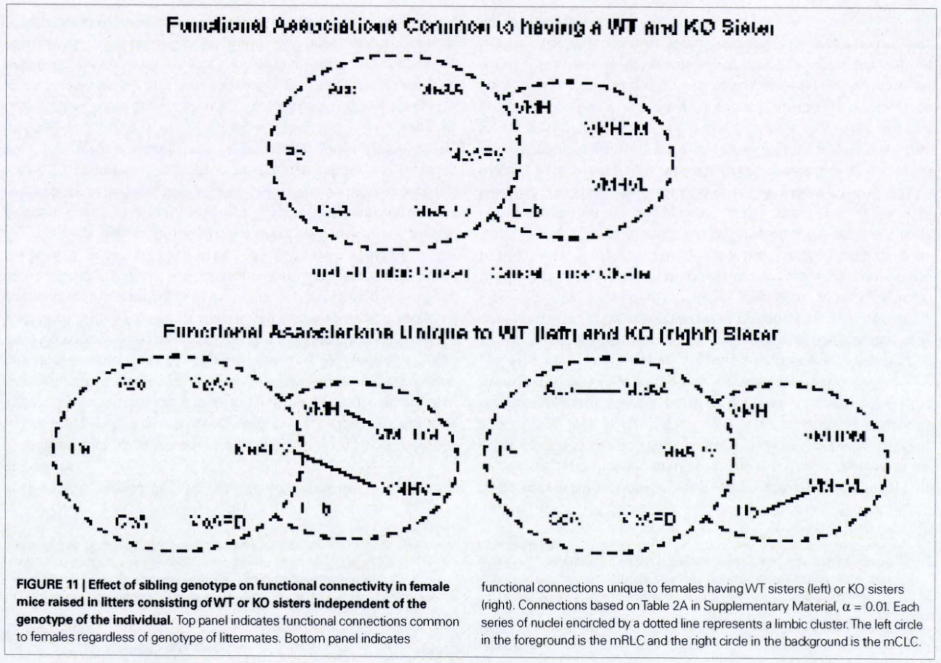

FIGURE 11 | Effect of sibling genotype on functional connectivity in female mice raised in litters consisting of WT or KO sisters independent of the genotype of the individual. Top panel indicates functional connections common to females regardless of genotype of littermates. Bottom panel indicates functional connections unique to females having WT sisters (left) or KO sisters (right). Connections based on Table 2A in Supplementary Material, $\alpha = 0.01$. Each series of nuclei encircled by a dotted line represents a limbic cluster. The left circle in the foreground is the mRLC and the right circle in the background is the mCLC.

in the CLC across all groups; three of these connections occurring between the PaN and VMH in the RLC. On the other hand, the VMH had nine connections in the CLC across all groups, three of which were shared with the PaN. There were six unique connections with the VMH in the CLC, compared to one in the PaN, suggesting that the VMH was actually functionally connected in both clusters of nuclei, whereas the PaN was likely just part of the RLC, the cluster in which it met selection criterion. The one connection of the PaN in the CLC with the MeAPV (**Figure 7**) is likely a functional circuit involving the VMH as this nucleus connects with both the PaN and the MeAPV. Consequently, the functional connection between the MeAPV and PaN could be mediated by the VMH.

The selection of clusters according to the results of PCA suggest a physically relevant cluster of nuclei, as the clusters were relatively conserved between Genotypes and the nuclei were placed in clusters without preconceptions about interactions between nuclei (excluding the placement of the PaN in the CLC). The nuclei considered in the RLC are associated with very different functions, albeit all of which have been related to mediating aggressive and sexual behaviors. The nuclei constituting the CLC have been implicated in sociosexual behaviors as well as feeding behavior. It is interesting that in certain instances anatomically associated nuclei were in different clusters. Nuclei that are very close to one another were usually quite similar in metabolic activity, so functional connectivity often follows. However, subnuclei in the medial amygdala – containing the MeAA, MeAPD, and MeAPV – and in the ventromedial hypothalamus – containing the VMH, VMHVL, and VMHDM, did not show strong correlations to one another. The MeAA was placed in the RLC and the MeAPD and MeAPV were placed in the CLC with little support for the MeAA to be placed with the posterior medial amygdaloid nuclei. This segregation of anterior and posterior medial amygdalar nuclei agrees with Newman (1999) who argued that the "medial extended amygdala" is divided into an anterior functional circuit and a posterior functional circuit. The VMH, on the other hand, divided independent of the VMHDM and VMHDL into the RLC, but also divided with these nuclei into the CLC. This suggests that two functional circuits, one which shares the anterior nuclei, that is the MeAA and VMH, and another posterior functional circuit that shares functional connectivity between the VMH, VMHVL, VMHDM, MeAPD, MeAPV. In support of the expected functional connectivity of proximal anatomic nuclei, the MeAPV connecting with the MeAPD, and the VMHVL connecting with the VMHDM, showed some of the most consistent functional connections among all groups. However, in the CLC the VMH showed functional connectivity with the MeAPV more frequently than either of the more caudal VMH subnuclei across groups. The reason for this functional dissociation of the VMH from the posterior VMH nuclei is not known, but may be related to the anterior–posterior division of the medial amygdala and ventromedial hypothalamus subnuclei discussed above.

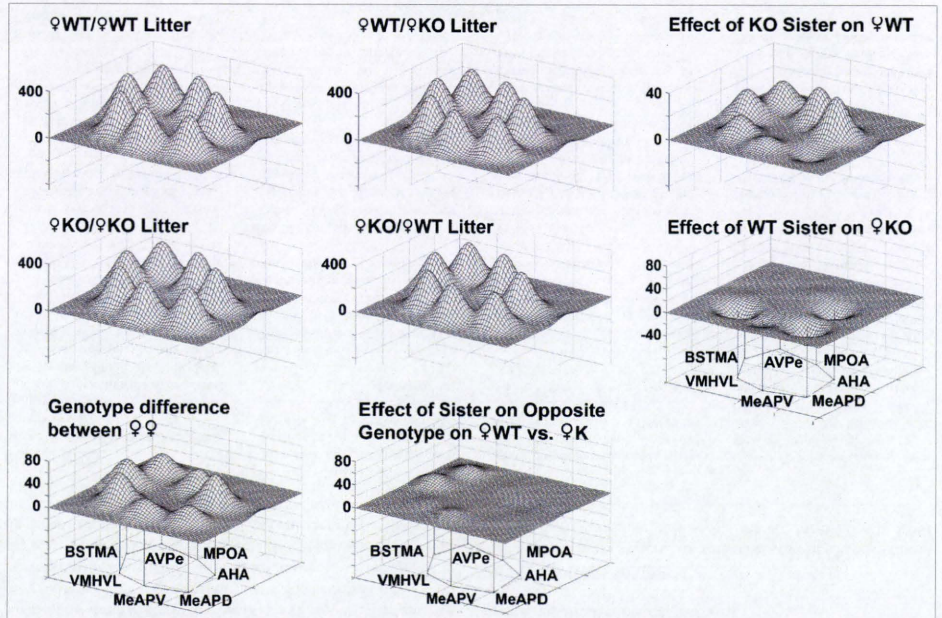

FIGURE 12 | Effect of genotype of sisters of on metabolic activity in a social behavior network in female wildtype (WT) and estrogen receptor a knockout (KO) mice. Groups are presented according to the type of sibling with which the WT (top row) or KO female (middle row) was raised with ♀KO or ♀WT sisters, respectively. The limbic landscape map on the upper right represents the difference in ♀WT raised with ♀KO sister. Below that is the complement, that is, the effect of a WT sister on the metabolic activity in ♀KO females. The bottom row indicates genotype differences between ♀WT and ♀KO females raised in same-sex, same-genotype litters. Right column is the effect of having a sister having the opposite genotype. Other details as in **Figure 8**.

The significant correlation coefficients (**Table 3B**) can be thought of as functional connections or associations, meaning that these correlations associate the COX activity in one nucleus with the COX activity in another nucleus, demonstrating that the two nuclei are functionally coupled in the animal. These associations do not imply directionality of an effect, or, even in the case of a demonstrated neuroanatomical connection, that the correlation is caused by direct activation of one nucleus upon another (Gonzalez-Lima and McIntosh, 1995). The correlation coefficients can only show how well the COX activity in one nucleus can predict the COX activity in another nucleus. The physiological meaning of this correlation, and, consequently, how the jackknifed correlations are to be interpreted depends on what a functional connection between nuclei reveals about the biological system. When a significant correlation occurs between two nuclei using COX activity, it may be understood that increased or decreased activity in one nucleus associates with the increased or decreased activity of the other nucleus. As COX activity shows the capacity of a cell to perform under metabolic demand, functional connectivity may be thought of as showing that the maximal metabolic capability of two nuclei to perform is associated (Sakata et al., 2005).

At the cellular level, this association is given greater meaning. Increased demand in energy by the cell has been shown to activate the bigenomic machinery responsible for the production of COX (Scarpulla, 2006), and, in the neuron, metabolic demand is mostly attributed to maintaining the proper membrane potential using the Na^+/K^+ ATPase (Wong-Riley, 1989). Since the depolarized neuron has a greater demand repolarize its membrane, it follows that energy consumption and the amount of COX present will depend upon the amount of tonic activation of a neuron due to excitatory signals from other neurons (Wong-Riley, 1989). Consequently, abundant COX activity within a given nucleus represents consistent activation by excitatory signals. Thus, functional connectivity reflects regions whose activity is correlated to some degree. Strong functional connections (high correlations ~0.8–0.9) can even be thought of as functional circuits due to the near complete coupling of activity between the two nuclei. However, such functional connections cannot be thought of as anatomical circuits due to the possibility of an unmeasured nuclei being involved. In fact, in the case of measurements made in the limbic brain, such as this study, it is almost impossible to consider every nucleus involved in a hypothesized circuit. Functional connections found between nuclei

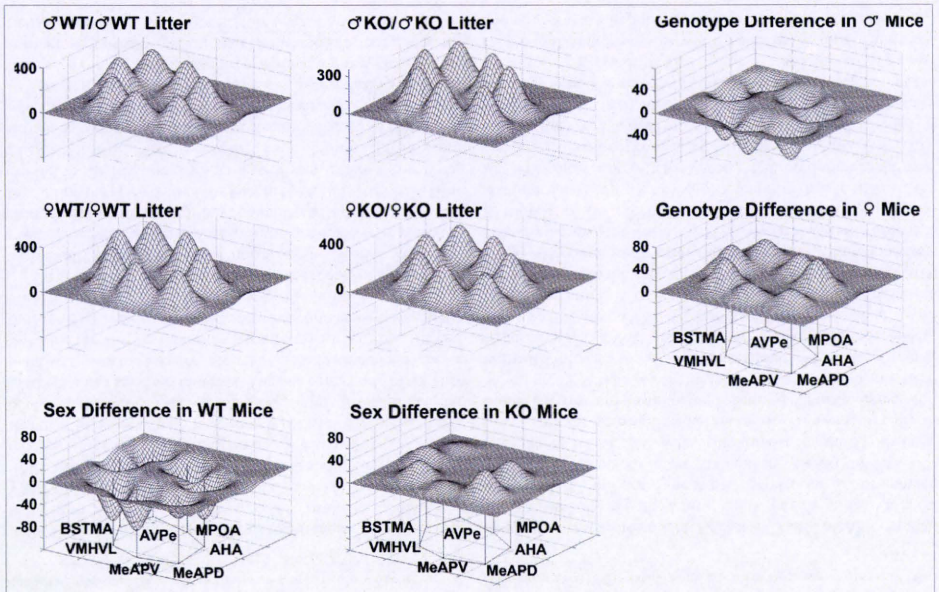

FIGURE 13 | Sex versus genotype differences in wildtype (WT) mice and mice having a dysfunctional estrogen receptor a (KO) mice. Groups consisted of same-sex, same-genotype litters (♂WT/♂WT; ♂KO/♂KO; ♀WT/♀WT; ♀KO/♀KO). The limbic landscape map on the upper right represents the genotype difference in male mice and below that is the genotype difference in female mice. The bottom row indicates sex differences between male and female WT mice and to the right is the sex difference in KO mice. Other details as in **Figure 8**.

in this study should then be considered as possible components of circuits.

Within each cluster certain functional connections are conserved independent of litter composition (i.e. Genotype or Sibling Type), suggesting that these functional connections are maintained independent of treatment. Both KO or WT males shared certain functional connections: MeAA with ACo, CeA, PaN, and VMH, and the ACo with CeA in the mRLC, and MeAPD with MeAPV and the VMHDM with VMHVL in the mCLC. Some of these functional connections are reasonable to expect due to the close proximity of each nuclei or subnuclei such as the functional connections between the MeAPD with MeAPV, and the VMHDM with VMHVL, or the slightly more distant functional connections between the MeAA, ACo and CeA. However the relatively distant functional connections between the MeAA and the PaN and VMH suggests that the MeAA is acting in a conserved functional connection or circuit. In comparing the effect of male Sibling Type on functional connectivity the following relationships maintained significance at $\alpha = 0.01$ level: the CeA with ACo and MeAA, MeAA with VMH, MeAPD with MeAPV, and VMHVL with VMHDM. This differs from the conserved connection between Genotypes in the loss of a functional connection of the MeAA with the ACo, CeA, and PaN. The above comparisons of functional connections are given at $\alpha = 0.01$, but there are some differences in correlations between comparable groups for Genotype or Sibling Type that are maintained at $\alpha = 0.05$. However, many other functional connections become significant for both Genotype and Sibling Type at $\alpha = 0.05$. Those new functional connections that are significant across treatments are the AHA with the PaN, the MeAA with the PaN, and the MeAPV with the VMHVL.

Large differences between groups in functional connectivity are possible indicators of connections and circuits that may contribute to differing behavioral phenotypes. In comparing Genotype, it appears that WT males have many more significant functional connections that appear in the mRLC in comparison to KO males, who have relatively few unique functional connections in this cluster (**Figure 6**). WT males show several strong connections that are not present in KO males (e.g., AHA with CeA, VMH, MeA, and cMPOA; cMPOA with CeA and AHA). Many of these nuclei are known to be involved in male sexual behavior and social interactions. The AVPe only shows one functional connection in the WT males (AVPe with VMH) and this connection is likely due to a large axonal connection between the AVPe and the BST that is known to be dependent upon estrogen for its formation. In KO animals, a unique functional connection between the VMH and the MeAPD and MeAPV was seen that had no counterpart in WT males.

The males having either WT or KO brothers shared certain functional associations: the ACo with the CeA, the CeA with the MeA, and the MeA with the VMH in the RLC and between the MeAPD and the MeAPV and between the VMHVL and the VMHDM (**Figure 7**). Males having WT brothers had many more functional associations than if they had KO brothers, although this may have been due to the number of males considered in each group.

Behavioral neuroscience has been characterized by a candidate nuclei/gene approach that is a gross oversimplification of what occurs in the brain during behavior. The fact that nuclei (or genes) do not operate in isolation but in networks may seem obvious, but this is not reflected in how most scientists write or communicate. Newman (1999) argued that an extended network of interconnected brain nuclei, including many of the nuclei measured in this study, functions in most basic social behaviors in rodents and, further, that this network is modulated by gonadal sex steroid receptors to function differently under different conditions. Another recent study taking alternative approaches has also emphasized a network approach to describe neural systems involved in responding to subordinate conspecific intruders (Motta et al., 2009). The present results indicate that an individual's experience in the litter influences its behavior as an adult by influencing the metabolic activity in integrated limbic circuits. This and previous research indicate that such experiences also shape how different individual respond to events later in life. This work is important for several reasons. First, it demonstrates a profound effect of the Sex and Genotype ratio of the litter on brain and behavior of genetically modified mice. Even in highly inbred rodents all litters are different, varying not only in the sex ratio and parity of the mother, but in the case of genetically modified animals, the genotype of the individual members. Such animals have been a mainstay in the area of molecular behavioral neuroscience, yet virtually every study to date has ignored the litter as a source of variation. By deconstructing the two variables (Sex and Genotype) that define the litter we show how the interplay of these factors shapes the neural substrates of behavior in the commonly used estrogen receptor KO mouse model. Second, we demonstrate how complex behavioral traits depend upon networks of nuclei whose functional relationships are altered fundamentally as a result of the litter environment in which the individual is raised. Third, we show how the potential for behavioral differences is based on the abundance of COX in specific brain nuclei and how the genotype (WT or KO) of brother and sister littermates modifies the amount of COX in limbic nuclei identified. Fourth, the work addresses key issues in how experiments in this very large field should be designed to yield a deeper understanding of how neural systems are organized early in life, particularly if the scientist's goal is to reveal more about how the individual is formed and functions. We argue that most studies today focus at the level of the control and consequences of gene action and hence miss the larger picture. Finally, this work touches on fundamental concepts of development of the neural substrates of behavior, such as how functional systems can be re-organized depending upon the composition of the litter in which the individual develops. Most important in this genocentric age, the ever-increasing use of genetically modified animals in behavioral neuroscience research makes in imperative that practitioners be aware of this important formative element.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/behavioralneuroscience/paper/10.3389/neuro.08/012.2009/

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Effect of ER-β gene disruption on estrogenic regulation of anxiety in female mice

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ABSTRACT

It has been shown that long-term estrogen treatment in gonadectomized female mice increases anxiety levels. On the other hand, a recent study has reported that estrogen may down-regulate the levels of anxiety by acting through estrogen receptor (ER) β. In the present study, we investigated the role of ER-β in the regulation of anxiety levels in female mice after long-term estrogen treatment. Gonadectomized ER-β knockout (βERKO) female mice and their wild type (βWT) littermates were implanted several different doses (experiment 1: 2.0 μg/day, experiment 2: 1.0, 0.4, 0.2 or 0.1 μg/day) of an estradiol benzoate (EB) or placebo pellet. Ten days after pellet implant, behavioral tests commenced to measure the anxiety levels (experiment 1: light–dark transition test (LDT), experiment 2: LDT, elevated plus maze test (EPM) and social investigation test (SIT)). We found that, at higher-doses, long-term treatment of EB had anxiogenic effects in both βWT and βERKO mice as indicated by a decrease of the time spent in the light side and the number of transitions between two sides during LDT. In contrast, several behavioral measurements indicated that the lower-doses treatment of EB might reduce the anxiety levels possibly through ER-β. Particularly, the anxiolytic effects of EB in the SIT were more pronounced in βWT mice than βERKO mice. Together, the findings in the present study suggest that estrogen may have both anxiolytic and anxiogenic effects in female mice, and that ER-β gene disruption did not affect anxiogenic regulation by estrogen in female mice, but partially affected anxiolytic regulation.

Estrogens are known to affect anxiety or fear-related behavior. Symptoms of depression, anxiety and cognitive dysfunction in postmenopausal women have been associated with reduced levels of estradiol; estrogen replacement therapy improved the mood disturbance [1]. In rodents, lower circulating levels of estradiol were often associated with an increase in anxiety-related behaviors. Female rats [2,3] and mice [4] in the diestrus stage during their estrous cycle show shorter time spent in open arms of elevated plus maze (EPM) than females in the proestrus or estrus stage. Acute estrogen treatment of female rats in the diestrus stage, which mimics the hormonal level that occurs during the proestrus, reduced anxiety, as indicated by a larger percentage of the spent time in open arms of EPM [2]. These results strongly suggest that an increase in estradiol level in estrus females may be responsible for the reduction of their anxiety.

On the other hand, the effects of estrogen replacement in ovariectomized mice and rats on anxiety-related behaviors are still controversial. An acute single injection of estrogen to ovariectomized rats 3 h before behavioral tests had no effect on the number of entries to open arms nor the percentage of time spent in the open arms during EPM tests [3,5]. Three consecutive daily injections of estradiol benzoate (EB) to ovariectomized rats, however, enhanced the time spent in the open arms of EPM [5]. This discrepancy of the effects of estrogens may not be simply due to the difference in the number of injections (single vs three daily injections). Rather, it may be due to the interval between estrogens injection and behavioral tests. A single EB injection could increase open-field activity [6] and time spent in open arms during EPM test [7] in ovariectomized rats when they were tested 48 h after the injection.

In contrast to these anxiolytic results of bolus treatment, chronic estrogen treatment in ovariectomized female mice is reported to increase anxiety levels. Female mice implanted with a capsule containing EB for 7 days prior to behavioral tests showed higher levels of fear responses in a range of fear and anxiety-provoking situations than the animals treated with a vehicle-containing capsule [8,9]. The anxiogenic effects of chronic estrogen treatment were also founded in humans. Postmenopausal women chronically treated with 17β-estradiol on 3 month exhibited a significant increase in negative mood and anxiety [10], and 10 years treatment with estradiol implants in healthy postmenopausal women impaired their cognitive

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function [11]. These results suggest that the effects of chronic estrogen treatments on mental situation differ from that of acute one. Additionally, chronic enhancements of estrogen level may also relate to mood disorders in pregnant and postpartum women [12].

Since the difference between the bolus injections and the chronic treatment was not only a treatment period but also the amount volume of estrogens during the period, these results suggest that estrogen may have different effects on anxiety-related behavior depending on the dose treated to the animals. Indeed, for several memory tasks, bipolar effects of estrogen were reported. High-dose estradiol injection impaired, whereas low-dose estradiol injection facilitated, the performances of ovariectomized female rats on spatial memory tasks [13,14] and non-spatial working memory task [15]. Additionally, many endocrine factors have unusual dose–response relationship, including U-shaped and inverted U-shaped curve, by interacting with the other endocrine mechanism. It may be possible that the dose effects of estrogens on anxiety-related behavior show nonlinear form.

Recent studies using genetically modified mice reported that estrogen receptor (ER) β might play an important role in the regulation of anxiety levels. Krezel et al. [16] reported that gonadally-intact female mice with disrupted ER-β gene spent shorter time in the open arms of the EPM. Ovariectomized female ER-β knockout (βERKO) mice also showed shorter spent time in the outer half of the open arms than their wild type, regardless of presence or absence of estrogen treatment [17]. Additionally, injection of diarylpropionitrile (DPN), which is a selective ER-β agonist, reduced anxiety-related behavior in ovariectomized female rats [18,19]. These results suggested that ER-β is involved in anxiolytic effects of estrogen.

In contrast, it is not known whether ER-β may also mediate anxiogenic effects of estrogen reported in chronically treated animals. In the present study, we investigated the role of ER-β in the regulation of anxiety levels in female mice after chronic EB treatment using female βERKO mice (experiment 1). Since it is still possible that differences in the behavioral effects of estrogen in animals treated with bolus injections and those treated chronically may be due to differences in the amount of estrogen administered, we tested the mice with several different doses of EB (experiment 2). In addition, we measured anxiety-related behaviors in several different behavioral paradigms in experiment 2, because the effects of estrogen on anxiety level may depend on the experimental context, such as testing paradigms. Measurements in behavioral tests commonly used to measure anxiety levels, such as open-field and EPM tests, are often confounded with general activity levels of animals and are affected by testing conditions, such as brightness of illumination [20]. In contrast, animals' behaviors in light–dark transition tests (LDT) are known to be relatively independent of activity levels and differences in testing conditions. Therefore, in the present study we tested the same animals both in EPM and LDT to compare the behavioral effects of various doses of estrogen and ER-β gene disruption in two types of anxiogenic situations. We also performed social investigation tests (SIT) to assess social anxiety levels, which were measured as responses to an intruder mouse presented in experimental animals' home cage.

1. Methods

1.1. Mice

βERKO female mice and their wild type (βWT) littermates were used. They were obtained from the βERKO breeding colonies maintained at the Rockefeller University by mating heterozygous male and female mice. Original breeding pairs (in C57BL/6J background) were obtained from the National Institute of Environmental Health Sciences [21]. From weaning to 10 days before the first behavioral tests, subjects were housed with same-sex littermates in plastic cages (30×20×12 cm). At the age of 9–17 weeks, mice were ovariectomized and received subcutaneous implants with either a β-estradiol 3-benzoate (EB) pellet or a placebo pellet (Innovative Research of America, Toledo, OH) under anesthesia with isoflurane inhalation. All pellets were on a 21-day release schedule and average amount released per day was calculated based on the total amount of EB in the pellets. In experiment 1, mice in each genotype were divided into two groups, (1) placebo (βWT: $n=11$, βERKO: $n=12$) and (2) EB 2.0 μg/day pellet (βWT: $n=12$, βERKO: $n=13$). In experiment 2, mice were treated with five different doses of EB, (1) EB 0 μg/day (placebo, βWT: $n=10$, βERKO: $n=11$), (2) EB 0.1 μg/day (βWT: $n=10$, βERKO: $n=9$), (3) EB 0.2 μg/day (βWT: $n=7$, βERKO: $n=7$), (4) EB 0.4 μg/day (βWT: $n=10$, βERKO: $n=9$), or (5) EB 1.0 μg/day (βWT: $n=9$, βERKO: $n=11$). After ovariectomy and pellet implants, all mice were individually housed in plastic cages (30×20×12 cm), except during the period of social investigation test described below. They were kept on a 12/12 h light/dark cycle (light off at noon) at constant temperature (22 °C) throughout the extent of the studies. Food and water were available *ad libitum*.

1.2. Behavioral tests

Starting 10 days after pellet implantation, all mice were tested for anxiety-related behaviors. In experiment 1, mice were tested once for light–dark transition on the 10th day. In experiment 2, mice were tested in three different behavioral paradigms, in the order of LDT (Day 10), EPM (Day 13) and SIT (Day 16). All behavioral tests were performed starting 2 h after lights off.

1.2.1. Light–dark transition (LDT) test

The test apparatus consisted of a clear plastic box (50×50×35 cm) with a black (light opaque) covered-plastic box (50×25×25 cm) in one side (the dark side). The black box had an open doorway (2×5 cm) that led to the light side of the apparatus, which was illuminated by a 40-W white bulb (about 420 lx on the floor). Mice were moved from the living room to the testing room at least 1 h before the test. At the beginning of the tests, mice were removed from their cages, gently placed at the doorway, and released into the dark side of the apparatus. A Digiscan analyzer and software (Digiscan Model RXYZCM, Accuscan Instruments, Columbus, OH) were used to collect and store horizontal-activity data, which was monitored by infra-red beams. For each mouse, the following measurements were recorded for 10 min: total moving time and total activity (total number of beam breaks) in each compartment, total time spent in the dark compartment, total time in the light compartment, number of transitions between the dark and light compartments, and the latency to the first emergence from the dark to the light compartment. After the test, the amount of defecation was counted. Between the tests, the apparatus was thoroughly wiped clean.

1.2.2. Elevated plus maze (EPM) test

Mice were tested in a maze consisting of a 5×5 cm center platform from which extended four acrylic arms in a cross formation, with two opposing arms enclosed by side walls and two left open (Rockefeller University Instrument Shop, New York, NY). Arms were 30×5 cm, with the enclosed arms having 15-cm-high opaque acrylic walls. The entire maze was elevated 40 cm above the floor and illuminated by a 50-W red light bulb suspended 100 cm above the maze. At the beginning of the test, mice were placed in the center of the maze, facing to one of the open arms. Number of entries into and time spent in open arms and closed arms were recorded manually during 5 min tests.

1.2.3. Social investigation test (SIT)

One day before the test, mice were transferred to larger housing cages (45×23×15.5 cm). They were tested against gonadally intact male mice of C57BL/6J strain for 10 min. Male stimulus mice were

placed in a clear perforated (28 small holes with a 5 mm diameter on the bottom 3 cm) plexiglass cylinder (7 cm diameter×12 cm height) at least 10 min before the testing. They were then introduced into the center of females' housing cages. Total moving distance of female mice in the housing cage, and total number and cumulative duration of females' sniffing toward perforated parts of the cylinder were analyzed by a Macintosh computer using a video tracking software, ImageSI (tube) version 1.13 (O'Hara & Co., Ltd., Tokyo, Japan) developed based on the NIH Image. All tests were performed in a testing room illuminated with a red ceiling light.

1.3. Measurement of serum estradiol levels

On the day after the SIT test (Day 17) of experiment 2, animals were sacrificed and their blood samples were collected. Serum estradiol levels were determined using Coat-A-Count Total Estradiol Kit (DPC, Los Angeles, USA) according to the manufacturer's instruction. All samples were run in a single assay. The cumulative intra-assay CV was 11.3%. Detection limit was 10 pg/ml. We provisionally substituted zero for the values under the limit.

1.4. Statistics

All data are presented as a mean+SEM, except estradiol levels which are indicated median+quartile deviation. All behavioral data were analyzed by two-way ANOVAs, for the main effects of genotype and EB doses, and their interaction. Significant main effects and interactions were tested with Bonferroni post hoc comparisons in order to identify significant variations among the variables. In SIT, we excluded three mice (one of each from the groups of βERKO-placebo, βWT-placebo and βWT-0.1 μg/day) from the analysis due to an equipment failure.

2. Results

2.1. Experiment 1

Overall, there were no main effects of genotype in any measurements during the LDT tests; the time spent in the light compartment (Fig. 1a), the number of transitions between two compartments (Fig. 1b), and the latency to the first emerge to the light compartment (Fig. 1c). EB treatment at the dose of 2.0 μg/day similarly affected these behavioral measurements in βWT and βERKO mice. Both the time spent in the light compartment [$F(1, 44)=8.75$, $P<0.01$] and the number of transitions [$F(1, 44)=16.53$, $P<0.001$] were significantly reduced by EB treatment compared to the placebo control groups regardless of genotype. EB treatment also tended to increase the latency to emerge to the light compartment in both genotypes, but did not reach a statistically significant level [$F(1, 44)=2.27$, $P=0.14$].

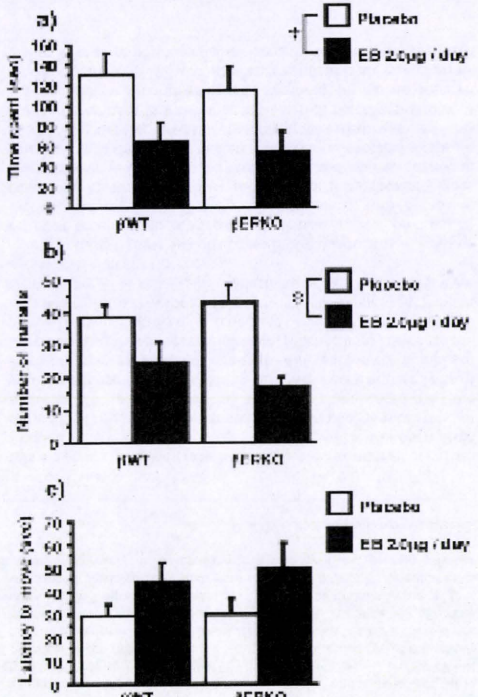

Fig. 1. The results (mean+SEM) of the light/dark transition (LDT) test in experiment 1. Regardless of genotype, mice treated with EB at the dose of 2.0 μg/day (βWT: $n=12$, βERKO: $n=13$) showed significantly higher levels of anxiety compared to the placebo treated mice (βWT: $n=11$, βERKO: $n=12$), as indicated by a large reduction of the total time spent in the light side of LDT apparatus (a) and the number of transitions between the dark and light sides (b). The latency to the first emergence from the dark to the light compartment also tended to increase in the EB treated groups. (*$P<0.05$).

2.2. Experiment 2

2.2.1. LDT

In contrast to anxiogenic effects of EB at a higher dose (2.0 μg/day) in experiment 1, EB treatment at lower-doses reduced the levels of anxiety. Overall, the mice implanted with a 0.2 μg/day EB pellet spent longer time in the light compartment than those implanted with a placebo pellet (Fig. 2a; main effect of EB treatment [$F(4, 83)=2.72$, $P<0.05$]). Though these behavioral effects of EB were more pronounced in βWT than βERKO mice, there was no main effect of genotype [$F(1, 83)=0.57$, $P=0.45$] and interaction between genotype and EB treatment [$F(4, 83)=1.24$, $P=0.30$]. Lower doses of EB treatment also increased the number of transitions between two compartments. The mice treated with an EB pellet at the doses of 0.2 μg/day showed a significantly higher number of transitions than those treated with a placebo pellet (Fig. 2b; main effect of EB treatment [$F(4, 83)=2.65$, $P<0.05$]). Again, there was no apparent genotype difference in terms of these anxiolytic effects of EB on this measurement. In contrast to these two measurements, the main effect of EB dose was not statistically significant in the latency to the first exit to the light compartment (Fig. 2c). Instead, there was a significant overall genotype difference: βERKO mice showed a significantly longer latency than βWT mice (Fig. 2c; main effect of genotype [$F(1, 83)=4.62$, $P<0.05$]. The genotype differences were more apparent in the mice treated with a placebo, 0.1, or 0.2 μg/day EB pellet compared to those in the other treatment groups, although the interaction between genotype and EB treatment was not statistically significant [$F(4, 83)=0.90$, $P=0.47$].

2.2.2. EPM

Most of the mice hardly enter open arms as indicated in the time spent in the open arms, and the main effects of genotype, EB treatment, and the interactions between them were not statistically significant in any behavioral measures (data not shown).

2.2.3. SIT

In this test, genotype specific dose-dependent effects of EB treatment were more clearly seen in all three measurements. Particularly,

a statistically significant interaction between genotype and EB treatment [$F(4, 80) = 3.06$, $P<0.05$] was found in the moving distance (Fig. 3a). Post hoc analysis revealed that the moving distance was highest in βWT mice treated with a 0.2 μg/day EB pellet [$P<0.05$ vs placebo group] and lowest in those treated with a 1.0 μg/day EB pellet [$P<0.05$ vs placebo or 0.2 μg/day EB pellet groups]. In βERKO mice, on the other hand, all five groups of mice showed similar levels of moving distance. Inverted U-shape effects of EB treatment were also seen in two other measurements, the number (Fig. 3b) and the total duration (Fig. 3c) of sniffing bouts, in βWT mice but not in βERKO mice, although the interaction between genotype and EB treatment did not reach a statistically significant level.

2.2.4. Plasma levels of estradiol

The plasma levels of estradiol of the animals treated with 1.0 μg/day EB pellet were 76 (+99) pg/ml and 114 (+124) pg/ml in βERKO and βWT mice, respectively. Those of the animals treated with 0.4 μg/day EB were 31 (+24) pg/ml (βERKO) and 11 (+35) pg/ml (βWT). In the mice treated with lower doses of estrogen (0.2 and 0.1 μg/day EB), the plasma concentrations were not at a detectable level. There were no genotype differences at any dose.

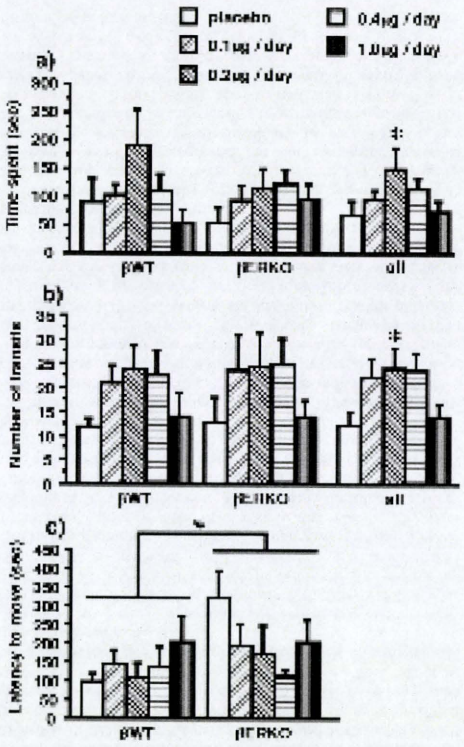

Fig. 2. Total time spent in the light side of the LDT apparatus (a), the number of transitions between the dark and light compartments (b) and the latency of the first move from the dark to the light compartment (c) in experiment 2 (*$P<0.05$, compared to the placebo group). In the latency to move from the dark to the light compartment, main effect of genotype was also significant (%$P<0.05$). All: each treatment group was collapsed across genotype. Placebo (βWT: $n=10$, βERKO: $n=11$), EB 0.1 μg/day (βWT: $n=10$, βERKO: $n=9$), EB 0.2 μg/day (βWT: $n=7$, βERKO: $n=7$), EB 0.4 μg/day (βWT: $n=10$, βERKO: $n=9$), and EB 1.0 μg/day (βWT: $n=9$, βERKO: $n=11$).

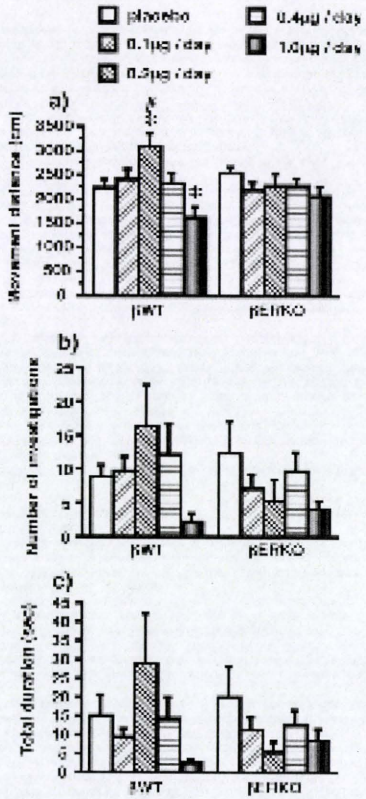

Fig. 3. The total moving distance of the subjects in the apparatus (a), total number (b) and duration (c) of investigation of stimulus mice during the SIT (*$P<0.05$, compared to placebo group of the same genotype; #$P<0.05$, compared to the 1.0 μg/day group of the same genotype). Placebo (βWT: $n=9$, βERKO: $n=10$), EB 0.1 μg/day (βWT: $n=9$, βERKO: $n=9$), EB 0.2 μg/day (βWT: $n=7$, βERKO: $n=7$), EB 0.4 μg/day (βWT: $n=10$, βERKO: $n=9$), and EB 1.0 μg/day (βWT: $n=9$, βERKO: $n=11$).

3. Discussion

In the present study, we examined the effects of estrogen on the levels of anxiety in both non-social (LDT and EPM) and social (SIT) situations. In experiment 1, we tested animals in the LDT apparatus and found that EB treatment at the dose of 2.0 μg/day decreased the time spent in the light side and the number of transitions between the dark and light sides. These results indicate that long-term EB treatment at a relatively high-dose, among the treatment dose used in this study, increases the anxiety level of the female mice. Especially, it is worthy to note that the EB treatment affected the time spent in the light side. That is considered a reliable index of the animal's anxiety level, because that does not directly depend on the animal's activity level. Actually, the total moving distance in the dark side, that is considered a direct index of the animal's activity level, did not show

a statistical difference between the EB-treated and placebo groups in experiment 1 (data not shown). Although we did not conduct EPM nor SIT in experiment 1, the results in the LDT tests strongly led us to hypothesize that estrogen might have anxiogenic effects, rather than anxiolytic effects, at higher-doses. Additionally, our findings of anxiogenic EB effects are consistent with those of previous studies reporting an increase of fear responses in female mice implanted with a silastic capsule containing EB (25–75 μg/capsule) 7 days before behavioral tests [8,9]. Since both βWT and βERKO mice responded similarly to EB treatment in experiment 1 (Fig. 1), ER-β may not be responsible for the behavioral effects of estrogen at this dose. On the other hand, ER-α may possibly play a crucial role for the anxiogenic regulation of estrogen, because βERKO mice have almost the same distribution of ER-α as βWT [22].

In contrast to the results in experiment 1, the mice implanted with a 0.2 μg/day EB pellet spent a longer time in the light compartment and showed a higher number of transitions between two compartments during the LDT tests than those implanted with a placebo pellet. Because these behavioral measures were commonly used for index of animal's anxiety level as described above, the enhancement in the mice implanted with a 0.2 μg/day EB pellet suggests that the chronic treatment of relatively lower-doses EB decreased the anxiety level of the mice. Similar inverted U-shape dose–response curve of estrogen effects were also shown in the SIT. For instance, the moving distance was highest in animals treated with a 0.2 μg/day EB pellet compared to those in four other treatment groups. Moving distance is directly affected by an activity level of animal. However, under ambiguous danger stimuli, or anxiogenic stimuli, depressing moving activity generally seems to be adaptive for the animal. While, less anxious animals may move more freely even when an anxiogenic stimulus such as a gonadally intact strange male mouse was introduced into their home cage. Actually, it was reported that the general activity level measured by running wheel in the OVX female mice treated with EB was enhanced compared to the animal treated with a vehicle oil, but the moving distance of the animals in the center area of open-field, that meant anxious situation, was decreased [9]. These results suggest that the moving distance in an anxious situation does not reflect general activity level of animals. Therefore, we hypothesize that moving distance in SIT also reflects animals' anxiety levels in social situations. It should be noted that the inverted U-shape dose–response curve with anxiolytic effects at lower-doses was only seen in βWT, but not βERKO, mice in this index. These results collectively suggest that ER-β activation may be more crucially involved in the regulation of animal behavior in social situations. While, the amount of sniffing to an intruder male mouse, more direct measurements of responsiveness to anxiogenic social stimulus, in the βWT mice treated with the lower-doses EB were not statistically different from the other groups of the genotype. The amount of sniffing may reflect the mechanism of a social attraction, or motivation of social investigation. Therefore, the anxiolytic effects of EB treatment on the moving distance on SIT in the present study cannot be explained directly from the mechanism of social attraction, or motivation.

Overall, the results in the present study suggest that the regulatory effects of chronic estrogen treatment on anxiety-related behaviors were dose-dependent; estrogen is anxiogenic at higher doses, whereas it has anxiolytic effects at lower doses. Only in the SIT of experiment 2, we found the effects of gene disruption of ER-β on anxiolytic action of estrogen, as the βWT mice treated with a 0.2 μg/day EB pellet showed increased moving distance, but not βERKO mice. Therefore, we can conclude that the disruption of ER-β, at least partially, affected anxiolytic actions of estrogen, but not anxiogenic actions. Recent studies have provided significant evidence to indicate that ER-β plays an important role in decreasing anxiety levels. βERKO mice showed higher anxiety-related behavior than βWT in EPM and open-field [16,17], and the injection of a selective ER-β agonist reduced anxiety-related behavior in ovariectomized female rats in several behavioral tests including EPM and open-field [18,19]. In the present study, there was a significant overall genotype difference between βERKO and βWT mice in the emergence latency to the light side of LDT, and the result also suggested overall higher anxiety of βERKO than βWT mice. Therefore, ER-β is possibly related to regulation of initial anxiety level of animal.

It is assumed that a number of mechanisms regulated by estrogen in an ER-β dependent manner may be responsible for behavioral effects of ER-β gene disruption found in the present study. It has been shown that oxytocin mRNA levels in the hypothalamic paraventricular nucleus are up-regulated by estrogen via ER-β [23]. Oxytocin is known to have anxiolytic effects since intracerebroventricular injections of oxytocin reduced anxiety levels [24–26] and disruption of oxytocin gene enhanced anxiety-related behaviors [27]. In addition, it is reported that the anxiolytic effects of oxytocin are enhanced by estrogen treatment [24,25]. Therefore, it is possible that ER-β activation suppresses the anxiety-related behaviors in female mice by regulating hypothalamic oxytocin levels. Moreover, it may be worth pointing out that in the present study, the clearest relationship between ER-β deletion and a lack of anxiolytic effects of estrogen treatment was shown in the SIT, which measured the levels of social anxiety. In the previous studies, we also found that social recognition was disrupted in both ER-β knockout mice and oxytocin knockout mice [28,29].

The brain serotonergic system is also implicated in the regulation of anxiety-related behaviors [30]. There is some evidence suggesting that estrogen regulates the mesencephalic ascending serotonergic pathway via ER-β. In mice, more than 90% of ER-β immunopositive cells in the dorsal raphe nuclei (DRN) co-express tryptophan hydroxylase (TPH), the rate-limiting enzyme of serotonin synthesis, whereas only 20% of ER-α positive cells express TPH [22]. Estrogenic regulation of serotonin contents in the DRN is greatly affected by ER-β gene disruption [17]. Therefore, it is possible that ER-β activation may modulate anxiety-related behavior by regulating the dorsal raphe-serotonergic pathway in female mice. In the DRN, ER-β, but not ER-α, plays a crucial role in the estrogenic induction of progestin receptors in TPH-positive cells [31]. Therefore, it is possible that ER-β dependent anxiolytic effects of estrogen may be further accentuated by progesterone administration.

It is also possible that ER dependent estrogenic regulation of neuronal activity in the medial amygdala may be responsible for the behavioral effects found in the present study. It has been reported that systemic EB treatment increased Fos immunoreactivity in the medial amygdala [32]. Furthermore, intra-amygdala EB administration decreased anxiety and fear related behaviors in ovariectomized rats [7]. In this brain region, both ER-α and ER-β are localized [33,34] and may possibly be involved in the estrogenic regulation of anxiety-related behaviors. While, a recent study [35] showed that hippocampus played an important role for anti-anxiety and anti-depressive effects of ER-β, but not ER-α, by administering the ER-α or ER-β selective agonists to the hippocampus, directly. These several mechanisms that were described above may act in compensate, rather than exclusive, manner for regulating the anxiety-related behavior.

One of the most important and remaining problems in the present study is the actual circulating level of estradiol responsible for the anxiolytic or anxiogenic effects. At the dose we found the most prominent anxiolytic effects, i.e., 0.2 μg/day, plasma levels of estradiol were not at a detectable level in the present study. Estradiol levels in mice treated with 0.4 μg/day of EB pellet were 31 (+24) pg/ml for βERKO and 11 (+35) pg/ml for βWT and similar to those found in estrus stage of naturally cycling mice (17 (+4) pg/ml) in our preliminary study using the same assay protocol. During pregnancy, females are exposed to higher level of estrogen for longer period, and the anxiety level in pregnant female was also reduced [36]. It was reported the serum level of estradiol in the pregnant female rodents

was approximately 30 pg/ml [37,38]. The estradiol level of pregnant female is lower than that of mice treated with 1.0 μg/day of EB pellet in our study, rather similar to that of mice treated with 0.4 μg/day of EB pellet. On the other hand, those in the mice treated with 1.0 μg/day EB were much higher than physiological levels, i.e., 76 (+99) pg/ml and 114 (+124) pg/ml in βERKO and βWT mice, respectively. Although we did not measure the plasma levels of estradiol in the mice which were treated with 2.0 μg/day EB pellet and showed high anxiety levels (experiment 1), the findings in the present study suggest that circulating levels of estradiol at the levels similar to or lower than those during estrus and/or pregnant female may have anxiolytic behavioral effects while super-physiological levels of estrogen may induce anxiogenic effects.

Another problem of this study is inconsistency of the results among the behavioral tests. In experiment 2, the effects of ER-β gene deletion on anxiety-related behavior showed in the SIT, but were not clear in LDT. The inconsistencies of anxiety-related behavior depend on the test paradigms also showed in many other studies [39,40]. These results may involve that anxiety and emotionality have multidimensional components and situation-dependant aspects. Especially, since ER-β is considered to play an important role for regulating social recognition mechanism [28,29], it is possible that such mechanisms underlie the background of the anxiolytic effects of EB treatment in social situation.

The differences of basal level of behavior between experiments were also a technical, but critical, problem. The animals treated with a placebo pellet in experiment 2 showed higher anxiety level compared to those of experiment 1, as indicated in shorter time spent in the light side, lower number of transitions, and longer latency to the first emerge in the LDT test. However, we do not think that the difference of basal level of the behavior is critical for our conclusion, since the anxiogenic effects of higher-dose EB treatment were also stable in experiment 2, as indicated that the animals treated with 1.0 μg/day EB pellet showed the highest anxiety level among the treatment groups in several behavioral measures.

In conclusion, our results suggest that estrogen may have both anxiolytic and anxiogenic effects in female mice and ER-β may be involved primarily in anxiolytic, but not anxiogenic, action of estrogen. We do not exclude the possibility, however, that ER-α may also be involved in the anxiolytic effects of estrogen. Indeed, in the LDT of experiment 2, the βERKO mice treated with a lower-dose of estrogen pellet also showed reduced levels of anxiety-related behaviors compared to those treated with placebo, as well as βWT. The exact role of ER-α in the regulation of anxiety levels needs to be determined in further studies.

Acknowledgments

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BEHAVIORAL NEUROSCIENCE

The cAMP response element-binding protein in the bed nucleus of the stria terminalis modulates the formalin-induced pain behavior in the female rat

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Keywords: dominant negative, estrogen, interphase, ovariectomy, proestrus

Abstract

Differences in male and female responses to pain are widely recognized in many species, including humans, but the cerebral mechanisms that generate these responses are unknown. Using the formalin test, we confirmed that proestrus female rats showed nociceptive behavior, modulated by estrogen that was distinct from male rats, particularly during the interphase period. We then explored the brain areas, which were involved in the female pattern of nociceptive behavior. We found that, after a formalin injection and at the time corresponding to the behavioral interphase, the number of phosphorylated cAMP response element-binding protein (pCREB)-immunoreactive neurons observed by immunocytochemistry increased in the dorsolateral division of the bed nucleus of the stria terminalis (BSTLD) in female but not male rats. There were no significant sex differences in pCREB expression following formalin in any region other than the BSTLD. The increased pCREB in female rats was eliminated after an ovariectomy and restored with 17β-estradiol treatment. Neither an orchidectomy nor 17β-estradiol treatment affected the pCREB response in male rats. The increase in pCREB expression in the BSTLD in female rats after formalin injection was confirmed with immunoblotting. To determine the role of CREB in the BSTLD, adenovirus-mediated expression of a dominant-negative form of CREB (mCREB) was carried out. The nociceptive behavior during interphase was significantly attenuated by injection of virus carrying mCREB into the BSTLD in female rats but not in male rats. These results suggest a novel role for CREB in the BSTLD as a modulator of the pain response in a female-specific, estrogen-dependent manner.

Introduction

Many studies have documented a higher sensitivity to pain in females compared with males. For example, female mice and rats exhibit a greater nociceptive response than males in the formalin test, a widely used model of tonic pain (Aloisi *et al.*, 1994; Kim *et al.*, 1999; Gaumond *et al.*, 2002). Furthermore, Spooner *et al.* (2007) found that nociceptive responses were reduced in estrogen receptor-β knockout females compared with wild-type females during the interphase and early tonic phase II of the formalin test, suggesting an essential role for estrogen receptor-β in pain modulation. In addition, compared with males, females showed less prominent opioid-induced analgesia (Mogil *et al.*, 2000; Craft *et al.*, 2004) and more potent adrenaline-induced hyperalgesia (Dina *et al.*, 2001). These results suggest that females are more sensitive to pain-related stimuli than males.

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Although estrogen is suggested to determine the sex difference in responses to many pain-test paradigms, the effects of gonadectomy and gonadal steroid replacement on pain activation were confounded and contradictory (Kuba & Quinones-Jenab, 2005; Aloisi & Bonifazi, 2006). The role of gonadal steroids in organizational action (McEwen, 1999; Schwarz & McCarthy, 2008) is also unclear. We recently reported that neonatally androgenized (treated with testosterone) female rats retained estrogen effects on the female nociceptive response (Hagiwara *et al.*, 2007). Additionally, although neonatally androgenized female rats displayed enhanced morphine analgesia relative to untreated females, they displayed significantly lower magnitudes of morphine analgesia compared with males. This result suggests that gonadal steroids exert limited organizational action in the mediation of morphine analgesia in female rats (Cataldo *et al.*, 2005).

Importantly, pain is not simply the perception of peripheral stimulation; rather, it is a series of events that include emotional processes controlled by supraspinal, cerebral mechanisms (Tracey & Mantyh, 2007). Although intracerebroventricular injections of

17β-estradiol demonstrated that estrogen plays a central role in nociceptive behavior (Aloisi & Ceccarelli, 2000), it remains unclear which neural substrates and cerebral structures are involved and how the sex difference in nociceptive behavior is generated. Therefore, this study focused on the cAMP response element-binding protein (CREB), a transcription factor that regulates important neural functions (Sheng & Greenberg, 1990; Carlezon *et al.*, 2005). In response to neural activity, CREB is activated by phosphorylation (pCREB), then pCREB binds to promoters to trigger the expression of specific genes (Mayr & Montminy, 2001), that results in long-lasting synaptic plasticity (Lonze & Ginty, 2002). Because pCREB activation only requires phosphorylation, CREB-mediated gene expression occurs more rapidly than cFOS-mediated expression. We examined whether CREB activation in cerebral regions plays a role in sex differences in nociceptive behavior.

Materials and methods

Animals

Eight-week-old adult male and female Wistar rats (Charles River, Yokohama, Japan) were maintained at a constant temperature of 24–26°C under controlled lighting conditions (lights on 05.00–19.00 h), with food and water available *ad libitum*. Daily vaginal smears were performed on female rats, and rats exhibiting two or more consecutive 4-day estrus cycles were used in the formalin test on the day of proestrus.

In experiments examining the effects of castration and steroid replacement, rats were anesthetized with isoflurane and castrated at 8 weeks of age and used in experiments 2 weeks after surgery. At the time of castration, some rats were subcutaneously implanted with a silicone tube (2.0 mm inside diameter, 3.0 mm outside diameter and 15 mm length) containing cholesterol alone as a control. The remaining rats were implanted subcutaneously with a silicone tube containing 20% 17β-estradiol crystals. We showed previously that this dose of 17β-estradiol results in serum estradiol levels that are equivalent to those observed on the day of proestrus in intact female rats (Butcher *et al.*, 1974) and induces a surge of luteinizing hormone secretion (Nishihara *et al.*, 1994). Implant sizes were assigned according to body weight. The silicone tubes were presoaked in saline for more than 24 h to facilitate rapid hormone release. Serum levels of estradiol were determined using an EIA kit (Cayman Chemical).

All animal housing and surgical procedures were in accordance with the guidelines laid out by the institutional animal care and use committee of Yokohama City University School of Medicine, and the experiments followed the Ethical Guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

Behavioral test

Nociceptive behavior was tested as reported previously (Hagiwara *et al.*, 2007). Rats were subcutaneously injected with 50 μL of 2% formalin or saline into the right hind paw. Immediately after the injection, rats were placed in a transparent Plexiglas box (30 × 30 × 30 cm) with a transparent floor positioned over a mirror angled at 45° to allow the observation of nociceptive behavior. Assessment of nociceptive behavior was carried out using a single parameter (Tjolsen *et al.*, 1992): the total time that the paw was kept elevated from the floor was determined every 5 min for 1 h. A mean response was then calculated for each phase. The first phase was defined as 0–5 min (phase I), the interphase as 5–15 min (interphase), and the second phase as 15–60 min. After the experiments, the rats were killed with an injection of sodium pentobarbital (100 mg/kg).

Immunohistochemistry

The immunohistochemical study was performed as reported previously (Mogi *et al.*, 2005). Twenty-four hours before injection, a silicone cannula for intravenous injection of an anesthetic was implanted into the right atrium under ether anesthesia. At varying times after formalin or saline injection, pentobarbital (100 mg/kg) was intravenously injected through the cannula to obtain rapid and sufficient analgesia. Thereafter, following heparinized phosphate buffer (PB), perfusion through the cardiac ventricle was performed with 4% paraformaldehyde in PB (pH 7.5) at approximately 4°C. After perfusion, the brains were removed from the cranium, fixed overnight at 4°C in PB containing 4% paraformaldehyde, and incubated overnight at 4°C in 25% sucrose in PB. The brains were then frozen with powdered dry ice and stored at −70°C until immunohistochemical processing.

Thirty-micrometer frozen coronal sections were cut with a Bright cryostat and every second section was used in the study. The sections were washed with 0.1 M phosphate-buffered saline (PBS), then incubated with 0.3% H_2O_2 in 20% methanol in PBS. Afterwards, sections were incubated overnight with anti-pCREB rabbit polyclonal antibody diluted 1 : 1000 (#9191; Cell Signaling, Beverly, MA, USA) in PBS containing 1.5% normal goat serum and 0.1% Triton X-100. The next day, the sections were incubated with biotinylated anti-rabbit IgG diluted 1 : 200 in PBS containing 1.5% normal goat serum and 0.05% Triton X-100, then incubated with streptavidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Lab, Burlingame, CA, USA). Bound peroxidase was visualized by incubating sections in 0.05% 3,3′-diaminobenzidine with H_2O_2 for 30 min. Finally, the sections were mounted on glass slides, dehydrated in graded alcohol, cleared in xylene and coverslipped with Permount.

Histological analysis

The counting was performed by an investigator unaware of the experimental conditions and expectations. Two–four sections per rat were carefully selected and matched across all animals in all experimental groups. The number of pCREB-immunoreactive cells per area in each rat was averaged for each rat and used in the statistical analysis.

Western blotting

In the immunoblotting study, female rats were killed 0, 5 and 30 min after subcutaneous saline or formalin injection, by intravenous injection of pentobarbital. The brain was quickly removed and placed on a brain slicer (Medical Agent Company, Kyoto, Japan) with the ventral side facing up. A stainless steel blade was used to make a 2-mm-thick coronal slice. The slice was placed in an ice-chilled dish with the caudal side facing up and the bed nucleus of the stria terminalis (BST), including the dorsomedial (BSTMD) and the dorsolateral divisions (BSTLD), was bilaterally isolated using a stainless steel pipe (internal diameter = 0.85 mm). T-PER tissue extraction reagent (Pierce Biotechnology, Rockford, IL, USA) containing Halt protease inhibitor mixture (Pierce Biotechnology) at a final concentration of 1× was added, and samples were homogenized on ice. Tissue debris was removed by centrifugation for 5 min at 15 000 ***g***. The supernatant was collected, and its protein concentration was determined with the Micro BCA protein assay kit (Pierce Biotechnology). Aliquots of the sample (approximately 10 μg/10 μL) were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Arlington Heights, IL,

USA). The membrane was blocked overnight with Blocking One (Nacalai Tesque, Kyoto, Japan), then incubated overnight with pCREB antibody (1 : 1000 dilution). This was followed by incubation with horseradish peroxidase-labeled secondary antibody (Amersham Biosciences; 1 : 50 000 dilution). The membrane was reprobed with β-actin antibody. The signal was analysed using ECL Plus Western blotting detection reagents (Amersham Biosciences). Quantification was performed with a computer-assisted densitometric analysis using MCID (Imaging Research, ON, Canada), and the arbitrary units of pCREB signal were corrected using the β-actin signal.

Adenovirus-mediated expression of a dominant-negative CREB

Adenovirus vectors were produced using the Adeno-X Expression and Adeno-X Tet-Off Expression Systems according to the manufacturer's protocol (Clontech Laboratories, Mountain View, CA, USA). mCREB is a dominant-negative form of CREB in which Ser133 is substituted with alanine (Gonzalez & Montminy, 1989). The adenovirus vector Ad-TRE/mCREB, expressing mCREB and a 6× His tag at its N-terminus in a tetracycline-regulated manner, was generated as reported previously (Ishida *et al.*, 2007). The Ad-TRE/β-gal and Ad-Tet-Off adenoviruses expressing β-galactosidase in a tetracycline-regulated manner and TetR-VP16, respectively, were purchased from Clontech Laboratories. The recombinant adenoviruses were propagated by serial infection in HEK293 cells. The adenoviruses obtained were purified with the Adeno-X Virus Purification Kit (Clontech Laboratories) and concentrated with the Centricon Centrifugal Filter Unit (Millipore, Bedford, MA, USA). The titers of adenovirus vectors (infectious units, ifu) were determined using the Adeno-X Rapid Titer Kit (Clontech Laboratories).

Rats were bilaterally injected (2 μL/side in 12 min) with a mixture containing 4×10^5 ifu Ad-Tet-Off and 4×10^5 ifu Ad-TRE/mCREB or Ad-TRE/β-gal. An injection cannula was acutely implanted (33-gage, outer diameter = 0.26 mm) into the BSTLD according to the atlas of Albe-Fessard *et al.* (1966) (stereotaxic coordinates: $A = 7.6$; $V = 5.0$; $L = 1.45$) under sodium pentobarbital anesthesia (31.5 mg/kg body weight) 2 weeks before the formalin test. Injection sites and adenovirus-mediated expression were verified by immunostaining with anti-6 × His tag antibody (Cell Signaling) and histochemistry for β-galactosidase in Ad-TRE/mCREB and Ad-TRE/β-gal-injected rats, respectively.

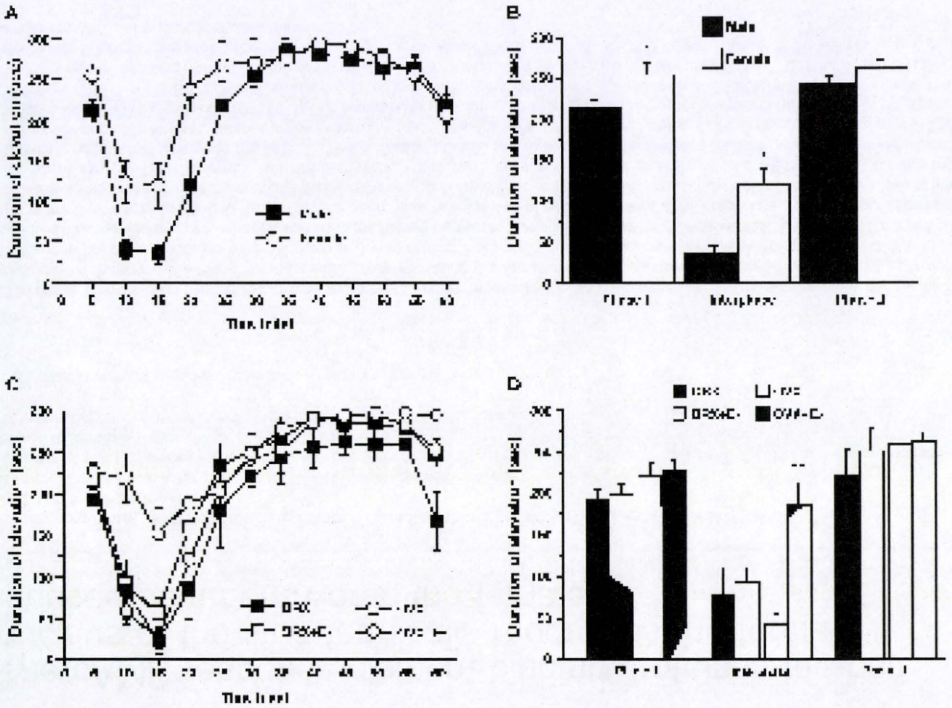

FIG. 1. Sex differences in nociceptive behavior after formalin treatment. (A) Behavioral responses to formalin injections in male (n = 9) and proestrus female rats (n = 10). (B) Nociceptive response during phase I, interphase and phase II based on the data shown in (A). (C) Behavioral responses to formalin injections in orchidectomized (ORX) rats implanted with a silicone tube containing cholesterol (n = 8) or 17β-estradiol (E_2, n = 7), and ovariectomized (OVX) rats implanted with a silicone tube containing cholesterol (n = 10) or E_2 (n = 12). (D) Nociceptive responses during phase I, interphase and phase II based on the data shown in (C). The data represent means ± SEM. *Indicates statistical significance when compared with the male group, **when compared with the other groups, within each phase ($P < 0.001$).

Data analysis

Data were presented as means ± standard error of the mean (SEM), and were analysed by repeated-measures analysis of variance (ANOVA) in behavioral studies, or one-way factorial ANOVA in pCREB expression and Western blotting studies, followed by Fisher's protected least significant difference (repeated-measures ANOVA) or Bonferroni (one-way factorial ANOVA) *post hoc* test for comparison of treatments. Differences were considered significant at $P < 0.05$ (behavioral studies), $P < 0.0014$ (time-course studies by pCREB expression), $P < 0.0083$ (17β-estradiol replacement studies by pCREB expression) or $P < 0.005$ (Western blotting studies).

Results

Sex differences in nociceptive behavior after formalin injection

As shown in Fig. 1A, formalin injection induced two phases of nociceptive responses separated by an interphase (a brief quiescent period; Tjolsen *et al.*, 1992; Henry *et al.*, 1999). This pattern was similar in both sexes, but there was a modest nociceptive response during phase I and a prominent increase during interphase (Henry *et al.*, 1999) in female rats (Fig. 1A), confirming the results of others (Gaumond *et al.*, 2007; Spooner *et al.*, 2007) and of our previous study (Hagiwara *et al.*, 2007). For statistical purposes, the data were separated into three phases: phase I, interphase and phase II, defined as 0–5 min, 5–15 min and 15–60 min after formalin injection, respectively. Repeated-measures ANOVA showed a significant effect of sex ($F_{1,17} = 11.6$, $P < 0.005$) and phase ($F_{1.2} = 146.4$, $P < 0.0001$) with a significant interaction ($P < 0.05$). Because the interaction was significant, the duration of elevation was further analysed at each phase. We found a modest nociceptive response during phase I ($F_{1,17} = 6.09$, $P < 0.05$) and a prominent nociceptive response in interphase ($F_{1,17} = 12.9$, $P < 0.005$) in female rats when compared with male

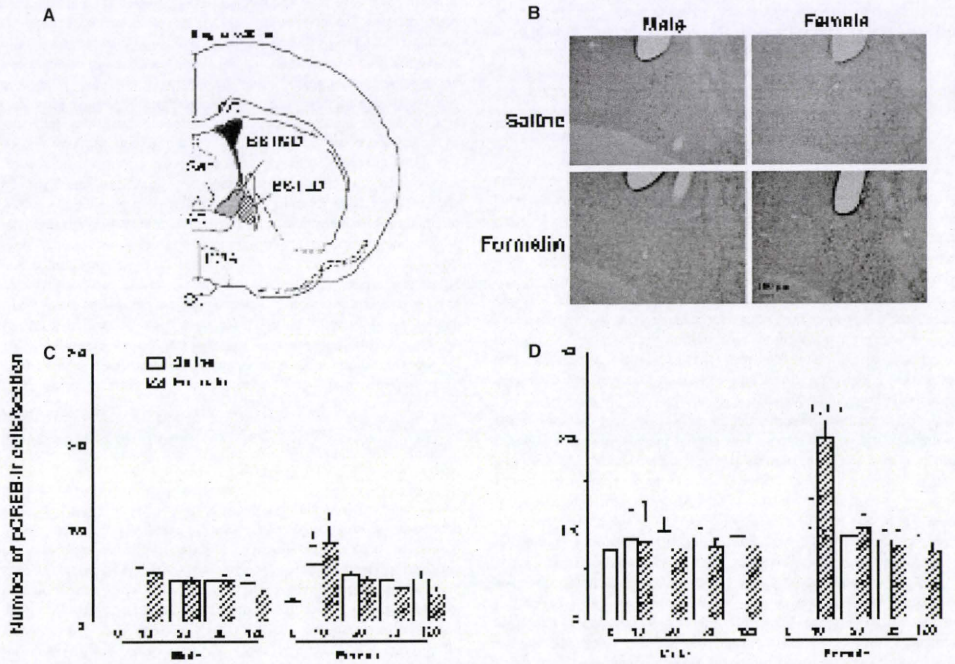

FIG. 2. CREB phosphorylation in cerebral regions after saline or formalin injections in male and female rats. (A) Illustration of the coronal region of the rat brain (modified from Paxinos & Watson, 1986) representing neural structures found in sections taken at −0.3, rostral to the bregma. The shaded areas indicate the regions in which the phosphorylated CREB-immunoreactive cells (pCREB-ir cells) were counted. AC, anterior commissura; BSTLD, dorsolateral division of the bed nucleus of the stria terminalis; BSTMD, dorsomedial division of the bed nucleus of the stria terminalis; CC, corpus callosum; POA, preoptic area; Sep, septum. (B) Representative photomicrographs of phosphorylated CREB in the BST 10 min after a formalin or saline injection in male and female rats. (C and D) Time-course for formalin- or saline-induced changes in the number of cells expressing pCREB. (C) Changes in the BSTMD and (D) the BSTLD. The data represent means ± SEM (n = 5–7). *Indicates statistical significance when compared with the time 0 group, **when compared with the saline group (Fisher's PLSD test following repeated ANOVA, $P < 0.05$).

rats, whereas there was no change during phase II ($F_{1,17} = 1.84$, $P > 0.1$).

To further confirm sex differences during the formalin test, we next examined the effects of castration and 17β-estradiol replacement on nociceptive behavior in female and male rats. Serum concentrations of estradiol in the cholesterol-implanted male, 17β-estradiol-implanted male, cholesterol-implanted female and 17β-estradiol-implanted female rats were 22.7 ± 4.2 ($n = 6$), 107.6 ± 13.1 ($n = 6$), 21.3 ± 2.1 ($n = 6$) and 93.5 ± 10.6 ($n = 6$), respectively. As shown in Fig. 1C and D, repeated-measures ANOVA showed a significant effect of groups ($F_{3,33} = 7.56$, $P < 0.001$) and phase ($F_{3,2} = 118.2$, $P < 0.0001$), with a significant interaction ($P < 0.001$). In phases I ($F_{3,33} = 1.8$, $P > 0.1$) and II ($F_{3,33} = 1.87$, $P > 0.1$), there was no significant effect on the nociceptive response. In interphase, however, ANOVA revealed a significant effect between groups ($F_{3,33} = 12.57$, $P < 0.0001$). The duration of elevation in the group of ovariectomized (OVX) female rats treated with 17β-estradiol was significantly longer than in any other group (Fisher's PLSD test, $P < 0.001$), showing that 17β-estradiol had a significant effect on OVX rats. This result suggests that the sex difference in nociceptive responses during interphase is estrogen dependent and female specific.

CREB phosphorylation in the cerebral regions after formalin injection in male and female rats

To identify cerebral regions exhibiting a sex-related response to formalin, the expression of pCREB was immunohistochemically examined at various times and in various regions, including the extended amygdala and the paraventricular nucleus, after saline or formalin injection in male and proestrus female rats. Within the areas we examined, the BST was the only region showing pCREB expression during interphase after formalin treatment in a female-specific manner. The shaded areas shown in Fig. 2A indicate the regions in which the number of pCREB-immunoreactive cells (pCREB cells) was reported in the BST (Paxinos & Watson, 1986).

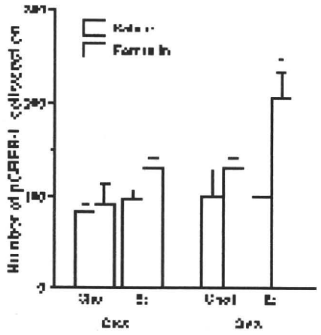

FIG. 3. Effects of castration and estrogen replacement on formalin-induced phosphorylation of CREB in the BSTLD in male and female rats. Formalin or saline was injected into orchidectomized (ORX) and ovariectomized (OVX) rats implanted with a silicon tube containing cholesterol (Chol) or 17β-estradiol (E_2). The number of phosphorylated CREB-immunoreactive cells (pCREB-ir cells) in the BSTLD was determined 10 min after injection. The data represent means ± SEM ($n = 4$–7). *Indicates statistical significance ($P < 0.05$) when compared with the saline group.

Figure 2B shows representative photographs of pCREB expression in the BST. We found that the number of pCREB cells in the bilateral BSTLD was significantly increased after formalin injection in females ($F_{8,39} = 7.13$, $P < 0.0001$) but not in males ($F_{8,34} = 1.76$, $P > 0.99$; Fig. 2C). These changes were selectively observed 10 min after formalin injection, a period corresponding to behavioral interphase, and were specific to formalin injection because saline injection did not affect pCREB expression ($P > 0.05$). In other cerebral regions, changes in the number of pCREB cells were either minimal or observed after both saline and formalin injections (data not shown). For example, in the BSTMD (Fig. 2D), the number of pCREB cells varied significantly in females ($F_{8,39} = 4.92$, $P = 0.0003$) but not in males ($F_{8,34} = 1.28$, $P = 0.28$). However, both saline and formalin injections increased the number of pCREB cells 5–10 min after injection ($P < 0.005$), suggesting that injection *per se* affected the neuronal activity of this nucleus in both males and females.

Next, we examined the effects of castration and 17β-estradiol replacement on the expression of pCREB in the BSTLD 10 min after injection (Fig. 3) in male and female rats, because we had observed that the sex difference in nociceptive response during interphase was estrogen dependent and female specific (Fig. 1C). Serum concentrations of estradiol in the cholesterol-implanted male, 17β-estradiol-implanted male, cholesterol-implanted female and 17β-estradiol-implanted female rats were 22.0 ± 3.0 ($n = 5$), 94.2 ± 9.3 ($n = 5$), 19.8 ± 3.7 ($n = 5$) and 99.2 ± 14.3 ($n = 5$), respectively. There was no change in the number of pCREB-expressing cells in the BSTLD

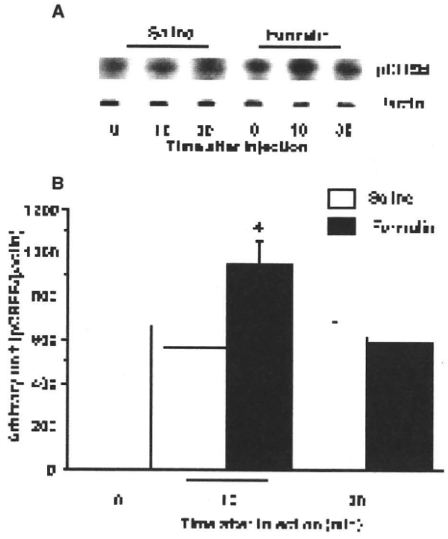

FIG. 4. Formalin-induced phosphorylation of CREB in the BST detected by immunoblotting. BST tissue was collected 10 min or 30 min after formalin or saline injections in female rats. (A) Representative immunoblots of phosphorylated CREB protein (pCREB). The band at 43 kDa is pCREB. (B) Quantitative analysis of pCREB levels was determined from the immunoblots. The data represent means ± SEM ($n = 8$–14). *Indicates statistical significance when compared with other groups (ANOVA followed by Fisher's PLSD test, $P < 0.001$).

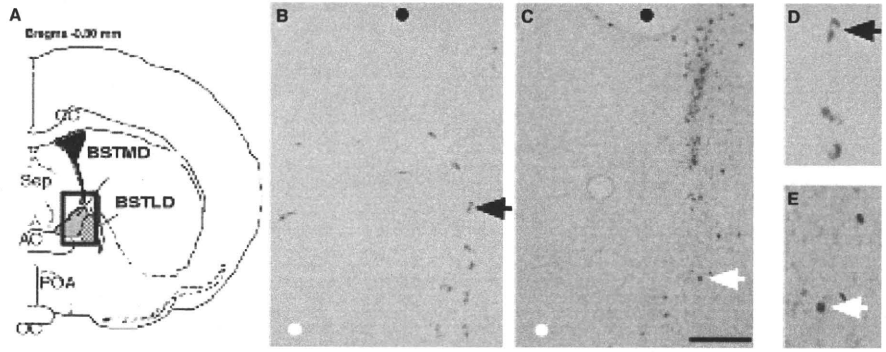

FIG. 5. Representative photomicrographs of β-galactosidase (control) or 6× His tag (mCREB) expression in the BSTLD of rats infected with the adenovirus vector. (A) Illustration of the coronal region of the rat brain, as shown in Fig. 2. The area with bold lines indicates the region taken for photographs. AC, anterior commissura; BSTLD, dorsolateral division of the bed nucleus of the stria terminalis; BSTMD, dorsomedial division of the bed nucleus of the stria terminalis; CC, corpus callosum; POA, preoptic area; Sep, septum. (B and C) Representative photomicrographs showing the expression proteins from the adenoviral vector at low and higher magnifications, respectively (D and E). A control rat expressed β-galactosidase (B and D; at the black arrow), and therefore histochemical cytoplasmic staining for β-galactosidase was evident (D). Likewise, the 6× His tag of virally expressed mCREB was detected in the mCREB group (C and E; at the white arrow) and only nuclear staining was seen for the His-tag by immunocytochemistry. The black and white dots indicate the area of the LV and the AC, respectively. Scale bars (B and D) indicate 200 μm.

10 min after formalin injection in male rats, irrespective of 17β-estradiol replacement (ANOVA $F_{3,20} = 1.985$, $P = 0.1487$; Fig. 3). Formalin injection failed to increase pCREB expression in the BSTLD in OVX rats without 17β-estradiol replacement ($P > 0.1$), but the formalin-induced increase in the number of pCREB-expressing cells was restored by 17β-estradiol replacement ($F_{3,15} = 4.49$, $P = 0.0194$). These results indicate that both behavioral response and pCREB expression in the BSTLD during the interphase change in parallel with each other, and that they are female specific and estrogen dependent.

To verify the changes in pCREB expression in the BSTLD of female rats as detected by immunohistochemistry, pCREB expression was determined by immunoblotting. pCREB levels in the BST in females increased only 10 min after formalin injection (ANOVA $F_{4,47} = 5.757$, $P = 0.0007$), and there was no change 30 min after formalin injection or 10 or 30 min after saline injection (Fig. 4A and B).

Effect of adenovirus-mediated expression of a dominant-negative CREB in the BSTLD on sex differences in the nociceptive response

To assess the role of CREB in the response to formalin, CREB activity in the BSTLD was blocked by adenovirus-mediated expression of mCREB, a dominant-negative form of CREB. Male and female rats were injected with Ad-TRE/mCREB, an adenovirus vector encoding mCREB, or Ad-TRE/β-gal, an adenovirus vector encoding β-galactosidase as a control, in combination with Ad-Tet-Off, into the bilateral sides of the BSTLD. Successful adenovirus-mediated expression of β-galactosidase and mCREB was verified by the

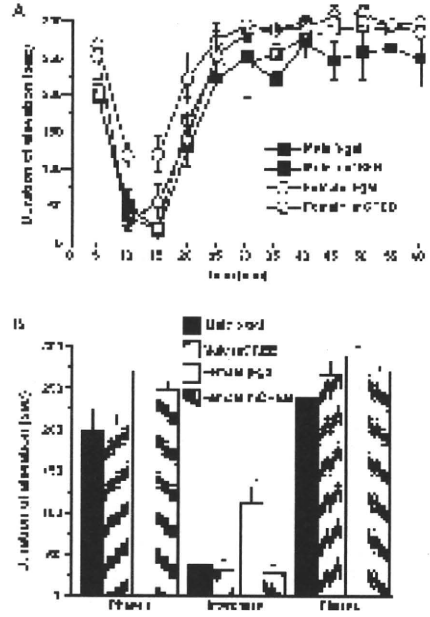

FIG. 6. Effect of adenovirus-mediated expression of dominant-negative mCREB in the BSTLD on sex differences in the nociceptive response. (A) Behavioral responses to formalin injections in male and female rats expressing β-galactosidase (β-gal; male $n = 5$, female $n = 10$) or dominant-negative mCREB (male $n = 6$, female $n = 11$) in the BSTLD. (B) Nociceptive responses during phase I, interphase and phase II based on the data shown in (A). The results represent means ± SEM. **Indicates statistical significance when compared with the other groups in the same phase (Fisher's PLSD test following a repeated ANOVA, $P < 0.05$).

presence of cells exhibiting β-galactosidase activity in the histochemistry (Fig. 5C), and 6× His tag-immunoreactivity (Fig. 5D) with immunocytochemistry, respectively. When the expression site was outside of the BSTLD, the data were discarded (data not shown). Repeated-measures ANOVA showed a significant effect between the groups ($F_{3,28} = 7.9$, $P < 0.001$) and between the times after formalin injection ($F_{3,2} = 638.1$, $P < 0.0001$), with a significant interaction ($P < 0.001$; Fig. 6A and B). At the interphase, ANOVA showed a significant difference between the groups ($F_{3,28} = 9.25$, $P < 0.001$), and a *post hoc* Fisher's PLSD test revealed that the nociceptive response in females after mCREB expression was significantly suppressed to the low level found in males ($P < 0.005$). In contrast, the higher level of nociceptive responses during phase I found in female rats was still found after mCREB expression ($F_{3,28} = 7.41$, $P < 0.001$, Fisher's PLSD test, $P < 0.05$). At phase II, there was no significant difference in nociceptive responses between the groups ($F_{3,28} = 2.25$, $P > 0.1$).

Discussion

We confirmed that nociceptive responses to formalin during phase I and interphase were greater in proestrus female rats than in male rats (Aloisi *et al.*, 1994; Kim *et al.*, 1999; Gaumond *et al.*, 2002), and the response during interphase was estrogen dependent (Aloisi & Ceccarelli, 2000; Aloisi & Bonifazi, 2006; Hagiwara *et al.*, 2007). Coinciding with the nociceptive responses to formalin, in the present study we found that CREB in the BSTLD was phosphorylated in a female-specific, estrogen-dependent manner. Furthermore, the blockade of CREB activity by adenovirus-mediated expression of a dominant-negative form of CREB in the BSTLD suppressed the interphase response only in females. These results suggest a novel role for CREB in the BSTLD as a modulator of the pain response that is female specific and estrogen dependent.

Our pCREB immunohistochemical studies showed several cerebral regions in which pCREB expression was increased after saline or formalin injection in female or male rats. However, among those regions, the BSTLD was identified as the sole region in which pCREB expression was selectively increased after formalin treatment in female rats only. The period of increased pCREB expression in the BSTLD corresponded exactly to the behavioral interphase. CREB is a transcription factor that plays important roles in a variety of neural functions (Carlezon *et al.*, 1998; Lonze & Ginty, 2002; Kovacs, 2008) and is phosphorylated by multiple protein kinases (Mayr & Montminy, 2001). Therefore, it is possible that the formalin-induced, rapid, transient stimulation of CREB phosphorylation in the BSTLD reflects a brief activation of the neurons by pain. This CREB activation may have affected the expression of target genes that resulted in the female-specific, estrogen-specific nociceptive response during interphase. In support of this hypothesis, we found that when CREB activity was blocked in the BSTLD by the expression of a dominant-negative form of CREB, the interphase response was suppressed in female but not male rats. Recent studies using viral vector techniques similar to those used here demonstrated that CREB was also involved in neuronal activity in the hippocampus and locus coeruleus (Marie *et al.*, 2005; Han *et al.*, 2006). Estrogen may affect the neural activity in the BSTLD by stimulating the phosphorylation of CREB. 17β-Estradiol treatment of OVX rats has been documented to induce the phosphorylation of CREB in the preoptic areas and in the BST (Gu *et al.*, 1996; Zhou *et al.*, 1996), and this effect was observed to be sex specific (Auger *et al.*, 2001).

The BSTLD neurons project into the central nucleus of the amygdala; thus, these neurons are considered a major component of the extended amygdala, a set of interconnected ventral forebrain structures with strong morphological, neurochemical and neuroanatomical similarities (Shammah-Lagnado *et al.*, 2000; Dong *et al.*, 2001). It has been suggested that the amygdala is involved in nociceptive behavior (Neugebauer *et al.*, 2004). Among the amygdaloid structures, the central nucleus plays a major role in modulating pain perception (Carrasquillo & Gereau, 2007). Furthermore, the basolateral nucleus of the amygdala mediates polymodal pain information sent from the cerebral cortex (Jasmin *et al.*, 2003). Less is known about the role of the BST in the regulation of nociceptive behavior. Deyama *et al.* (2007) demonstrated that bilateral lesions in the BST increased nociceptive responses during interphase after formalin injection in male rats, suggesting that the BST was involved in inhibiting the interphase response. Recently, pain-induced neural activation in the BST was demonstrated by the expression of cFos immunoreactivity as a marker of neural activation (Morano *et al.*, 2008). Our finding that female-specific, formalin-induced changes in CREB phosphorylation were observed in the BSTLD but not in the amygdala suggests that the BST and the amygdala are involved in pain-related behavior through distinct mechanisms. In support of this notion, the BST receives nociceptive information from the spinal cord in a manner distinct from that of the amygdala (Braz *et al.*, 2005).

It has been suggested that the interphase component of the formalin test is caused by active inhibition mechanisms rather than by a resting state lying between the two phase I and II activated peaks of the nociceptive response (Henry *et al.*, 1999). Gaumond *et al.* (2007) documented that the opioid antagonist, naloxone, preferentially blocked the formalin-induced behavioral interphase in female rats and produced a slight blockade in male rats; this suggested that sex differences in the inhibitory interphase may involve opioidergic neural systems.

An alternative speculation of the present results is that the BST affects emotions, like anxiety, that can alter the pain response; as noted in the Introduction, pain is not simply a perception but an event that includes emotional processes (Tracey & Mantyh, 2007). Anxiety has been shown to affect pain responses (Andre *et al.*, 2005; Boccalon *et al.*, 2006), and the BST is involved in anxiety (Walker *et al.*, 2003).

In conclusion, the present study suggests that the BSTLD and CREB activation in this region are crucial for determining the formalin-induced nociceptive behavior in female rats. The novel role of CREB in the BST in the modulation of nociception suggests that the BSTLD could be a new target for pain control in females.

Acknowledgement

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Abbreviations

BST, bed nucleus of the stria terminalis; BSTLD, dorsolateral division of the BST; BSTMD, dorsomedial division of the BST; CREB, cAMP response element-binding protein; OVX, ovariectomized; PB, phosphate buffer; PBS, phosphate-buffered saline; pCREB cells, pCREB-immunoreactive cells; pCREB, phosphorylated CREB.

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Behavioral/Systems/Cognitive

Gonadal Steroids Maintain 24 h Acetylcholine Release in the Hippocampus: Organizational and Activational Effects in Behaving Rats

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Extracellular acetylcholine (ACh) levels in the dorsal hippocampus increases during learning or exploration, exhibiting a sex-specific 24 h release profile. To examine the activational effect of gonadal steroid hormones on the sex-specific ACh levels and its correlation with spontaneous locomotor activity, we observed these parameters simultaneously for 24 h. Gonadectomy severely attenuated the ACh levels, whereas the testosterone replacement in gonadectomized males or 17β-estradiol replacement in gonadectomized females successfully restored the levels. 17β-Estradiol-priming in gonadectomized males could not restore the ACh levels, and testosterone replacement in gonadectomized females failed to raise ACh levels to those seen in testosterone-primed gonadectomized males, revealing a sex-specific activational effect. Spontaneous locomotor activity was not changed in males by gonadectomy or the replacement of gonadal steroids, but 17β-estradiol enhanced the activity in gonadectomized females. Gonadectomy severely reduced the correlation between ACh release and activity levels, but the testosterone replacement in gonadectomized males or 17β-estradiol replacement in gonadectomized females successfully restored it. To further analyze the sex-specific effect of gonadal steroids, we examined the organizational effect of gonadal steroids on the ACh release in female rats. Neonatal testosterone or 17β-estradiol treatment not only increased the ACh levels but also altered them to resemble male-specific ACh release properties without affecting levels of spontaneous locomotor activity. We conclude that the activational effects of gonadal steroids maintaining the ACh levels and the high correlation with spontaneous locomotor activity are sex-specific, and that the organizational effects of gonadal steroids suggest estrogen receptor-mediated masculinization of the septo-hippocampal cholinergic system.

Introduction

Cholinergic activation of the hippocampus is necessary for both spatial and contextual memory consolidation (Gale et al., 2001; Wallenstein and Vago 2001; Herrera-Morales et al., 2007). Extracellular acetylcholine (ACh) levels in the hippocampus increase during exploration or learning (Ragozzino et al., 1996; Stancampiano et al., 1999; Giovannini et al., 2001), exhibiting a clear diurnal rhythm (Mitsushima et al., 1998). The episodic activation of septo-hippocampal cholinergic system is important for the generation of theta oscillations (Lee et al., 1994; Buzsáki 2002) that modulate the induction of long-term potentiation (LTP) in the hippocampal CA1 neurons (Hyman et al., 2003). Moreover, the released ACh not only enhances synaptic plasticity via the muscarinic M_1/M_2 receptors (Seeger et al., 2004; Shinoe et al., 2005), but is also responsible for neurogenesis in the dentate gyrus (Mohapel et al., 2005; Kotani et al., 2006).

Spatial memory in rats requires the dorsal hippocampus (Moser et al., 1995; O'Keefe and Burgess 1996), which exhibits sex-specific extracellular ACh levels (Masuda et al., 2005). Male rats have significantly higher ACh levels in the dorsal hippocampus than females, which is consistent with superior spatial ability in males (Jones et al., 2003; Jonasson 2005). We previously hypothesized that sex-specific ACh levels could be attributed to the organizational and/or activational effects of gonadal steroids (Mitsushima et al., 2008b). Although neuroanatomical, neurochemical, and behavioral evidence have suggested an activational effect of gonadal steroids on the septo-hippocampal cholinergic neurons for decades (Luine et al., 1986; Gibbs and Pfaff 1992; Daniel et al., 1997; Mufson et al., 1999; Kritzer et al., 2001; Markowska and Savonenko 2002; Miettinen et al., 2002; Nakamura et al., 2002), conclusive evidence has been lacking in freely behaving rats. Further, the organizational effect of gonadal steroids on ACh levels is completely unknown.

Acetylcholine levels episodically change with spontaneous movement (Day et al., 1991; Mitsushima et al., 1996) that stimulates electrical activity of cholinergic neurons in the basal forebrain (Buzsáki et al., 1988). Moreover, voluntary running enhances neurogenesis, spatial learning, and synaptic plasticity in mice (van Praag et al., 1999). In contrast, a restriction of exploratory behavior reduces ACh levels as well as the spatial learning (Mitsushima et al., 1998, 2001). Therefore, it is necessary to rule out the possibility that the effects of gonadal steroids reflect the change in spontaneous locomotor activity. By simultaneous monitoring of ACh levels and spontaneous locomotor activity, we revealed a sex-specific activational effect of gonadal steroids on the 24 h ACh release profile in freely behaving rats. Moreover, the sex-specific activational effect was affected by neonatal estradiol injection, suggesting sexual differentiation of septo-hippocampal cholinergic system.

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Materials and Methods

Subjects

Male and female Wistar-Imamichi rats were obtained from Animal Reproduction Research at 7–8 weeks of age. Same sex groups of 2–3 rats were housed in plastic cages (length 31 cm, width 47 cm, height 20 cm) at a constant temperature of 23 ± 1°C under a constant cycle of light and dark (lights on: 5:00 A.M. to 7:00 P.M.). Food and water were available *ad libitum* in all experimental periods. All animal housing and surgical procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Animal Research Center, Yokohama City University Graduate School of Medicine.

Experimental design

Experiment I. To examine the activational effect of gonadal steroids on sex-specific extracellular ACh levels, each sex was divided into 4–5 groups. Male groups were (1) gonadally intact male rats (Males), (2) orchidectomized rats (ORX), (3) orchidectomized testosterone-primed rats (ORX+T), and (4) orchidectomized 17β-estradiol-primed rats (ORX+E); female groups were (1) gonadally intact diestrous female rats (Diestrous Females), (2) gonadally intact female rats from proestrus to estrus (Proestrous Females) (3) ovariectomized rats (OVX), (4) ovariectomized testosterone-primed rats (OVX+T), and (5) ovariectomized 17β-estradiol-primed rats (OVX+E). Gonadectomy was performed 14.8 ± 0.6 d before the *in vivo* experiment, and a testosterone or 17β-estradiol capsule was subcutaneously implanted on the day of the gonadectomy. To make a 17β-estradiol capsule, we packed a 1:4 mixture of 17β-estradiol (Sigma) and cholesterol crystals in a piece of SILASTIC tubing (15 mm length per 250 g body weight, i.d. 2.0 mm, o.d. 3.0 mm; Dow Corning). To make a testosterone capsule, we packed testosterone crystals (Sigma) in a piece of SILASTIC tubing (30 mm length per 250 g body weight, i.d. 2.0 mm, o.d. 3.0 mm; Dow Corning). Using the testosterone capsule, we maintained serum testosterone levels at the levels in intact males. Using the 17β-estradiol capsule, we maintained serum 17β-estradiol levels at the levels in proestrous female rats (Mitsushima et al., 2008b). We previously confirmed the efficacy of the hormone treatments at the behavioral level: OVX+E females showed high lordosis quotient (~90%) and persistent vaginal cornification ($N = 10$). In contrast, ORX+T males displayed vigorous mounting and anogenital sniffing toward the receptive females ($N = 10$).

Experiment II. To examine the organizational effect of gonadal steroids on the ACh levels, an *in vivo* microdialysis study was performed in neonatally steroid-treated female rats. On the day of birth and 24 h later, testosterone propionate (100 μg/50 μl, Sigma), 17β-estradiol benzoate (100 μg/50 μl, Sigma), 5α-dihydrotestosterone (100 μg/50 μl, Fluka Chemie), or sesame oil (50 μl) was subcutaneously injected into the back skin of pups as previously described (Mong et al., 1999; Amateau et al., 2004). After maturation (8 weeks old), the rats were ovariectomized and a testosterone capsule was subcutaneously implanted. We performed *in vivo* microdialysis 16.2 ± 0.4 d after the procedure. To confirm that the neonatal steroid treatment was effective in inducing sexual differentiation, we observed behavioral responses to a sexually receptive female for 10 min after the dialysate sampling.

Surgery

Under sodium pentobarbital anesthesia (30–50 mg/kg, i.p.), a stainless-steel guide cannula (outer diameter, 0.51 mm) was implanted stereotaxically into the right side of the dorsal hippocampus. The coordinates were 4.0–4.3 mm anterior from the ear-bar, 3.0 mm lateral to the midline, and 2.1–2.2 mm below the surface of the brain according to the brain atlas of Paxinos and Watson (1997). The coordinates were adjusted based on sex and body weight. After cannula implantation, a stylet was inserted into the guide until the microdialysis was performed. Although rats were reared and housed in group cages, each rat was individually housed in a cylindrical plastic cage (diameter = 35 cm, height = 45 cm) for 10.5 ± 1.4 d. During this period, vaginal smears were taken from the female rats to confirm expression of the normal estrous cycle. Male rats were handled for a short time daily.

In vivo *microdialysis*

The experiment was performed in an electromagnetic- and sound-shielded room (Mitsushima et al., 2006; Jitsuki et al., 2009) (length 1.2 m, width 2.2 m, height 2.3 m). The stylet was replaced with a microdialysis probe the day before the experiment (outer diameter = 0.31 mm, A1-8-1; Eicom Co.). A two-channel fluid swivel device (SSU-20; Eicom Co.) was connected to the inlet and outlet of the probe. During the experiment, an artificial CSF solution (containing, in mM: 147 NaCl, 4 KCl, 1.2 $CaCl_2$, 0.9 $MgCl_2$) was infused through the dialysis probe with 1.0-mm-long semipermeable membrane at a rate of 1.2 μl/min using a microdialysis pump (CMA/102; Carnegie Medicin). The rats were housed individually in their cage, and the dialysis was performed under unanesthetized, freely moving conditions (Mitsushima et al., 2006, 2008b). After the overnight stabilization period, dialysates were automatically collected in an auto-injector (24 μl) (EAS-20; Eicom Co.) every 20 min for 24 h and the same volume of ethylhomocholine solution (100 nM) was mixed in as the internal standard. This mixture was injected directly into a HPLC column every 20 min (Takase et al., 2007; Mitsushima et al., 1998). In intact female rats, we collected dialysates on diestrous days (i.e., from diestrous 1 to diestrous 2) or the days from proestrus to estrus.

Biochemical analysis of ACh

To examine *in vivo* ACh levels without artificial chemicals, no eserine was used in the present study (Takase et al., 2007). ACh was quantified by a combination of HPLC column, enzyme reaction, and electrochemical detection (HTEC-500; Eicom Co.). A solution consisting of 0.1 mM Na_2HPO_4, pH 8.5, containing 200 mg/L sodium 1-decanesulfonate (Aldrich Chemical Company) was delivered as the HPLC mobile phase at a rate of 150 μl/min. After sample separation in a styrene polymer column (AC-GEL; Eicom Co.), ACh was converted to hydrogen peroxide by a postcolumn enzyme reactor (AC-ENZYMPAK; Eicom Co.) containing immobilized acetylcholinesterase and choline oxidase. The hydrogen peroxide was detected with an electrochemical detector with a least detectable amount of 5–10 fmol/sample.

To calculate the recovery rate of each dialysis probe, *in vitro* experiments were also performed. The amount of ACh collected every 20 min was divided by the *in vitro* recovery rate to estimate the extracellular ACh levels. The *in vitro* recovery rate was determined for individual probes and applied to the results from individual rats (mean ± SEM; 13.4 ± 0.5%).

Measurement of spontaneous locomotor activity

During dialysate collection, the rats were individually housed in cylindrical plastic cages (diameter 35 cm, height 45 cm) placed on dielectric constant sensors with counters (ACTMONITOR II; Dia Medical System Co.). The spontaneous locomotor activity was evaluated by changes in the dielectric constant and recorded in a personal computer every 20 min for 24 h (VAIO PCG-Z1/P, Sony) using an interface unit (DAS-008; Neuroscience Inc.) (Mitsushima et al., 1996, 1997). Activity counts were individually normalized by body weight using a previously described formula (Takase et al., 2007).

Histology

After the sampling, the animals were deeply anesthetized and perfused with a 10% formalin solution. Frozen coronal sections (50 μm thick) were sequentially cut from the forebrain using a microtome (MA-101; Yamato Koki Co.). The location of the dialysis probe was microscopically verified in the frozen sections (Fig. 1).

Statistics

In experiment I, extracellular ACh levels or spontaneous locomotor activity were analyzed by three-way ANOVA with repeated measures; between group factors were sex and steroid, and within group factor was time points. Correlation coefficient or slope was analyzed by two-way factorial ANOVA, where the variables were sex and steroid. These were

followed by *post hoc* ANOVAs with the Fisher protected least significant difference test. Simple linear regression was used to evaluate the relationship between ACh levels and spontaneous locomotor activity. Pearson's correlation coefficient (Mitsushima et al., 1996) and slope of the best fit line (Takase et al., 2009) were calculated for each individual rat. To perform the ANOVAs, proestrous and diestrous female groups were combined as cycling females.

In experiment II, ACh levels or spontaneous locomotor activity were analyzed by two-way ANOVA with repeated measures; between group factor was neonatal steroid treatment, and within group factor was time points. This was followed by *post hoc* ANOVAs with the Fisher protected least significant difference test. The effect of neonatal gonadal steroids on correlation coefficient, slope, or sexual behavior was analyzed by one-way factorial ANOVA followed by the *post hoc* test. $p < 0.05$ was considered statistically significant.

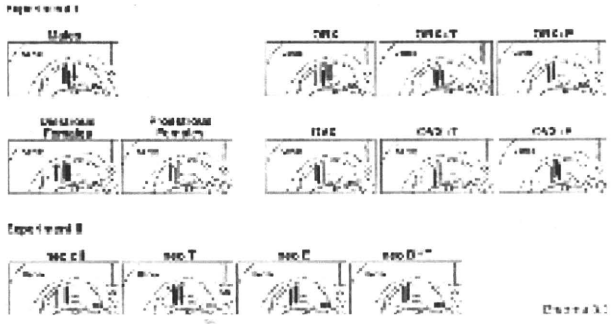

Figure 1. Location of the microdialysis probes within the dorsal hippocampus. Vertical lines represent the 1.0 mm length of the dialysis membrane. CC, Corpus callosum; DG, dentate gyrus; LP, lateral posterior nucleus. The number indicates the distance posterior to the bregma.

Results

Extracellular ACh levels

All groups exhibited an episodic ACh release profile in the dorsal hippocampus throughout the day, confirming the presence of a significant diurnal rhythm. In male groups (Fig. 2*A*), the orchidectomy clearly reduced extracellular ACh levels, which were successfully restored by testosterone replacement. However, 17β-estradiol replacement could not restore ACh levels in ORX males. In female groups (Fig. 2*B*), the extracellular ACh levels were clearly reduced by ovariectomy, and 17β-estradiol replacement successfully restored them. Testosterone replacement slightly, but significantly, increased the levels. Three-way ANOVA found the main effects of steroid ($F_{(3,3763)} = 9.159$, $p < 0.01$) and time ($F_{(71,3763)} = 56.026$, $p < 0.01$). Significant interactions were also observed between sex and steroid ($F_{(3,3763)} = 2.977$, $p < 0.04$), between steroid and time ($F_{(213,3763)} = 1.780$, $p < 0.01$), and among sex, steroid, and time ($F_{(213,3763)} = 2.098$, $p < 0.01$). Intact male rats had significantly greater ACh levels than cycling female rats ($p < 0.01$). Moreover, ORX+T males had significantly greater ACh levels than OVX+T females ($p < 0.01$), whereas ORX+E males had significantly smaller ACh levels than OVX+E females ($p < 0.01$). The results of the statistical analysis are summarized in Figure 2, *C* and *D*.

Spontaneous locomotor activity

All groups exhibited an episodic locomotor activity profile throughout the day, showing a clear diurnal rhythm. In male groups (Fig. 2*A*), orchidectomy and hormone replacement after orchidectomy did not alter observed locomotor activity. In contrast, female groups (Fig. 2*B*) exhibited some differences in response to ovariectomy and hormone replacement. Although proestrous and OVX+E females had relatively higher locomotor activity than diestrous females, the levels in OVX or OVX+T females were similar to the levels in diestrous females. Three-way ANOVA found the main effects of sex ($F_{(1,3763)} = 7.318$, $p < 0.01$) and time ($F_{(71,3763)} = 46.084$, $p < 0.01$). Significant interaction was also observed among sex, steroid and time ($F_{(213,3763)} = 1.252$, $p < 0.01$). OVX+E females had significantly greater locomotor activity than cycling, OVX, or OVX+T females ($p < 0.01$). The results of the statistical analysis are summarized in Figure 2, *C* and *D*.

Correlation between the ACh and locomotor activity

Both sexes exhibited a positive correlation between the ACh levels and the spontaneous locomotor activity. Representative cases of 2 gonadally intact and 2 ORX males are shown in Figure 3*A*. Although the ACh levels in intact males (#100 and #102) were highly correlated with the spontaneous locomotor activity, the ACh levels in ORX males (#88 and #107) were not always high when they are moving. The individual results of simple linear regression analysis are shown in Figure 3*B*. Intact males had a higher correlation coefficient and a steeper slope of the fit line than ORX males. All correlation and slope data were summarized in Figure 3, *C* and *D*. For the correlation coefficient, two-way ANOVA showed that the main effect of steroid ($F_{(3,53)} = 16.328$, $p < 0.01$) and the interaction between sex and steroid ($F_{(3,53)} = 3.501$, $p < 0.02$) were significant. For the slope of the fit line, the main effects of sex ($F_{(1,53)} = 13.220$, $p < 0.01$) and steroid ($F_{(3,53)} = 7.137$, $p < 0.01$), as well as the interaction between sex and steroid ($F_{(3,53)} = 6.568$, $p < 0.01$) were significant. In the male groups, reduced correlation and slope in ORX males ($p < 0.01$) were successfully restored in ORX+T males (Fig. 3*C*). Similarly, in the female groups, a reduced correlation in OVX females ($p < 0.01$) was successfully restored by treatment with either 17β-estradiol or testosterone, although the slope was relatively small and without difference (Fig. 3*D*). Moreover, data from intact males had a significantly steeper slope than cycling females ($p < 0.01$), showing a sex-specific ACh release property.

Neonatal gonadal steroid treatments

To examine the organizational effect of gonadal steroids on 24 h ACh release, we subcutaneously injected oil, testosterone, 17β-estradiol, or 5α-dihydrotestosterone into neonatal female rats (Experiment II, see Materials and Methods). At 8 weeks of age, either dihydrotestosterone- or oil-treated rats showed normal 4 d estrous cycles, whereas 17β-estradiol-treated rats showed clear constant estrus. The vagina did not open in all testosterone-treated rats. Then, the four groups of rats were bilaterally ovariectomized, and a testosterone capsule was implanted.

The ACh levels exhibited a clear diurnal rhythm in all 4 groups. Although neonatal testosterone or estradiol treatment significantly increased the ACh levels, the effect was not clear in neonatal dihydrotestosterone treatment (Fig. 4*A*). Two-way ANOVA found the main effect of time ($F_{(71,1633)} = 26.201$, $p < 0.01$) and the interaction between treatment and time ($F_{(213,3763)} = 1.252$, $p < 0.01$). The results of the statistical analysis are summarized in Figure 4*B*.

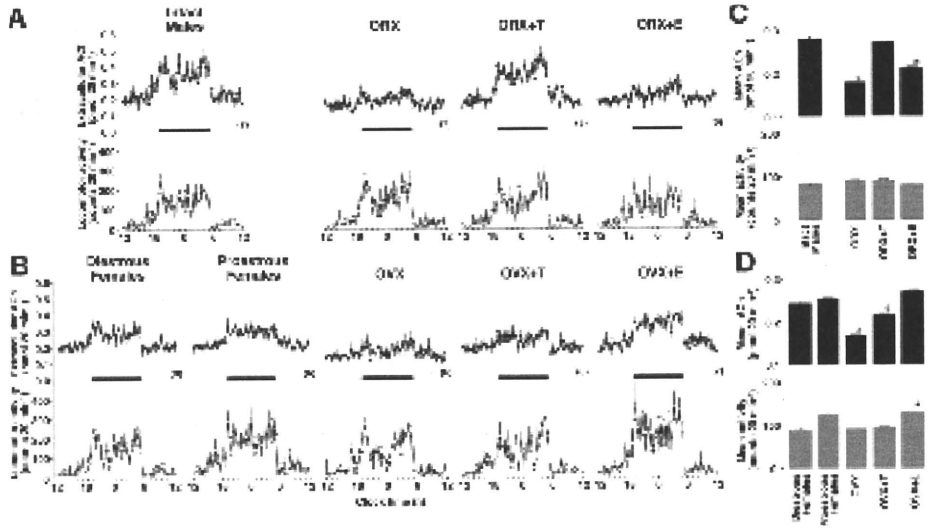

Figure 2. *A–D*, Activational effects of gonadal steroids on the extracellular ACh levels and spontaneous locomotor activity. In male groups (*A*), ORX reduced the ACh levels without changing the spontaneous locomotor activity. Replacement of testosterone (+T) but not 17β-estradiol (+E) restored the ACh levels. In female groups (*B*), OVX reduced the ACh levels. Testosterone replacement in OVX females failed to raise ACh levels to those seen in testosterone-primed ORX males. Replacement of 17β-estradiol not only maintained ACh levels, but also enhanced spontaneous locomotor activity. Horizontal black bars indicate the dark phase. Overall levels of ACh and spontaneous locomotor activity were statistically summarized for male (*C*) and female (*D*) groups. $^{*}p < 0.01$ compared with gonadally intact males. $^{\#}p < 0.01$ compared with cycling females (diestrous and proestrous groups were combined for statistical analysis). The number of rats in each group is shown in parentheses. Data are expressed as the mean ± SEM.

Locomotor activity exhibited a clear diurnal rhythm in all 4 groups, but the effect of neonatal treatment was not clear (Fig. 4*A*). Two-way ANOVA found the main effect of time ($F_{(71,1633)} = 20.406$, $p < 0.01$), but the main effect of treatment or the interaction was not significant (Fig. 4*B*).

The results of regression analysis are shown in Figure 4*C*. Although there was no difference in the correlation coefficient ($F_{(3,23)} = 1.405$, $p > 0.05$), data from neonatally testosterone (neo T)- or 17β-estradiol-treated (neo E) rats had a steeper slope of the fit line than oil-treated rats (neo oil), revealing male-specific ACh release ($F_{(3,23)} = 5.230$, $p < 0.01$). In addition, neonatal dihydrotestosterone treatment may masculinize the ACh release property (neo DHT, $p = 0.06$ vs neo oil). Moreover, the neonatal testosterone or estradiol treatment enhanced male-like copulatory behavior in the rats (Table 1, $F_{(3,23)} = 10.139$, $p < 0.01$).

Discussion

In experiment I, we found an activational effect of gonadal steroids on the extracellular ACh levels in the dorsal hippocampus of behaving rats; the levels were severely reduced after gonadectomy and testosterone replacement in ORX males or 17β-estradiol replacement in OVX females successfully restored them. Moreover, testosterone replacement in OVX females failed to increase ACh to the levels seen in ORX+T males. In experiment II, neonatal androgenization not only increased ACh levels but also altered ACh release to resemble that of males without affecting spontaneous activity levels. This is the first report showing an organizational effect of gonadal steroids on sex-specific ACh release profiles in behaving rats.

The activational effects of gonadal steroids on cholinergic neurons are consistent with previous neuroanatomical and neurochemical findings. For example, orchidectomy decreases the density of cholinergic fibers in the dorsal hippocampus, whereas testosterone replacement in ORX male rats maintains fiber density (Nakamura et al., 2002). Also, 17β-estradiol increases the induction of choline acetyltransferase in the basal forebrain in OVX female rats (Luine et al., 1986; McEwen and Alves, 1999). A previous *in vitro* study demonstrated that 17β-estradiol treatment increases both high affinity choline uptake and ACh synthesis in basal forebrain neurons (Pongrac et al., 2004). Furthermore, we recently reported an activational effect of gonadal steroids on the maintenance of stress-induced ACh release in the dorsal hippocampus in immobilized rats (Mitsushima et al., 2008b). Despite all of this evidence suggesting the activational effect of gonadal steroids on ACh release in the dorsal hippocampus, conclusive evidence such as dynamic ACh changes under physiological conditions has not been presented in behaving animals. Because spontaneous movement increases extracellular ACh levels (Day et al., 1991; Mitsushima et al., 1996), we simultaneously analyzed ACh levels and spontaneous locomotor activity to determine the precise effect of gonadal steroids. In the present study, we found that gonadectomy impaired ACh levels without affecting spontaneous locomotor activity levels. More-

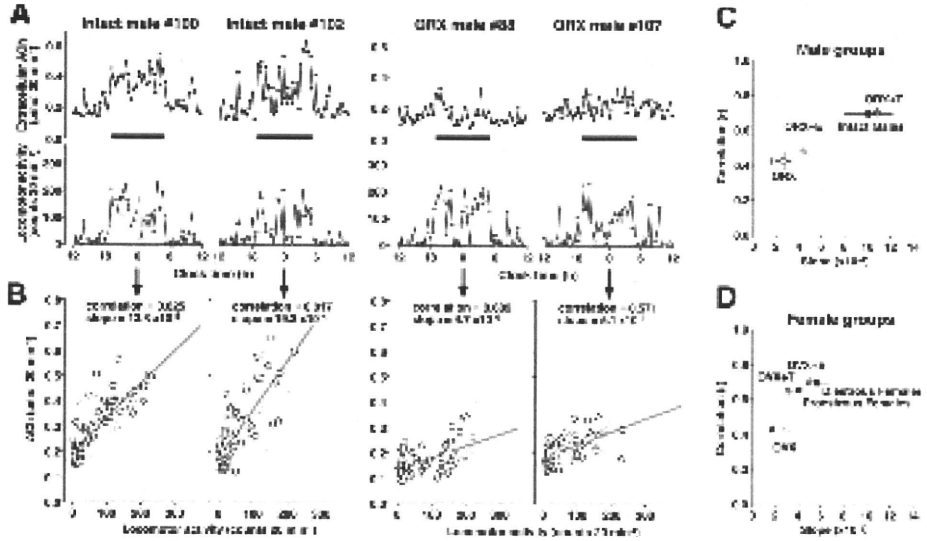

Figure 3. ***A***, Correlation between ACh levels and spontaneous locomotor activity in 2 gonadally intact and 2 ORX male rats. ***B***, Simple linear regression analysis showed that the data from intact males (#100 and #102) had a steep slope of the fit line, whereas the data from ORX males (#88 and #107) had a gentle slope. ***C***, Orchidectomy reduced both correlation and slope, with testosterone replacement (+T) restoring the property in male groups. ***D***, OVX reduced the correlation, with steroid replacement either testosterone or 17β-estradiol (+E) restoring the property in female groups. $^{*}p < 0.01$ compared with males (both correlation and slope). $^{\#}p < 0.01$ compared with cycling females (correlation only). Moreover, intact males had significantly steeper slope than cycling females ($p < 0.01$). Data in ***C*** and ***D*** represent the means ± SEM.

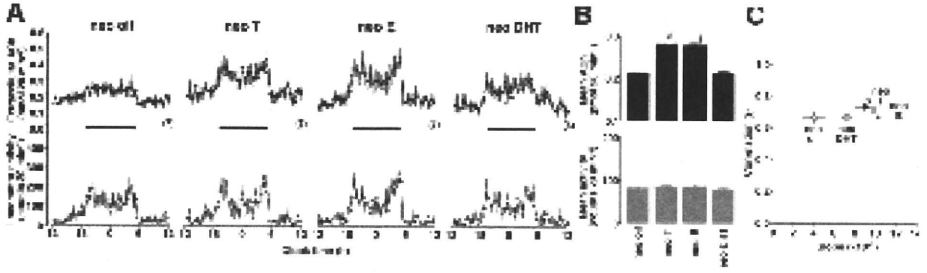

Figure 4. Organizational effects of gonadal steroids on ACh levels in female rats. At postnatal days 0 and 1, female pups were given oil (neo oil), testosterone (neo T), 17β-estradiol (neo E), or 5α-dihydrotestosterone (neo DHT). After maturation, the rats were bilaterally ovariectomized and given testosterone replacement. ***A***, Neo oil or neo DHT rats exhibited relatively low ACh levels, whereas neo T or neo E rats enhanced the ACh levels compared with male rats. ***B***, Overall levels of ACh and spontaneous locomotor activity were statistically summarized for neonatally steroid-treated female rats. Neo T or neo E treatment significantly enhanced the mean ACh levels in female rats without changing the spontaneous locomotor activity. ***C***, In simple linear regression analysis, neo T or neo E data had significantly steeper slope than neo oil data. $^{*}p < 0.01$ compared with neo oil. The number of rats in each group is shown in parentheses. Data are expressed as the mean ± SEM.

over, the activational effect on ACh levels was clear especially during active period, although it is not clear during resting period (Mitsushima et al., 2008b). Our results provide the first evidence that the 24 h extracellular ACh levels in the dorsal hippocampus are dependent on the presence of gonadal steroids.

Acetylcholine levels were still sex-specific under the comparable gonadal steroids levels. For example, testosterone replacement in OVX females failed to increase ACh levels to those seen in ORX+T males. Moreover, 17β-estradiol replacement was unable to restore ACh levels in ORX males. These results may be consistent with previous neuroanatomical reports at the spine synapse level. First, estradiol benzoate consistently increases the NMDA receptor binding and spine density in the CA1 area of OVX females, although the treatment fails to increase the parameters in ORX males (Romeo et al., 2005; Parducz et al., 2006). Second, androgens are important in maintaining normal spine

Table 1. Mounting behaviors in neonatally steroid-treated rats

Group	Number of animals	Number of mounts per 10 min
neo oil	7	8.3 ± 1.4
neo T	7	23.0 ± 3.3*
neo E	7	23.9 ± 2.5*
neo DHT	6	10.3 ± 2.8

*Significantly different from neo oil ($p < 0.01$).

synapse numbers on CA1 pyramidal neurons, whereas estrogen is important only in females (MacLusky et al., 2006). These findings suggest that the activational effects of gonadal steroids are sex-specific and essential for maintaining hippocampal functions. Based on the reports, together with the present data from experiment I, we hypothesized that the action of sex-specific steroids is the result of neonatal sexual differentiation rather than the activational effect of gonadal steroids in adult rats.

In experiment II, we examined the organizational effects of testosterone under the comparable gonadal steroids levels. Because ACh levels exhibited distinct sex difference in gonadectomized testosterone-primed condition (see ORX+T vs OVX+T), we chose the steroid condition to evaluate the organizational effects. Without gonadal steroid-priming, gonadectomized rats did not show a clear sex difference. In gonadectomized 17β-estradiol-primed condition, it would be difficult to interpret the sex-specific ACh levels, because 17β-estradiol increases spontaneous locomotor activity in OVX females. We found that neonatal testosterone injection not only increased the ACh levels but also altered the ACh release property to resemble the male-specific profile without changing spontaneous activity levels. These results provide the first evidence that neonatal testosterone exposure sexually differentiates septo-hippocampal cholinergic neurons.

We further analyzed the organizational effect of testosterone using 17β-estradiol and 5α-dihydrotestosterone. 17β-Estradiol is the aromatized product of testosterone, acting as an estrogen receptor agonist. In contrast, 5α-dihydrotestosterone is the 5α-reduced metabolite of testosterone, acting as an androgen receptor agonist. Because testosterone can be converted to 17β-estradiol or 5α-dihydrotestosterone in neonatal rat brain (Zwain and Yen, 1999), neonatal testosterone can activate both estrogen and androgen receptors (McEwen, 1981). Concerning other sex-specific functions, estrogen receptor is known to mediate the activation of male copulatory behavior and the disruption of estrous cyclicity (McEwen, 1981; Herath et al., 2001), whereas androgen receptors mediate the masculinization of social play (Meaney et al., 1983). In experiment II, not only testosterone but also 17β-estradiol treatment in neonatal female pups masculinized ACh release property in adults, suggesting estrogen receptor-mediated masculinization of septo-hippocampal cholinergic systems. Consistently, testosterone or estradiol treatment in neonatal female pups improves their adult spatial performance, whereas neonatal gonadectomy in male pups causes decrements in the performance (Williams and Meck 1991). In contrast, neonatal 5α-dihydrotestosterone treatment may partly masculinized the ACh release property, but failed to increase the overall ACh levels. Although 5α-dihydrotestosterone has been classically considered a prototypical androgen receptor agonist, a metabolite of 5α-dihydrotestosterone, 5α-androstane-3β,17β-diol (3β-diol), has higher affinity for estrogen receptor β (Lund et al., 2006). Therefore, 5α-dihydrotestosterone and its metabolites (3β-diol) may stimulate both androgen receptor and estrogen receptor β, whereas 17β-estradiol stimulates estrogen receptor α and β. Considering the action of gonadal steroids and their metabolites, estrogen receptor α may mediate the organizational effect on septo-hippocampal cholinergic system and spatial learning performance.

Acetylcholine in the dorsal hippocampus has physiological relevance. Behavioral studies have demonstrated that the level of extracellular ACh increases during learning (Ragozzino et al., 1996; Stancampiano et al., 1999) and is positively correlated with memory performance (Gold, 2003). Endogenous ACh in the hippocampus plays an important role in both spatial and contextual memory consolidation. Bilateral injections of scopolamine into the dorsal hippocampus impair both spatial learning (Herrera-Morales et al., 2007) and contextual encoding in male rats (Wallenstein and Vago 2001). At the cellular level, both pyramidal and nonpyramidal neurons in the hippocampal CA1 area receive direct cholinergic afferents mediated by the muscarinic receptors (Cole and Nicoll 1983; Widmer et al., 2006).

Interestingly, both *in vivo* learning (Whitlock et al., 2006) and *in vitro* bath application of carbachol, a cholinergic agonist, induce LTP in the hippocampal CA1 region without artificial tetanus stimulus (Auerbach and Segal 1996). Furthermore, gene disruption of muscarinic M_2 receptors not only impairs spatial learning but also blocks the carbachol-induced LTP in the Schaffer collateral pathway to the hippocampal CA1 region (Seeger et al., 2004), suggesting the involvement of muscarinic M_2 receptors. Although the molecular mechanism of carbachol-induced LTP was unknown, Shi et al. (2001) demonstrated that synaptic delivery of AMPA receptors is a mechanism of electrically induced LTP. Moreover, *in vitro* patch clamp recording demonstrated that focal application of ACh induces long-lasting enhancement of Schaffer collateral EPSCs at the CA1 pyramidal neurons (Fernández de Sevilla et al., 2008). Based on these findings, we hypothesized that the ACh released in the hippocampus enhances glutamatergic transmission in CA1 pyramidal neurons, playing a principal role in hippocampal learning. Finally, by combining Herpes virus-mediated *in vivo* gene delivery with *in vitro* patch-clamp recordings (Takahashi et al., 2003), we previously revealed that learning-induced ACh release mediates synaptic delivery of AMPA receptors in CA1 pyramidal neurons (Mitsushima et al., 2008a).

In the present study, we found reduced correlation between ACh levels and locomotor activity levels in gonadectomized rats, suggesting that hippocampal function in rats may not always be activated at a subthreshold level of gonadal steroids. High positive correlation between ACh levels and activity levels depends on the presence of gonadal steroids. It is therefore possible, that the learning impairment in gonadectomized rats (Gibbs and Pfaff 1992; Kritzer et al., 2001; Markowska and Savonenko 2002; Luine et al., 2003) may be attributable to insufficient ACh levels in the hippocampus when it is required for memory. These results suggest that circulating gonadal steroids strengthen the coupling between spontaneous behaviors and ACh levels, which in turn, may activate the learning function in the hippocampus at the appropriate time.

In humans, circulating levels of gonadal steroids decline with age. Moreover, a reduction in ACh synthesis is known as a common feature of Alzheimer's disease (Coyle et al., 1983), afflicting >18 million people worldwide (Ferri et al., 2005; Mount and Downtown 2006). The disease is the most common form of dementia (Cummings 2004) and is frequently accompanied by insomnia, poor concentration, or day/night confusion (McCurry et al., 2004; Starkstein et al., 2005). Centrally active acetylcholinesterase inhibitor (donepezil) is effective in not only mild, but also

moderate to severe cases (Petersen et al., 2005; Winblad et al., 2006), proving the importance of endogenous ACh in humans. In addition, women are twice as likely to develop the disease (Swaab and Hofman 1995), and estradiol seems to play a protective role (Zandi et al., 2002; Norbury et al., 2007). A recent study using single photon emission tomography showed that estrogen replacement therapy in healthy women increases muscarinic M_1/M_4 receptor binding in the hippocampus (Norbury et al., 2007). Conversely in men, testosterone but not estradiol seems to play a protective role (Moffat et al., 2004; Rosario et al., 2004) and testosterone supplementation clearly improved hippocampus-dependent learning deficits in men with Alzheimer's disease (Cherrier et al., 2005). These results suggest a sex-specific activational effect of gonadal steroids on the cholinergic system in humans. Thus, there are many similarities between the rat model and the human studies, supporting the idea that the gonadal steroids replacement or an increase in their bioavailability is necessary when there is a subthreshold level of the hormone. Based on the neonatal sexual differentiation of the septo-hippocampal cholinergic system, we may have to search for sex-specific clinical strategies for Alzheimer's disease.

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