Cell, Volume *139*

Supplemental Data

Estrogen Masculinizes Neural Pathways and Sex-Specific Behaviors

Melody V Wu, Devanand S Manoli, Eleanor J Fraser, Jennifer K Coats, Jessica Tollkuhn, Shin-Ichiro Honda, Nobuhiro Harada, and Nirao M Shah

SUPPLEMENTAL TEXT

Serum estrogen and testosterone are unchanged in mice bearing the aromatase-IPIN allele

A comparison of serum hormone levels for testosterone and estrogen did not reveal significant differences between wildtype (WT) animals and mice homozygous for the aromatase-IPIN allele. (Testosterone: WT males, 5.75 ± 1.4 nM; *aromatase*$^{IPIN/IPIN}$ males, 5.43 ± 1.2 nM; WT females, 0.54 ± 0.2 nM; *aromatase*$^{IPIN/IPIN}$ females, 0.5 ± 0.17 nM. Estrogen: WT males, 0.12 ± 0.02 nM; *aromatase*$^{IPIN/IPIN}$ males, 0.15 ± 0.02 nM; WT females, 0.14 ± 0.03 nM; *aromatase*$^{IPIN/IPIN}$ females, 0.17 ± 0.02 nM; Mean ± SEM; $n \geq 4$; $p > 0.29$ for within sex and hormone comparisons.) Individual females of either genotype often had 2 - 3 fold elevations above the mean in their serum estrogen at the time of sacrifice, presumably reflecting the rise in estrogen preceding estrus.

Similar proportions of neurons express aromatase in the BNST and MeA in both sexes

The dimorphisms in aromatase expression could reflect an absolute difference in cell number in these regions between the sexes. Such dimorphisms could also arise from an increase in the fraction of neurons expressing aromatase in males. We find a similar proportion of NeuN positive cells co-labeled with βgal in the two sexes in the BNST (males, 36.57 ± 1.3%; females, 28.2 ± 5.9%; n = 3; p = 0.17, Student's t test) as well as the MeA (males, 40.4 ± 2.3%; females, 41.4 ± 2.6%; n = 3; p = 0.78; Student's t test), indicating an increase in the total number of neurons in both regions in males compared to females.

The anterior and ventromedial hypothalamic regions contain a small, equivalent number of aromatase expressing cells in both sexes

We examined aromatase expression in the anterior and ventromedial hypothalamic regions of adult, age matched male and female mice bearing the aromatase-IPIN allele. We find a small, equivalent number of cells labeled for βgal activity in these regions in both sexes (Anterior hypothalamus: Male, 25 ± 2; Female, 24 ± 1; n = 4; p > 0.5, Student's t test; Ventromedial hypothalamus: Male, 21 ± 4; Female 20 ± 3; n = 4; p > 0.9, Student's t test).

Serum estrogen and testosterone are similar between adult females treated neonatally with estrogen or vehicle

A comparison of serum hormone levels for testosterone and estrogen did not reveal significant differences between adult females treated neonatally with estrogen (NE) or vehicle (NV). (Testosterone: NV, 0.33 ± 0.17 nM; NE, 0.38 ± 0.17 nM. Estrogen: NV, 0.23 ± 0.06 nM; NE, 0.24 ± 0.06 nM; $n \geq 5$; $p \geq 0.848$.) Individual NE and NV females often had 2 - 3 fold elevations above the mean in their serum estrogen at the time of sacrifice, presumably reflecting the rise in estrogen preceding estrus. We present serum estrogen levels of eight adult individuals of each treatment group to illustrate such elevations above the mean in some females. (Serum estrogen in nM: NV, 0.07, 0.09, 0.12, 0.14, 0.17, 0.33, 0.42, 0.49; NE, 0.06, 0.08, 0.1, 0.13, 0.26, 0.42, 0.42, 0.46.)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of mice bearing the aromatase-IRES-PLAP-IRES-nLacZ allele

Genomic clones containing the last exon of *aromatase* were obtained by screening a 129/SvJ lambda phage library from Stratagene. A ~5.8 kb *Pml*I genomic clone containing the last two exons of the *aromatase* gene was used to design the targeting vector. An *Asc*I restriction site was inserted 3 bp 3' of the stop codon of the *aromatase* gene using site-directed mutagenesis (Stratagene). This mutagenized targeting vector has 4.5 kb and 1.3 kb of homology 5' and 3' of the *Asc*I restriction site, respectively. As described previously, we utilized the self-excising neomycin cassette, ACN, which was inserted 3' of IRES-PLAP-IRES-nLacZ (Bunting et al., 1999; Shah et al., 2004). This IRES-PLAP-IRES-nLacZ-ACN cassette was inserted into the targeting vector as an *Asc*I fragment. The aromatase targeting vector containing the reporters and the neomycin selection cassette was electroporated into a 129/SvEv mouse ES cell line.

We obtained a targeting frequency of 5% for homologous recombinants, which were detected using PCR for the 3' arm for the targeting vector. We used a primer (5'-CATCGCCTTCTATCGCCTTCTTGAC) that was complementary to the ACN cassette and an external primer (5'-CTTGATCATTGGAGCCAAATCTGGATG) that was complementary to genomic sequence located 3' of the 3' homology arm of the targeting vector. A subset of positive clones was tested by PCR for homologous targeting of the 5' arm using an external primer (5'-CCAGCTGGATTTCTTGGGATCAAATTCAGG) and a primer unique to the modified allele (5'-GAATTCGGCGCGCCTCTTCACTGTTG). ES clones harboring the homologously recombined modified aromatase allele were injected into blastocysts to obtain chimeric mice which were crossed to C57Bl/6J females to obtain germline transmission. Chimeric mice that transmitted the aromatase-IPIN allele were obtained from one positive clone. ACN contains a *neomycin*^{R} gene that is self-excised upon passage through the male germline, and F1 progeny obtained by crossing the chimeric males to C57Bl/6J females had deleted ACN as determined by PCR. The resulting progeny (backcrossed > 3 generations in C57Bl/6J) were used for experimental analysis.

Other mouse strains

Mice bearing the tfm allele were obtained from Jackson Laboratories and backcrossed extensively to the C57Bl/6J strain (> 10 generations) prior to experimental use. Mice bearing a null allele of aromatase (deletion of exons 1 and 2) have been described previously (Honda et al., 1998).

Estrus induction

Females aged 8 - 16 weeks were ovariectomized, and allowed to recover from the surgery for one week. Estrus was induced by sequential daily injections of 10 μg and 5 μg of EB followed by 50 μg of progesterone. The females were used for sexual behavior 4 - 6 hours after progesterone injection. Females were allowed to recover for ≥ 1 week between assays.

Immunostaining

The primary antisera used in this study are: rabbit anti-βgal (Cortex Biochem, 1:2000 or ICL, 1:5000), mouse anti-βgal (Promega, 1:2500), mouse anti-NeuN (Chemicon, 1:300), rabbit anti-ERα (Upstate, 1:50000), rabbit anti-activated caspase-3 (Cell Signaling Tech, 1:100; Chemicon, 1:100; R&D Systems, 1:200), rabbit anti-activated caspase-9 (Cell Signaling Tech, 1:50), and rabbit anti-Apaf-1(Epitomics, 1:5000). The fluorophore conjugated secondary antisera are: Cy3 donkey anti-rabbit, Cy3 donkey anti-mouse (Jackson ImmunoResearch, 1:800), AlexaFluor 488 donkey anti-mouse, and AlexaFluor 488 donkey anti-rabbit (Invitrogen, 1:300). Sections were exposed to the primary antisera for 12 - 16 hours at 4^{o}C, and to the secondary antisera for 2 hours at room temperature. The buffers, washes, and mounting media used in these experiments have been described previously (Shah et al., 2004).

TUNEL

Brains were dissected from paraformaldehyde - perfused animals and fixed for an additional 30 minutes at 4^{o}C. The brains were incubated in 20% sucrose for 12 - 18

hours at 4°C, and embedded in 1:1 mix of Tissue-Tek OCT (Sakura) and Aquamount (VWR). The brains were sectioned at 16 μm, and adjacent sections were collected on alternating slides. One set of slides was immunolabeled for βgal as described above. The second set was stained for apoptotic cells using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon) in accordance with the manufacturer's protocol. These TUNEL stained sections were imaged using an upright epifluorescence microscope. For every animal, TUNEL positive cells were enumerated bilaterally in 6 - 7 representative sections containing the BNST or MeA each. An identical approach was used to enumerate the apoptotic cells (labeled with antibodies to Caspase-3, 9 and Apaf-1) that also expressed aromatase.

In situ hybridization

We performed in situ hybridization (ISH) using RNA probes to detect expression of ERα and ERβ mRNA in the neonatal brain, and aromatase message in the adult brain. The probes used to detect ERα and ERβ mRNA correspond to those utilized by the Allen Institute for Brain Science for the mouse brain (Lein et al., 2007). We prepared RNA sense and anti-sense probes corresponding to 396 - 728 bp of aromatase mRNA (accession D00659). ISH on neonatal brains using digoxigenin labeled RNA probes was performed as described previously (Kurrasch et al., 2007). For ISH on adult animals, we dissected the brain of mice perfused with 4% paraformaldehyde (PFA), and fixed it for an additional 14 - 18 hours at 4°C in 4% PFA. The brains were embedded in 3% bacto-agar and sectioned at 90 μm. These sections were treated with proteinase K (10 μg/mL, Roche) for 30 minutes, rinsed, and fixed in 4% PFA for 30 minutes at room

temperature. We treated these sections with acetylation buffer for 10 minutes and equilibrated them in hybridization solution for 2 - 5 hours at 65^{o}C. These sections were subsequently incubated for 14 - 18 hours at 65^{o}C in fresh hybridization buffer containing 0.5 μg/mL RNA probe. The sections were subsequently washed in high stringency buffers and subjected to electrophoresis to remove unhybridized probe as described previously (Kobayashi et al., 1994). These washes were followed with incubation for 12 - 18 hours at 4^{o}C in buffer containing alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:2000, Roche). After extensive washing, we incubated the sections for 6 hours at 37^{o}C in staining solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Roche). The sections were subsequently washed, fixed in 4% PFA, and mounted on glass slides. Detailed protocols are available upon request.

Quantitation of histological data

We estimated the cell number and volume for both sides of the brain (left and right) individually, and obtained the mean for each animal. All imaging and data analysis were performed by an experimenter blind to sex, age, genotype, and hormone treatment. When using unbiased stereology based methods for quantitation, cells were enumerated with an Optical Fractionator probe, and projection fiber volume was estimated with a Cavalieri Estimator probe, using parameters set per the manufacturer's instructions (StereoInvestigator, MicroBrightField) and published stereology protocols (Keuker et al., 2001).

To enumerate immuno-labeled βgal$^+$ cells in adult animals, 65 μm thick sections encompassing the BNST and MeA were imaged with confocal microscopy to collect Z-stacks (3.9 μm step) through the entire section, and processed as described previously (Shah et al., 2004). All immunolabeled cells in the central three optical slices in each histological section were enumerated through the rostrocaudal extent of the BNST and MeA. Similar results were also obtained using unbiased stereology as follows. In each Z-stack, the region of interest was outlined, 4 μm guard zones were established to preclude overcounting, and βgal$^+$ cells were enumerated with a 20x20 μm^2 counting box in a 70x70 μm^2 sampling grid. To determine whether βgal$^+$ cells expressed NeuN, images of the sections were collected as Z-stacks (3.4 μm steps) using a 63X lens. Guard zones of 3 μm were established, and a 20x20 μm^2 counting box was used in a 45x45 μm^2 grid to enumerate cells. The same parameters were used to determine whether βgal+ cells expressed ERα or the androgen receptor.

Sections through the entire BNST and MeA of P1 animals were collected at 12 μm, stained for βgal activity, and viewed with brightfield optics (10X) on an upright microscope with a motorized stage. We used 1 μm guard zones and a 20x20 μm^2 counting box in a 40x40 μm^2 sampling grid to enumerate βgal$^+$ cells in alternate sections.

PLAP stained sections spanning the rostrocaudal extent of the anterior hypothalamus or the VMH were imaged using 5X brightfield optics on an upright

microscope. A lattice with nodes spaced 12 μm apart was superimposed on the images, and each node that overlapped $PLAP^+$ fibers was manually marked to determine the area occupied by these projections. The software estimated the volume filled by $PLAP^+$ fibers as we used sections of defined thickness (80 μm).

Behavioral Assays

We used 10 - 24 week old mice in behavioral assays. Mice were group housed by sex after weaning, and were sexually naive when the behavioral testing was initiated. Animals were tested at least one hour after the lights were switched off. The behaviors were recorded using an infrared sensitive video recorder. Animals were isolated two days before testing started and maintained in individual housing for the duration of the tests. The animals were rested for ≥ 2 days between behavioral tests, and residents were always exposed to a novel intruder.

SUPPLEMENTAL REFERENCES

Bunting, M., Bernstein, K.E., Greer, J.M., Capecchi, M.R., and Thomas, K.R. (1999). Targeting genes for self-excision in the germ line. Genes Dev *13*, 1524-1528.

Honda, S., Harada, N., Ito, S., Takagi, Y., and Maeda, S. (1998). Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. Biochem Biophys Res Commun *252*, 445-449.

Keuker, J.I., Vollmann-Honsdorf, G.K., and Fuchs, E. (2001). How to use the optical fractionator: an example based on the estimation of neurons in the hippocampal CA1 and CA3 regions of tree shrews. Brain Res Brain Res Protoc *7*, 211-221.

Kobayashi, S., Saito, H., and Okada, M. (1994). A simplified and efficient method for in situ hybridization to whole Drosohpila embyros, using electrophoresis for removing non-hybridized probes. Development Growth and Differentiation *36*, 629-632.

Kurrasch, D.M., Cheung, C.C., Lee, F.Y., Tran, P.V., Hata, K., and Ingraham, H.A. (2007). The neonatal ventromedial hypothalamus transcriptome reveals novel markers with spatially distinct patterning. J Neurosci *27*, 13624-13634.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J.*, et al.* (2007). Genome-wide atlas of gene expression in the adult mouse brain. Nature *445*, 168-176.

Shah, N.M., Pisapia, D.J., Maniatis, S., Mendelsohn, M.M., Nemes, A., and Axel, R. (2004). Visualizing sexual dimorphism in the brain. Neuron *43*, 313-319.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Expression of β-galactosidase mirrors the distribution of aromatase mRNA

(A-L) Coronal sections through the adult BNST, MeA, septum (Sep), POA, anterior olfactory nucleus (AON), and thalamus (Thal) of a mouse heterozygous for the aromatase-IPIN allele. Adjacent sections were processed for in situ hybridization to reveal aromatase mRNA (A-F) or βgal activity (G-L). The expression of βgal mirrors that of aromatase message. There are no βgal positive cells in the AON and the thalamus, regions with no detectable aromatase message. There are small clusters of aromatase mRNA expressing and βgal positive cells in adjacent sections through the BNST, MeA, septum, and POA. **(S)** There is no sex difference in the number of βgal expressing cells in the POA or septum. (**M-R**) Nissl stained (grayscale) sections depicting locations of the BNST, MeA, septum, POA, AON, and thalamus. Error bars represent SEM; $n = 4$; $p \geq 0.164$. Scale bars equal 250 μm.

Figure S2. β-galactosidase and PLAP activity offer excellent resolution of the distribution of aromatase positive neurons and their fiber tracts

(A, B, D, E) Coronal sections through the adult brain of male mice bearing the WT (B, E) or genetically modified aromatase (aromatase-IPIN) (A, D) allele stained for βgal (A, B) or PLAP (D, E) activity. These representative sections through the BNST (A, B) and VMH (D, E) demonstrate robust labeling of the cell bodies and fibers of aromatase expressing neurons in mice bearing the aromatase-IPIN allele. There is no visible background labeling for βgal or PLAP activity in the WT brain. Note that sections from

the WT and genetically modified mice were processed in parallel for the histochemical stainings in this figure and for all the studies presented in this study. **(C, F)** Nissl stained (grayscale) sections depicting locations of the BNST and VMH. Scale bars equal 500 μm (A, B) and 250 μm (D, E).

Figure S3. Sexual dimorphism in aromatase expressing neural pathways

Coronal sections through the adult brain of male and female mice bearing the aromatase-IPIN allele stained for PLAP activity. Adjacent sections through the BNST (A-D), MeA (E-H), and caudal hypothalamus (CH) (I-L) are shown, with the top panel in each instance representing the more rostral section. PLAP stained fibers occupy a larger volume in the male BNST and MeA. There are more PLAP labeled fibers in the female caudal hypothalamus. Note that PLAP not only labels the cell bodies that express aromatase, but also the fibers emanating from these neurons. The arrow (F) shows the PLAP labeled stria terminalis, a fiber tract that contains projections from the BNST and MeA, both of which contain aromatase expressing neurons. Scale bars equal 250 μm.

Figure S4. ERα and ERβ are expressed in the neonatal BNST and MeA

(A-H) Coronal sections through the BNST and MeA of WT newborn male and female mice processed for in situ hybridization to reveal ERα or ERβ mRNA. There are ERα and ERβ expressing cells in the BNST and MeA in both sexes. **(I-T)** Representative coronal sections through the BNST and MeA of newborn male and female mice bearing the aromatase-IPIN allele immunostained for βgal (red) and ERα (green). The merged

images show that individual cells co-express (yellow) aromatase (βgal) and ERα in the BNST and MeA. The vast majority (>99%) of βgal positive cells co-express ERα. n ≥ 2. Scale bars equal 250 μm (A-H) and 100 μm (I-T).

Figure S5. Estrogen administration in adulthood does not masculinize aromatase expressing neurons

Representative coronal sections through the brain of adult females bearing the aromatase-IPIN allele treated with estrogen or vehicle as adults and stained for βgal (A-J) or PLAP activity (K-R). There appears to be no significant difference in aromatase positive neural pathways between estrogen treated females and their controls. Adult females were injected subcutaneously every other day with 5μg of estrogen or vehicle for three weeks. These animals were sacrificed and their brains processed for βgal or PLAP activity. Arrows (A-D) indicate the POA visible in these low magnification views of the brain at the level of the BNST. n ≥ 2. Scale bars equal 250 μm.

Figure S6. Testosterone masculinizes aromatase expressing neural pathways and territorial behaviors

(A) Adult females treated neonatally with testosterone (NT) have significantly more aromatase expressing (βgal+) cells in the BNST and the MeA compared to NV controls. The number of cells in these regions is comparable between NT females and WT males. The volume occupied by PLAP stained fibers in the AH and surrounding the VMH is greater in NT compared to NV females and similar to that observed in WT males. PLAP labeling also revealed the cluster of cells in the caudal hypothalamus in NV but not NT females, indicating a masculinization of this region of the brain (not

shown). The horizontal dashed lines (red) represent the mean of the number of βgal+ cells or the volume occupied by PLAP+ fibers in these regions in WT males, and were determined from the data in Figures 2 and 3 in the main Text. **(B-D)** NT females display male pattern aggression (B, C) and urine marking (D). The majority of NT residents attack intruder males in most assays of resident-intruder aggression whereas none of the NV females display aggression (B). The frequency and duration of the attacks initiated by NT females is comparable to that observed in WT males (C). NT females deposit more urine spots than NV mice, with a greater proportion of these urine marks being deposited in the cage center (D). The horizontal dashed lines (red) represent the mean values of such behavioral displays by WT males observed in the data presented in Figure 6 in the main Text. Error bars represent SEM; n = 3 (A), n = 5 (B-D); * $p < 0.04$, ** $p < 0.03$, *** $p < 0.008$; these p values were obtained for the comparisons between NT and NV females.

Figure S1

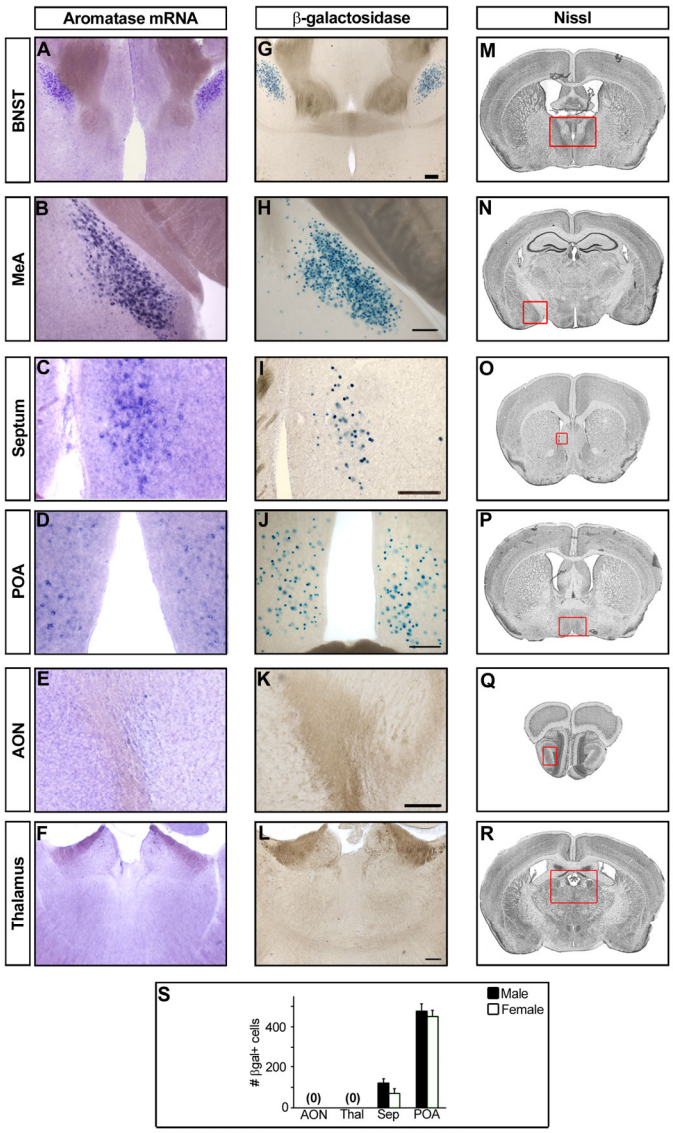

Aromatase mRNA
β-galactosidase
Nissl
BNST
MeA
Septum
POA
AON
Thalamus
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
Male
Female
βgal+ cells
400
200
0
(0)
(0)
AON
Thal
Sep
POA

Figure S2

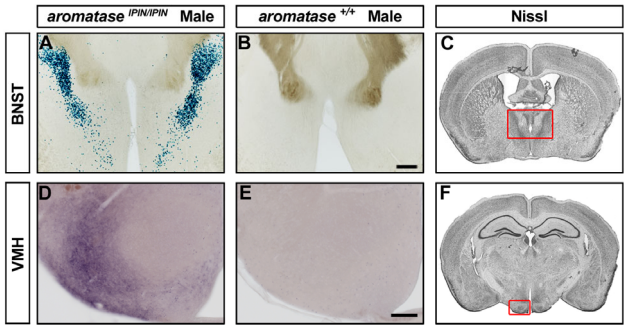

aromatase IPIN/IPIN Male
aromatase +/+ Male
Nissl
BNST
VMH
A
B
C
D
E
F

Figure S3

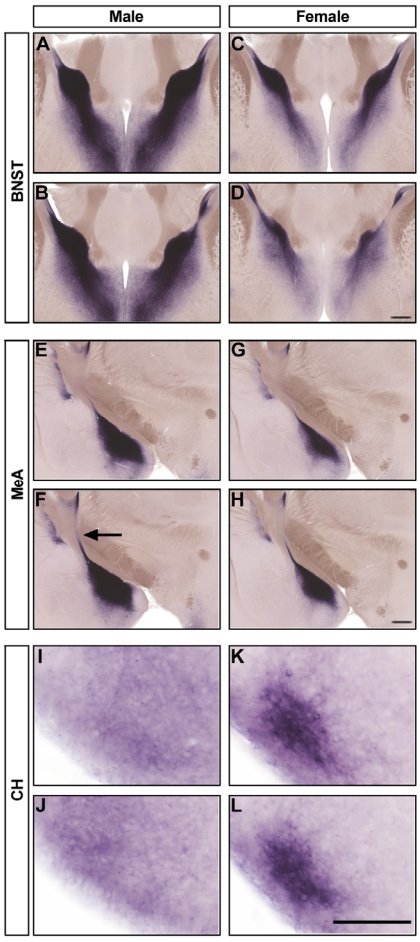


Figure S4

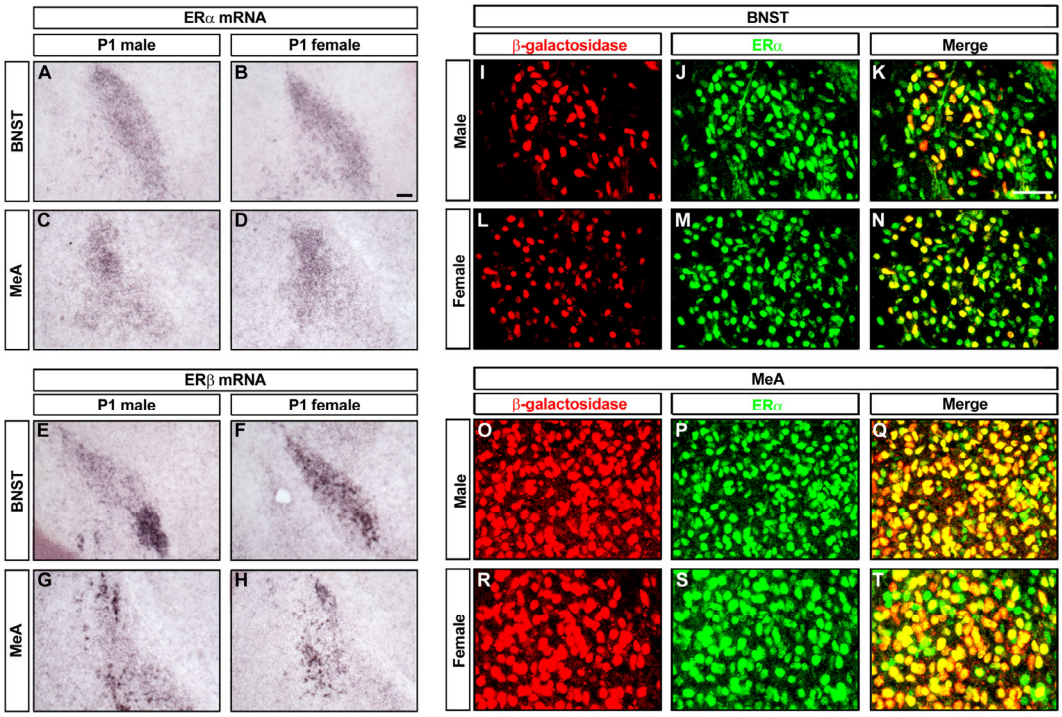

ERα mRNA
P1 male
P1 female
BNST
MeA
A
B
C
D
BNST
β-galactosidase
ERα
Merge
Male
Female
I
J
K
L
M
N
ERβ mRNA
P1 male
P1 female
BNST
MeA
E
F
G
H
MeA
β-galactosidase
ERα
Merge
Male
Female
O
P
Q
R
S
T

Figure S5

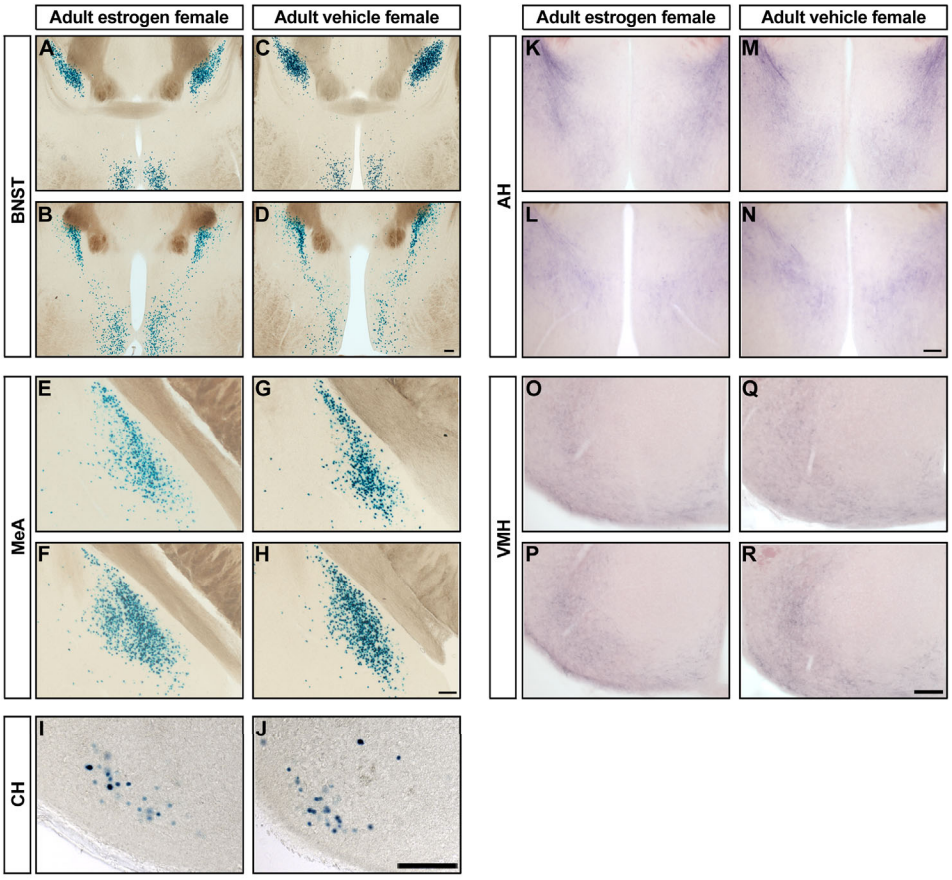
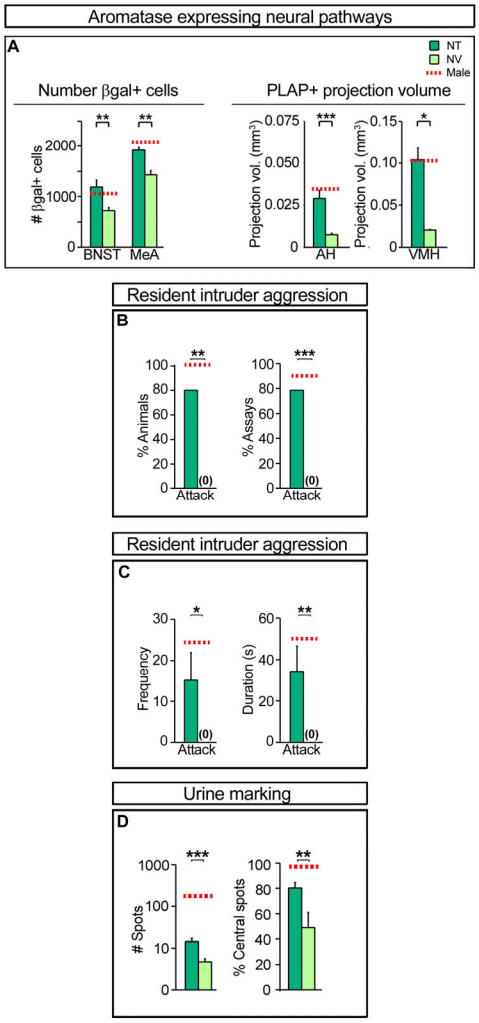

Adult estrogen female
Adult vehicle female
Adult estrogen female
Adult vehicle female
BNST
A
C
B
D
AH
K
M
L
N
MeA
E
G
F
H
VMH
O
Q
P
R
CH
I
J

Figure S6


Aromatase expressing neural pathways
A
NT
NV
Male
Number βgal+ cells
PLAP+ projection volume
βgal+ cells
2000
1000
0
BNST
MeA
Projection vol. (mm³)
0.075
0.050
0.025
0
AH
0.15
0.10
0.05
VMH
Resident intruder aggression
B
% Animals
% Assays
100
80
60
40
20
Attack
(0)
Resident intruder aggression
C
Frequency
30
10
Duration (s)
Urine marking
D
Spots
1000
100
10
% Central spots