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The effects of 17α-estradiol treatment on endocrine system revealed by single-nucleus transcriptomic sequencing of hypothalamus

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eLife Assessment

This study demonstrates the potential role of 17α-estradiol in modulating neuronal gene expression in the aged hypothalamus of male rats, identifying key pathways and neuron subtypes affected by the drug. While the findings are **useful** and provide a foundation for future research, the strength of supporting evidence is **incomplete** due to the lack of female comparison, a young male control group, unclear link to 17α-estradiol lifespan extension in rats, and insufficient analysis of glial cells and cellular stress in CRH neurons.

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Abstract

In this study, we investigated the role of 17α-estradiol in lifespan extension and its potential side effects from long-term administration. Pooled hypothalami from aged male Norway brown rats treated with 17α-estradiol (O.T), aged male controls (O), and young male controls (Y) were subjected to single-nucleus transcriptomic sequencing (snRNA-seq). To evaluate the effects of 17α-estradiol on aging neurons, supervised clustering of neurons based on neuropeptides and their receptors were used to evaluate the responses of each neuron subtype during aging and after 17α-estradiol treatment. The elevated cellular metabolism, stress and decreased expression levels of pathways involved in synapse formation in neurons initiated by aging were significantly attenuated by 17α-estradiol. Assessment of changes in neuron populations showed that neurons related to food intake, reproduction, blood pressure, stress response, and electrolyte balance were sensitive to 17α-estradiol treatment.

17α-estradiol treatment not only increased serum Oxytocin (Oxt), but also heightened the activity of hypothalamic-pituitary-gonadal (HPG) axis, as evidenced by significantly elevated levels of plasma Gnrh, total testosterone, and decreased estradiol. Elevated Gnrh1 was confirmed to be one of the causal effects mediating the role of 17α-estradiol in energy homeostasis, neural synapse, and stress response. Notably, *Crh* neurons exhibited prominent stressed phenotype among all the checked neuron subtypes in O.T, which may indicate a potential side effect of 17α-estradiol treatment. Therefore, the HPG axis and energy metabolism may be key targets of 17α-estradiol in male hypothalamus. Additionally, supervised clustering of neurons was shown to be a useful method for assessing treatment responses among different neuron subtypes in the hypothalamus.

Background

The hypothalamus serves as the central hub for controlling energy homeostasis, stress response, temperature, learning, feeding, sleep, social behavior, sexual behavior, hormone secretion, reproduction, osmoregulation, blood pressure, visceral activities, emotion, and circadian rhythms [1]. The hypothalamic energy-sensing system, particularly the circuits that regulate food intake, plays a crucial role in life span extension [2]. Elevated metabolic activity in the aged hypothalamus has been reported in aged hypothalamus, including increased mTor signaling [3, 4]. Additionally, decreases in gonadotropin-releasing hormone (GnRH), Ghrh, Trh, monoamine neurotransmitters, and blood supply are hallmarks of aging hypothalamus [5].

Previous studies have demonstrated that 17α-estradiol extends the lifespan of male mice and has beneficial effects on metabolism and inflammation, similar to those of rapamycin and acarbose [6–8]. Recent study indicated that 17α-estradiol also extends the lifespan of male rats [9].Further investigations revealed certain unique features of 17α-estradiol in life extension distinct to rapamycin and acarbose [10, 11]. Moreover, it has been shown that 17α-estradiol targets hypothalamic *POMC* neurons to reduce metabolism by decreasing feeding behavior through anorexigenic pathways [12]. Interestingly, the lifespan extension effect has only been observed in male animals [13]. The safety of 17α-estradiol is key for translation into clinical treatment, and the potential side effects on reproduction and feminization by 17α-estradiol treatment must be considered. However, contradictory results have been reported regarding its side effects on reproduction and feminization [6, 14, 15]. Therefore, further investigation and verification are needed to understand the underlying mechanisms of lifespan extension and the safety of 17α-estradiol.

In this report, we utilized single-nucleus transcriptomic sequencing and performed supervised clustering of neurons based on neuropeptides, hormones, and their receptors. Supervised clustering offers better resolution in cell cluster screening compared to traditional unsupervised clustering. We assessed the effects of 17α-estradiol on metabolism, stress responses, ferroptosis, senescence, inflammation, and pathways involved in synaptic activity in each neuron subtype, ranking the most sensitive neurons. The effects of 17α-estradiol on reversing aging-related cellular processes were evaluated by two opposing regulatory networks involved in hypermetabolism, stress, inflammation, and synaptic activity. Several key endocrine factors from serum were examined, and the potential side effects of 17α-estradiol on specific neurons were also evaluated.

Materials and methods

Animals, treatment and tissues

Twelve Norway brown male rats (12 months old) were acquired from Charles River, including 8 12-months old and 4 1-month old (Beijing). Aged rats were randomly allocated into control and 17α-estradiol-treated groups. Four Aged rats treated with 17α-estradiol (Catalog #: E834897, Macklin Biochemical, Shanghai, China) were fed freely with regular diet mixed with 17α-estradiol at a dose of 14.4 mg/kg (14.4 ppm), starting at 24 months of age for 6 months according to prior reports [16, 17]. The young rats were fed a regular diet without 17α-estradiol continuously for 3 months until 4 months old. All rats had ad libitum access to food and water throughout the experiments. The rats were then euthanized via CO_2, hypothalami, testes and blood serum were collected for subsequent experimental procedures. All blood samples were collected at 9:00-9:30 a.m to minimize hormone fluctuation between animals. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Nantong University.

Enzyme immunoassays

Enzyme immunoassays kits for rat Oxt (Catalog #: EIAR-OXT), Corticotropin Releasing Factor (Catalog #: EIAR-CRF), and gonadoliberin-1 (Catalog #: EIAR-GNRH) were obtained from Raybiotech (GA, USA). Enzyme immunoassay kits for rat serum total testosterone (Catalog #: ml002868), estradiol (Catalog #: ml002891), aldosterone (Catalog #: ml002876), and cortisol (Catalog #: ml002874) were obtained from Enzyme-linked Biotechnology (Shanghai, China). Sera from 3 animals per group were used and each was diluted 10 or 20 times for immunoassays.

Seminiferous tubule inflammation test

8 testes were obtained from each sample group and then subjected to fixation in 4% formalin for at least 1 week. Formalin-fixed paraffin-embedded mouse testis sections of 5 μm thickness were used for HE Staining. At least 30 seminiferous tubules in each slide were examined for inflammation test. Testis with at least 1 inflammatory seminiferous tubule was set as 1, and normal testis was set as 0 for inflammation index calculation.

snRNA-seq data processing, batch effect correction, and cell subset annotation

Intact hypothalami were cryopreserved in liquid nitrogen from sacrificed rats. Two (O) or three (Y and O.T) hypothalami were pooled within each group and homogenized in 500 μL ice-cold homogenization buffer (0.25 M sucrose, 5 mM $CaCl_2$, 3 mM $MgAc_2$, 10 mM Tris-HCl [pH 8.0], 1 mM DTT, 0.1 mM EDTA, 1× protease inhibitor, and 1 U/μL RiboLock RNase inhibitor) with Dounce homogenizer. Then, the homogenizer was washed with 700 μL ice-cold nuclei washing buffer (0.04% bovine serum albumin, 0.2 U/μL RiboLock RNase Inhibitor, 500 mM mannitol, 0.1 mM phenylmethanesulfonyl fluoride protease inhibitor in 1× phosphate buffer saline). Next, the homogenates were filtered through a 70-μm cell strainer to collect the nuclear fraction. The nuclear fraction was mixed with an equal volume of 50% iodixanol and added on top of a 30% and 33% iodixanol gradient. This solution was then centrifuged for 20 min at 10 000 ×g at 4 °C. After the myelin layer was removed from the top of the gradient, the nuclei were collected from the 30% and 33% iodixanol interface. The nuclei were resuspended in nuclear wash buffer and resuspension buffer and pelleted for 5 min at 500 ×g at 4 °C. The nuclei were filtered through a 40-μm cell strainer to remove cell debris and large clumps, and the nuclear concentration was manually assessed using trypan blue counterstaining and a hemocytometer. Finally, the nuclei were adjusted to 700–1200 nuclei/μL, and examined with a 10X Chromium platform.

Reverse transcription, cDNA amplification and library preparation were performed according to the protocol from 10X Genomics and Chromium Next GEM Single Cell 3' Reagent Kits v3.1. Library sequencing was performed on the Illumina HiSeq™ 4000 by Gene Denovo Biotechnology Co., Ltd (Guangzhou, China). 10X Genomics Cell Ranger software (version 3.1.0) was used to convert raw

BCL files to FASTQ files, and for alignment and counts quantification. Reads with low-quality barcodes and UMIs were filtered out and then mapped to the reference genome. Reads uniquely mapped to the transcriptome and intersecting an exon at least 50% were considered for UMI counting. Before quantification, the UMI sequences were corrected for sequencing errors, and valid barcodes were identified using the EmptyDrops method. The cell × gene matrices were produced via UMI counting and cell barcodes calling. Cells with an unusually high number of UMIs (≥8000) or mitochondrial gene percent (≥15%) were filtered out. Batch effect correction was performed by SCTransform function built in Seurat V4.4.0.

Pathways, gene signatures, TFs and TF cofactors, cell communication

Gene sets and pathways were derived from Hallmark gene sets of MSigDB collections, the KEGG pathway database, Reactome pathway database, and WikiPathways database, and some ontology terms derived from the Gene Ontology (GO) resource. Mitochondrial pathways were derived from MitoCarta3.0 [18]. Pathways, gene sets, and gene signatures were evaluated with the PercentageFeatureSet function built into R package Seurat. TFs and TF cofactors were obtained from AnimalTFDB 3.0 [19]. TFs and TF cofactors were further filtered with mean counts >0.1. The ligand–receptor pairs were calculated via R package CommPath [20].

Correlation analysis and ROC analysis

Pearson correlation coefficient was calculated with the linkET package ($p < 0.05$). Fast Wilcoxon rank sum test and auROC analysis was performed with the wilcoxauc function in R package presto. The minimal cell number in either one of the comparing pairs should be no less than 15. Ranks of area under the curve (AUC) values were in descending order. A total of 431 pathways from Hallmark, KEGG and PID databases were used for correlation analysis with MitoCarta OXPHOS subunits in neurons and non-neural cells (Figure 2—figure supplement 1). A total of 97 pathways related to synapse activity were derived from GO, including GO cellular components, GO biological processes and GO molecular functions (Table S1).

The division of expression level-dependent clusters in each pathway and their gene signatures

The quarters of the mixed cell populations from O, O.T, and Y hypothalamic neurons were equally divided using the R function fivenum from the R package stats, based on pathway expression levels. Thus, the total number of neurons was evenly divided into four clusters (c1-c4) in terms of cell number. The cell proportions from O.T, O, and Y neurons in each cluster were weighted against the total number of neurons in the three groups. The unique markers of each cluster were calculated using the FindAllMarkers function from the Seurat package. The intersection of the unique markers from the six pathways was obtained for heatmap plotting. Nineteen genes that were highly expressed in c1 were identified as c1.up.signature via the PercentageFeatureSet function in the Seurat package. Twelve genes that were highly expressed in c4 were identified as c4.up.signature. There were no intersecting unique markers in clusters c2 and c3 among the six selected pathways.

TF and pathway activities

The TF resources were derived from CollecTRI, the pathway resource was from PROGENy, and the enrichment scores of TFs and pathways were performed with the Univariate Linear Model (ulm) method according to the pipeline in R package decoupleR [21].

Subtypes of neurons generated by supervised clustering

Vast majority of these subtypes were clustered by neuropeptides, hormones, and their receptors within all the neurons with the subset function from R package Seurat (the target gene expression level > 0). A total of 121 neuron subtypes were obtained, comprising 80 neuropeptide-secreting neurons and 41 neurons expressing a unique neuropeptide receptor or hormone receptor. Further groupings may exist within the identified neuron subtypes, and the category of excitatory or inhibitory neurons was not discriminated further. The cell proportion of each neuron subtype was weighted according to the total number of neurons in O.T, O, and Y samples. The mean values ± standard deviation of pathways and gene signatures were performed for each subtype. The top 10 and the bottom 10 items were calculated, and the top 10 subtypes ranked within each of 16 pathways or gene signatures were collected for word clouds annotation via R package wordcloud2.

Differential expression and pathway enrichment analysis

DEGs between groups were identified via FindMarkers (test.use = bimod, min.pct = 0.1, logfc.Threshold = 0.25, avg_diff > 0.1 or < −0.1). DEGs were then enriched in redundant GO terms via WebGestalt and filtered with false discovery rate < 0.05 [22].

Bidirectional Mendelian randomization (MR) study

The protein quantitative trait locus (pQTL) GWAS summary data of 204 human endocrine-related GWAS summary data with European ancestry were obtained from open-access MRC Integrative Epidemiology Unit (IEU) (Table S4) [23, 24]. Independent genome-wide significant SNPs for exposure OXT (id:prot-a-2159) or GNRH1 (id: prot-a-1233) were used as instrumental variables with genome-wide significance ($P < 1 \times 10^{-5}$), independence inheritance (r2 < 0.001) without linkage disequilibrium (LD) with each other for MR. For the reverse MR, independent genome-wide significant SNPs from 203 endocrine-related GWAS summary data ($P < 1 \times 10^{-5}$, r2 < 0.001) without LD with each other were obtained as exposures and human GWAS summary data of OXT (id:prot-a-2159) or GNRH1 (id:prot-a-1233) were used as outcomes. Weak instruments less than 10 were discarded via F-statistics.

MR and reverse MR analysis were conducted with method inverse-variance weighting (IVW), MR Egger, Weighted median, Simple mode, and Weighted mode. The screening criteria: all of the odds ratio (OR) values of the 5 methods should be simultaneously either >1 or <1 and the significant p value of IVW was <0.05. The heterogeneity via IVW method and the horizontal pleiotropy were also evaluated with R package TwoSampleMR [25].

Results

The overall changes in aged hypothalamus with or without long-term 17α-estradiol treatment via snRNA-seq profiling

To investigate the hypothalamus as a potential key target of 17α-estradiol's effects on life extension, we performed snRNA-seq on the entire hypothalamus of aged and 17α-estradiol-treated aged Norway brown rats, using the hypothalamus from young adult male rats as a control. We identified 10 major cell types based on specific cell markers of the hypothalamus (**Figure 1A-B**). Notably, the proportions of all non-neural cells changed in O versus Y (**Figure 1C**). For instance, Oligo, OPC, and Micro were found to be increased, while Astro, Tany, Fibro, PTC, and Endo were decreased in O compared to Y. The proportions of Oligo, OPC, and Micro were also increased in 17α-estradiol-treated aged group (O.T) compared to those in Y. Furthermore, Endo was increased

in O.T compared to both Y and O. The proportions of Astro, Tany, Epen, and PTC decreased more in O.T than those in O when compared to Y. These results indicated that 17α-estradiol treatment had extensive effects on the proportions of non-neural cells in hypothalamus.

Cell communication analysis revealed significant changes in the ligand-receptor pairs between neurons and other cell types, particularly in Endo, Fibro, Tany, and Astro (**Figure 1D**). Significant ligand–receptor interactions among neurons also changed in O.T and O groups, especially in O (**Figure 1E**). Notably, among the significant ligand–receptor pairs in neurons, *Bmp2–Acvr1/Acvr2a/Acvr2b/Bmpr*, *Gdf11–Acvr2a/Acvr2b*, *Inhba–Acvr1/Acvr2a/Acvr2b*, *Nrg1/Nrg2/Nrg4–Erbb4*, *Rspo1–Lgr5/Lrp6*, and *Rspo3–Lgr5* were exclusively and significantly increased in neurons of the O group compared to those in O.T and Y, suggesting enhanced TGF superfamily-mediated signaling activity and canonical Wnt signaling during aging. The significantly changed ligand–receptor pairs *Nlgn1–Nrxn1/Nrxn2*, *Nlgn2–Nrxn1/Nrxn2/Nrxn3*, *Nlgn3–Nrxn1/Nrxn2/Nrxn3*, *Nxph1–Nrxn1/Nrxn2/Nrxn3*, *Nxph3–Nrxn1/Nrxn2/Nrxn3*, *Pomc–Oprd1/Oprk1/Oprm1*, and *Vip–Adcyap1r1/Avpr1a/Vipr2* were exclusively increased in neurons of O.T compared to O and Y (**Figure 1E**). These ligand–receptor pairs were associated with synaptic activity, cellular adhesion, the opioid system, and vasodilation, indicating unique roles of 17α-estradiol in restoring certain physiological functions in the aging hypothalamus. The increased *Pomc* signal in O.T neurons aligns with previous reports suggesting that 17α-estradiol treatment decreases food uptake in mice, potentially correlated with *Pomc* neurons (**Figure 1E**) [12].

Gene set enrichment analysis (GSEA) based on DEGs also corroborated the expression profiles related to stress responses and synapse-associated cellular processes in neurons (**Figure 1F**). ROC analysis of significantly differently expressed pathways related to neural synapses, manually selected from Gene Ontology databases, indicated that most top-ranked pathways related to synapses, according to AUC values, were downregulated in aged neurons, while 17α-estradiol treatment reversed this trend (**Figure 1G**, Table S1).

Overall, these findings suggest that 17α-estradiol broadly reshapes cell populations, cellular communication, neuropeptide secretion, and synapse-related cellular processes in the aging hypothalamus, distinguishing it from both the young hypothalamus and the untreated aged hypothalamus.

The two opposing signaling networks in regulating metabolism and synapse activity, which can be balanced effectively by 17α-estradiol

To monitor the metabolism and neural status affected by 17α-estradiol, we utilized the energy metabolism pathway MitoCarta OXPHOS subunits to calculate the positively or negatively correlated pathways in hypothalamic neurons (Figure 2—figure supplement 1). Our findings revealed that energy metabolism and synapse activity represent two opposing regulatory signaling networks in hypothalamic neurons, with 17α-estradiol strongly playing a significant role in balancing these networks (**Figure 2A**). At the core of these opposing signaling pathways are two categories of contrasting TFs (**Figure 2B**). For example, *Calr*, *Clu*, *Peg3*, *Prnp*, *Ndufa13*, *Actb*, *Ywhab*, *Nfe2l1*, *Mtdh*, *Npm1*, *Bex2*, *Aft4*, and *Maged1* were positively correlated with pathways involved in OXPHOS subunits, lysosome function, protein export, mTorc1 signaling, and the unfolded protein response (UPR) in O, O.T and Y neurons, while showing negative correlations with pathways related to ubiquitin-mediated proteolysis, endocytosis, tight junctions, focal adhesion, axon guidance, and MAPK signaling. Additionally, TFs *Myt1l*, *Ctnnd2*, *Tenm4*, *Camta1*, *Med12l*, *Rere*, *Csrnp3*, *Erbb4*, *Jazf1*, *Dscam*, *Klf12*, and *Kdm4c* exhibited opposite correlation patterns with these selected pathways in O, O.T and Y neurons. These TFs may take conserved roles in regulating the two opposing biological processes in hypothalamic neurons.

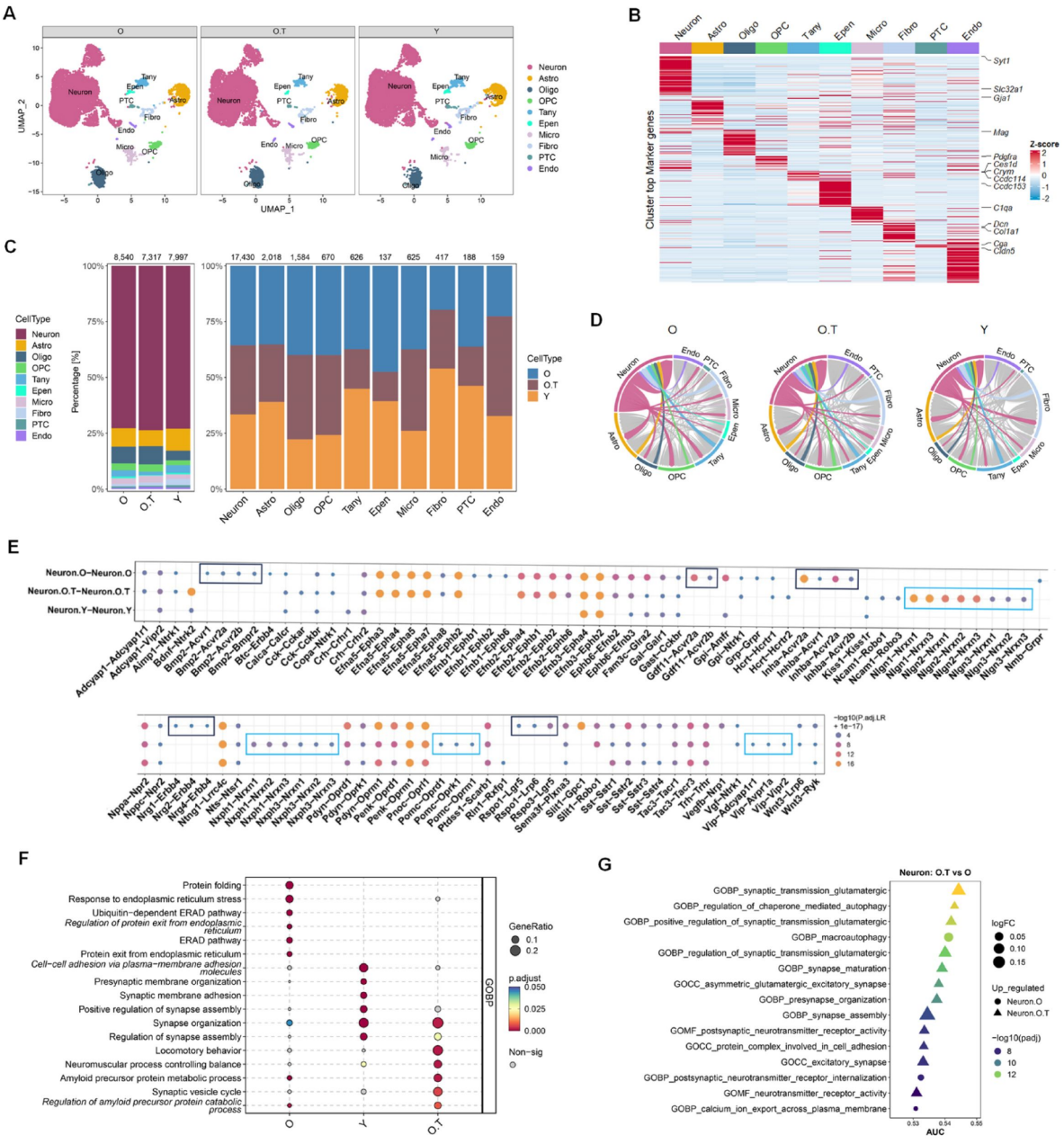


Figure 1.

snRNA-seq profiling of the hypothalamus from O, O.T and Y samples.

(A) UMAP visualization of nuclei colored by 10 cell types: neuron (Neu), astrocyte (Astro), oligodendrocyte (Oligo), oligodendrocyte precursor cell (OPC), tanycyte (Tany), ependymocyte (Epen), microglia (Micro), fibroblast (Fibro), pars tuberalis cell (PTC), and endothelial cell (Endo), from hypothalamus of aged rats (O), 17α-estradiol-treated aged rats (O.T) and young rats (Y). **(B)** Heatmap showing the classic markers of 10 major cell types in hypothalamus. **(C)** Cell-type compositions by groups (left panel) or by major cell types with the total cell numbers shown above each column. **(D)** Circos plot depicting the number of ligand–receptor pairs between Neu and other cell types (color strips) for each group. **(E)** Dot plot showing significant ligand–receptor interactions between Neurons for each group. Boxes showing the unique ligand–receptor interactions between Neuron.O (black boxes) or between Neuron.O.T (blue boxes). **(F)** Dot plot of the top 6 enriched GO biological process terms across three groups of neurons via GSEA analysis. **(G)** The top 15 changed pathways/gene sets according to the ranks of AUC values in selected pathways related to neuronal synapses and axons from Gene Ontology (GO) biological process, GO molecular function and GO cellular component.

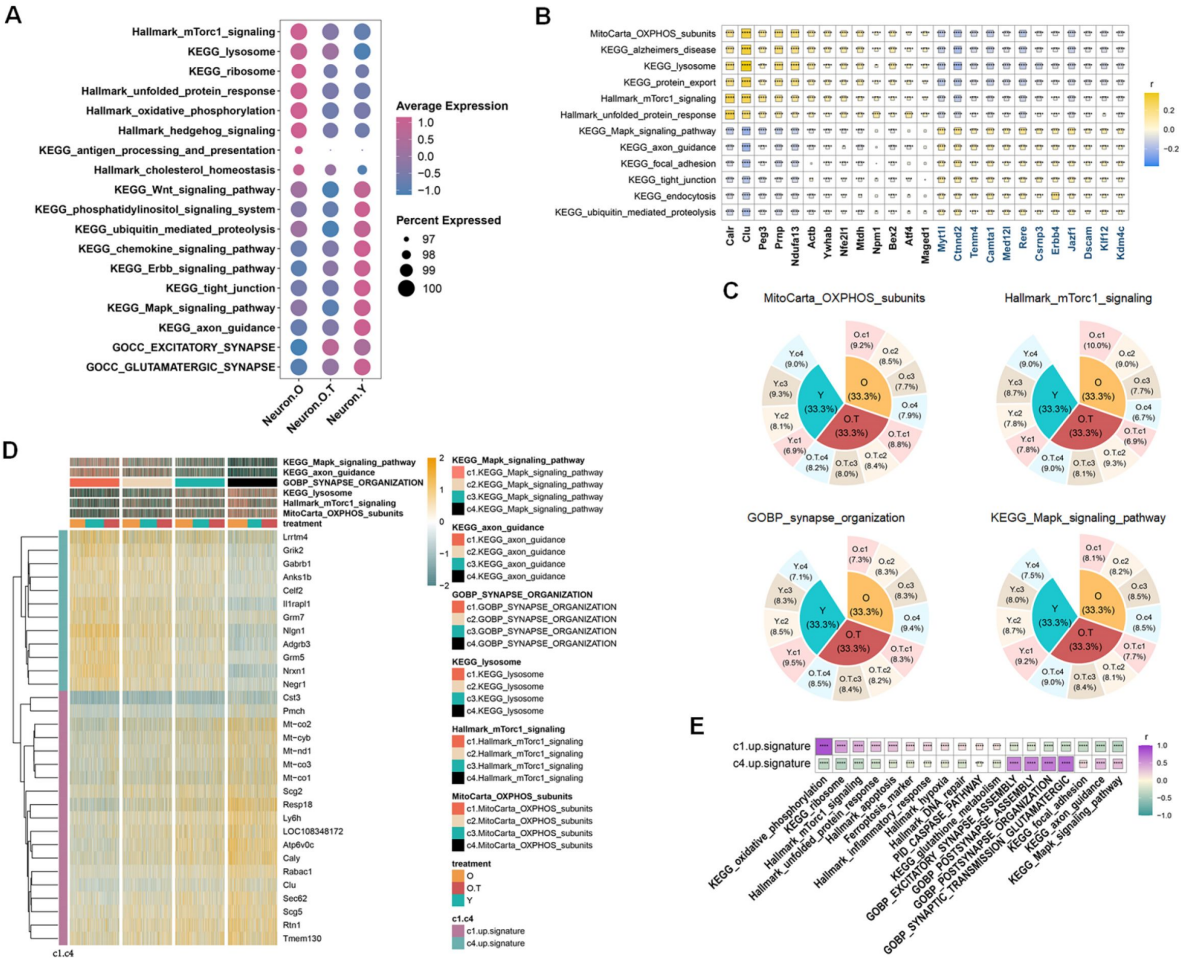


Figure 2.

Two opposing regulatory signaling networks in neuron metabolism.

(A) Dot plot of the selected pathways representing the prominent changes of overall expression levels across Neuron.O, Neuron.O.T and Neuron.Y in metabolism, signaling and synaptic activity. **(B)** Correlation heatmap showing transcription factors (TFs) that correlated with the two opposing regulatory signaling networks in the mixed neurons of O, O.T and Y. **(C)** The shared unique markers of each quarter (c1-c4) in 6 pathways in hypothalamic neurons (O, O.T, and Y). The markers were then collected as c1.up.signature (19 genes) and c4.up.signature (12 genes). **(D)** The aging-related cell proportions of each quarter shown by 4 pathways. **(E)** The correlation of c1.up.signature and c2.up.signature with the two opposing regulatory signaling networks.

We then attempted to establish gene signatures to represent these two opposing signaling networks, thereby displaying the cell status of aging and evaluating the effects exerted by 17α-estradiol. To achieve this, we evenly divided the expression levels of each of the six selected pathways from the two opposing signaling networks into four quarters (c1-c4) among the mixed neurons from O, O.T, and Y, calculating the shared unique markers in each quarter (**Figure 2C, D**). From the distribution patterns, we observed that the proportion of neurons in O decreased from c1 to c4 in metabolic pathways (MitoCarta OXPHOS subunits and Hallmark mTorc1 signaling), while this trend was reversed in the opposing signaling pathways (GOBP synapse organization and KEGG MAPK signaling pathway) (**Figure 2C**). In contrast, in Y, this trend was opposite, suggesting the expression levels from the 4 quarters (c1-c4) of the two opposing signaling networks can be used to monitor aging status. Treatment with 17α-estradiol alleviated this trend or even reversed it in O.

We then screened the shared unique markers of each quarter from the six selected pathways in an attempt to establish the gene signatures representing the two opposing signaling networks. Unique markers in c1 (19 genes, c1.up.signature) and c4 (12 genes, c4.up.signature) were identified; however, c2 and c3 lacked unique markers shared by the 6 pathways (**Figure 2D**). Consequently, the 19 genes in c1.up.signature displayed an inversed correlation pattern with the 12 genes in c4.up.signature, indicating the two opposing gene signatures are capable of reflecting the two opposing signaling networks in hypothalamic neurons (**Figure 2E**). Conversely, the balance of the two opposing signaling networks affected by 17α-estradiol in non-neural cell types was less pronounced than in neurons, showing variable effects on non-neural cells (Figure 2—figure supplement 2). GOBP pathway enrichment analysis revealed that Micro exhibited lower levels of synapse-related cellular processes in O.T compared to O, which was distinct from the observations in neurons (Figure 2—figure supplement 3). Therefore, in this report, we primarily focused on hypothalamic neurons and their responses to aging and 17α-estradiol.

Supervised clustering revealed distinct responses of different subtypes of hypothalamic neurons to aging and 17α-estradiol

The hypothalamus contains numerous neuron subtypes that release various neuropeptides and hormones to regulate fundamental body functions. To differentiate the changes occurring during aging and the effect of 17α-estradiol on each neuron subtype, we performed supervised clustering based on neuropeptides, hormones, or their receptors (**Figure 3A**). Most of the top 10 neuron subtypes involved in UPR, ferroptosis, mTorc1 signaling, insulin signaling pathway, immune response pathways, OXPHOS subunits, and senescence signature during aging were attenuated by 17α-estradiol treatment (**Figure 3B**, Figure 3—figure supplement 1). For example, Serpine1-secreting neurons exhibited very high expression levels of UPR, ferroptosis signature, mTorc1 signaling, Tnfα signaling via NF-kB, and OXPHOS subunits during aging, all of which were attenuated by 17α-estradiol.

To further identify the most sensitive neurons in response to aging and 17α-estradiol, we calculated the frequency of each neuron subtype by combining the top 10 neurons across each of the 16 pathways and gene signatures (**Figure 3C**, Table S2). Serpine1-secreting neurons were exclusively enriched in aged hypothalamus, suggesting they are effective targets of 17α-estradiol.

Additionally, *Mlnr*, *Nmb*, *Pomc*, *Ednra*, *Serpine3*, *Gast*, and *Pcsk6* neurons were all affected by 17α-estradiol (**Figure 3D**). *Galp*, *Glp1r*, *Serping1*, *Sstr2*, *Sstr3*, and *Vip* neurons were enriched in O, O.T and Y hypothalamus, indicating a lack of effect of 17α-estradiol on these neuron types. The unique neurons in O.T (*Crh*, *Serpinb9*, *Cckbr*, *Pth2r*, *Kiss1*, *Prpr*, *Hcrtr1*, *Npb*, *Nppa*, *Nxph3*, and *Npff*) may be a consequence of compensatory effects from 17α-estradiol treatment.

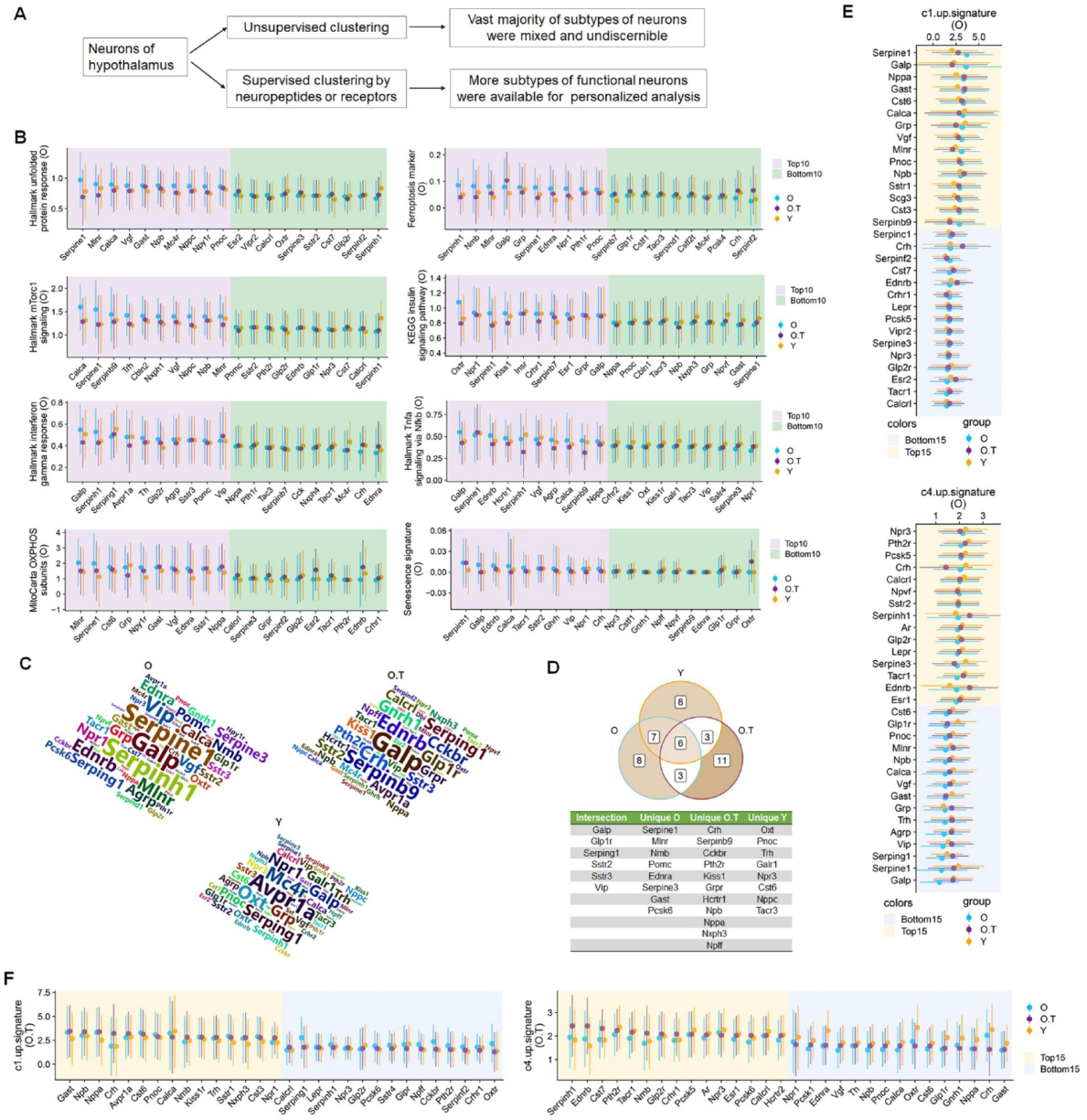


Figure 3.

Screening of neuron subtypes by supervised clustering that responded distinctly to aging and 17α-estradiol treatment.

(A) Diagram outlining the features of supervised clustering of neurons in hypothalamus compared with the traditional unsupervised clustering. **(B)** The top 10 and bottom 10 neurons of 121 neurons according to the mean expression values of eight signaling pathways from stress, apoptosis, metabolism, immune response, and senescence in Neuron.O. The expression levels of these neuron subtypes from Neuron.O.T and Neuron.Y were also indicated. **(C)** Word clouds displaying the frequency of neurons that were among the top 10 in each of the 16 signaling pathways as shown in Table S2. The 16 pathways were from oxidative stress, apoptosis, metabolism, immune response, and senescence. **(D)** Venn diagram showing the number of neuron subtypes exclusively in each group or shared by the groups in (C). The neurons with frequency >3 were selected for calculation in (C). **(E)** The top 15 and bottom 15 neurons among 121 neurons according to the mean expression levels of c1.up.signature and c4.up.signature in Neuron.O. **(F)** The top 15 and bottom 15 neurons among 121 neurons according to the mean expression levels of c1.up.signature and c4.up.signature in Neuron.O.T.

We then evaluated the most and least sensitive neurons to aging by ranking the neuron subtypes according to the levels of c1.up.signature and c4.up.signature (**Figure 3E**). *Serpine1*, *Galp*, *Calca* neurons were among the most affected subtypes in the aged hypothalamus, consistent with the combined ranks of several pathways (Figure 3—figure supplement 1). *Npr3*, *Crh*, *Ar*, *Esr1*, and *Esr2* neurons were among the least affected subtypes in the aged hypothalamus (**Figure 3E**). In O.T, neuron subtypes ranked by c1.up.signature and c4.up.signature indicated that *Gast*, *Npb*, *Nppa*, and *Crh* were affected most by 17α-estradiol treatment (**Figure 3F**). Neurons *Oxtr*, *Glp1r*, *Gnrh1*, and *Crh* also showed the lowest levels of c4.up.signature, indicative of an aging phenotype. Meanwhile, neurons *Glp2r*, *Ar* and *Esr1* ranked among the top neurons based on c4.up.signature, suggesting higher expression levels of synapse-related processes and relatively less stress status in these types of neurons in O.T.

These results indicate that supervised clustering of each neuron subtype of neurons facilitated the visualization of the different responses from distinct subtypes of neurons in response to aging and medical treatments. Furthermore, it strongly suggests that *Crh*, *Oxtr*, *Glp1r*, *Gnrh1*, *Glp2r*, *Ar*, and *Esr1* neuron subtypes are particularly sensitive to long-term 17α-estradiol treatment, which is associated with appetite regulation, glucose metabolism, stress response, and, as expected, sex hormone secretion and signaling.

The potential side effects on hypothalamic–pituitary–adrenal (HPA) axis by long-term 17α-estradiol treatment in the males

To further investigate the potential side effects or compensatory effects of 17α-estradiol treatment, we performed stricter screening by intersecting the top 10 ranks in the UPR, OXPHOS subunits, and ferroptosis signature (**Figure 4A**). *Crh* and *Nppa* were the only two intersected neuron subtypes identified through this strict screening. The treatment with 17α-estradiol elevated several key metabolic pathways in *Crh* neurons compared to those in Y and O (**Figure 4B**). Additionally, 17α-estradiol treatment increased the c1-up-signature while simultaneously reducing many pathways associated with synapse activity and the c4-up-signature in *Crh* neurons of O.T, indicating a potent stressed phenotype in *Crh* neuron. In contrast, in *Nppa* and *Nppc* neurons, the decreased c1-up-signature in O.T implied a lesser extent of stressed phenotype in these neurons compared to *Crh* neurons. The aberrant changes in *Crh* neurons were also evidenced by the increased number of DEGs related to mitochondria-expressed genes in O.T, along with a reduced number of DEGs in the adherens junction pathway (**Figure 4C**). The status of Crh neurons in O.T may be associated with elevated TF activities of *Esr2*, *Usf2*, *Hdac5*, *Creb3l1*, *Tfam*, *Preb*, *Pou3f2*, and *Hoxb5* (**Figure 4D**).

Notably, the HPA axis was altered by 17α-estradiol treatment, as evidenced by the elevated cortisol levels in O.T compared to O ($p = 0.078$) (**Figure 4E**). The correlation between elevated cortisol production and the heightened stress in *Crh* neurons by 17α-estradiol treatment needs further investigation. Additionally, as a crucial component of the renin-angiotensin-aldosterone system, the significantly increased serum aldosterone in O.T and its potential role in sodium reabsorption and cardiovascular health also warrant more detailed investigation (**Figure 4E**). In summary, 17α-estradiol treatment altered the activity of HPA axis in male BN rats and introduced increased potential side effects in *Crh* neurons.

17α-estradiol increased Oxt neuron proportion and secretion and its possible role in mediating the effect of 17α-estradiol on endocrine system

Aging and 17α-estradiol treatment also altered the proportions of various neuron subtypes among O, O.T, and Y (**Figure 5A, B**, Table S3). The proportions of *Grp*, *Pmch*, *Npb*, *Serpinb9*, *Sstr2*, *Agrp*, *Sstr3*, *Mlnr*, and *Hcrt* neurons ranked in the top 10 in O, while *Oxt*, *Vip*, *Avp*, *Calca*, *Glp2r*, *Tacr1*, *Trh*, *Serping1*, *Npff*, and *Npy1r* were in the top 10 in O.T. *Galp*, *Calcrl*, *Ednra*, *Oxt*, *Serpinh1*, *Pomc*,

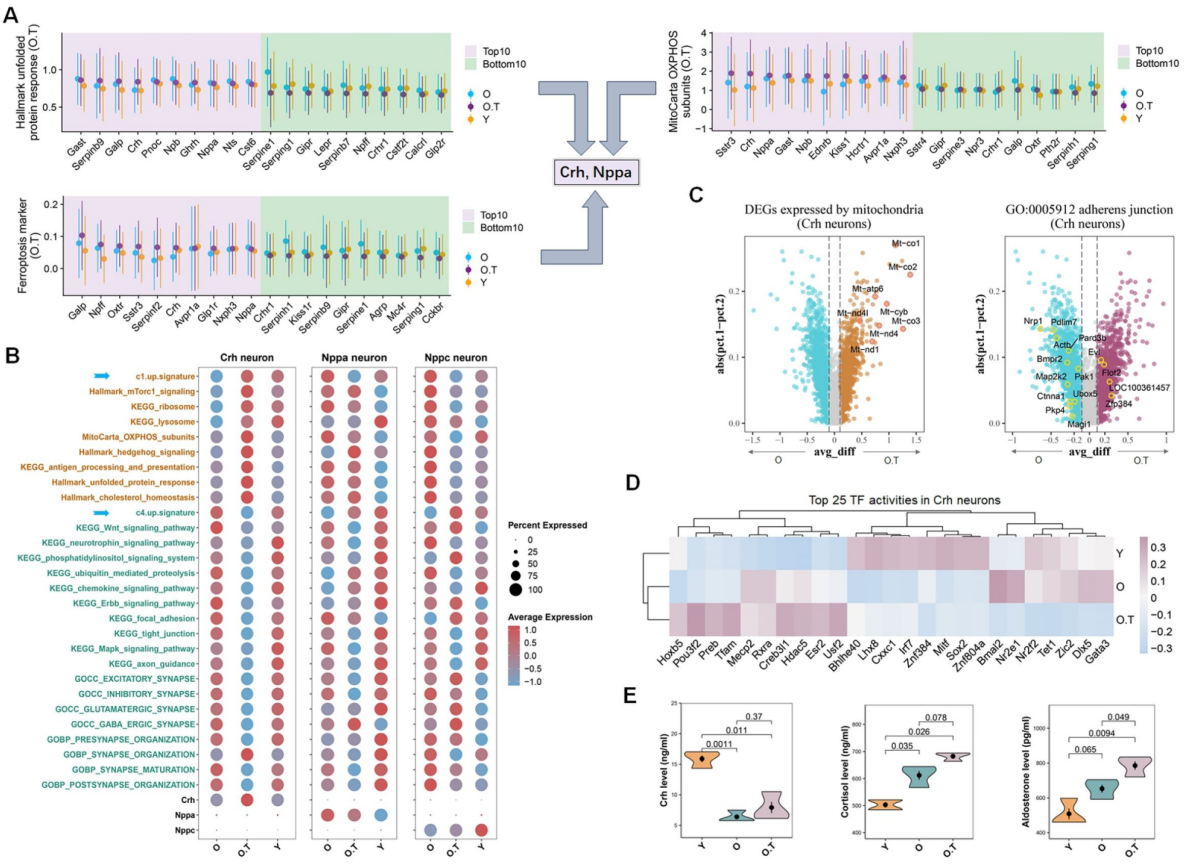


Figure 4.

The responses of Crh neurons to long-term 17α-estradiol treatment.

(A) The two intersected neurons among the top 10 neurons according to the mean expression levels of the three senescence-related pathways. **(B)** The expression profiles of selected pathways from the two opposing signaling networks in Neuron *Crh*, *Nppa* and *Nppc*. Neuron *Crh* and *Nppa* were the only two types of neurons shared by the three top 10 neurons in (A). **(C)** The downregulated and upregulated DEGs expressed by mitochondria or in pathway adherens junction between O.T and O in Crh neurons. **(D)** The top 25 TF activities in *Crh* and *Gnrh1* neurons. **(E)** Enzyme immunoassay of the serum levels of Crh, cortisol, and aldosterone in Y, O and O.T. Two-tail unpaired T-test was performed. p values were labeled.

Cck, *Crh*, *Tacr1*, and *Kiss1* neurons were among the bottom 10 in O, whereas *Oxtr*, *Galp*, *Agrp*, *Serpinb9*, *Npvf*, *Serpinh1*, *Ednrb*, *Agt*, *Gipr*, and *Pomc* neurons ranked among the bottom 10 in O.T. *Agrp*, *Pomc*, *Oxt*, *Oxtr*, *Gipr*, and *Glp2r* neurons are well-known for their roles in regulating food intake and energy homeostasis. *Agrp* neurons are activated by hunger, while *Pomc* neurons are activated by satiety in the ARC of the hypothalamus. 17α-estradiol treatment effectively elevated the expression levels of the c4.up.signature and synapse-associated processes in neuron subtypes *Agrp*, *Pomc*, *Oxt* and *Glp2r* in O.T compared to O. This may mitigate the adverse effects of reduced cell populations in Pomc and *Agrp* neurons in aging hypothalamus (**Figure 5C**). This finding indicates a potential role of 17α-estradiol in appetite control, as previously reported [12]. Additionally, 17α-estradiol treatment resulted in elevated proportions of *Vip*, *Avp*, *Npff*, *Calca*, and *Tacr1* neurons, which ranked in the top 10, while the proportions of Agt neurons decreased. These are all associated with blood pressure regulation (Figure 5—figure supplement 1). Notably, the proportions of *Oxt* and *Glp2r* neurons, both of which have anorexigenic effects [26, 27], increased in O.T. In addition to the increased number of *Oxt*-positive neurons, the expression level of *Oxt* also rose in O.T. The elevated expression of synapse-related pathways was supported by the increased DEGs in the enriched synaptic membrane pathway in *Oxt* neurons (**Figure 5D**).

More importantly, the serum level of Oxt was significantly elevated in O.T compared to O ($p=0.04$), although it remained lower than that in Y (**Figure 5E**). Notably, the top TF activities in O.T and O differed markedly from those in Y (**Figure 5F**). The elevated levels of *Hopx* and *Xbp1* may be associated with the response to 17α-estradiol treatment.

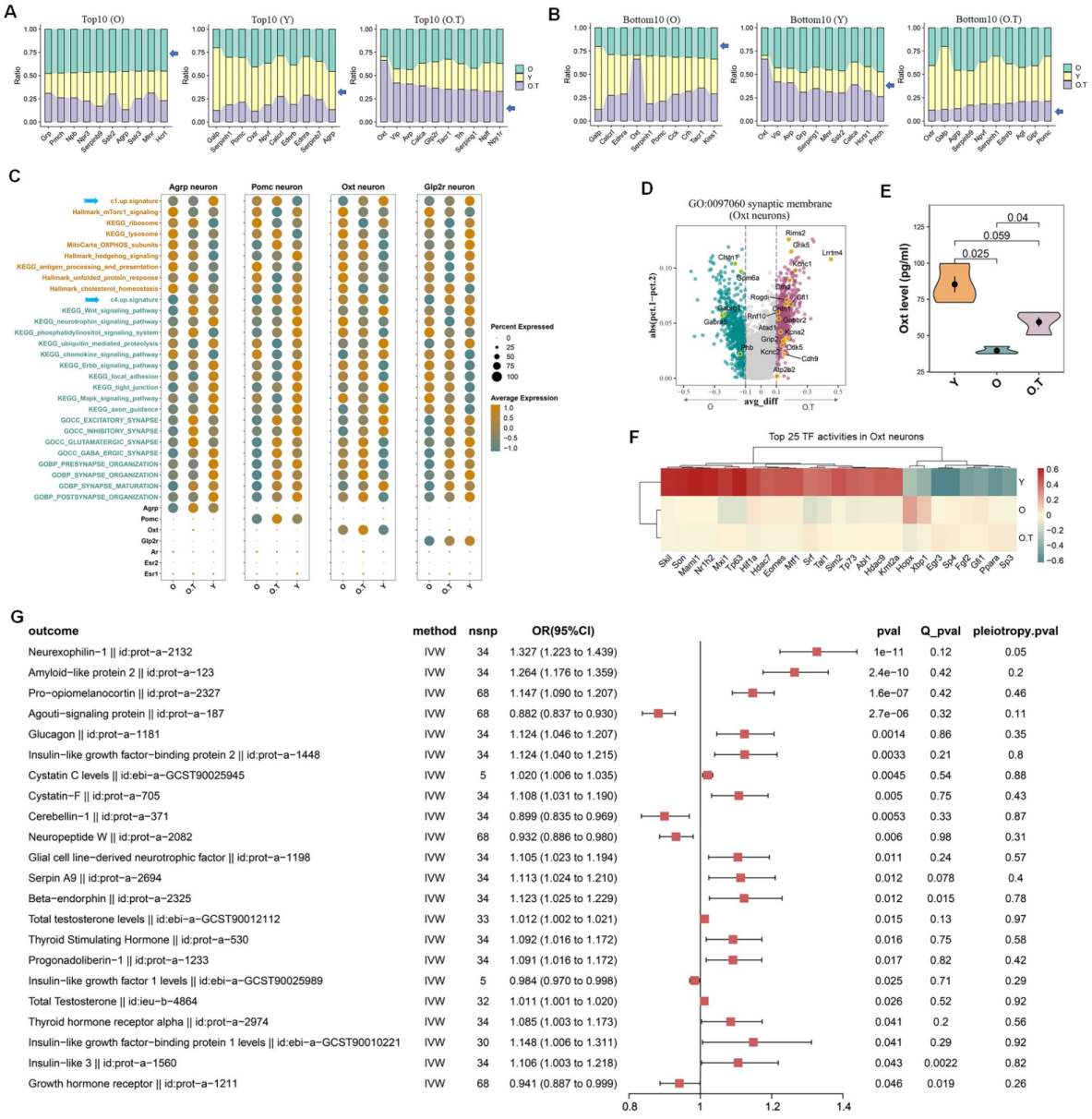
Due to the intricate regulatory networks among various endocrine factors, elucidating the causal effect of Oxt on other endocrine factors is quite complex using traditional methods. MR analysis, employing variant SNPs as genetic tools, is advantageous for such task. We performed a bidirectional MR analysis of the GWAS summary data of human plasma OXT and 203 endocrine-related and hypothalamus-related factors, most of which are protein quantitative trait loci (pQTL) data from the IEU (Table S4). As an exposure, OXT revealed a significant causal effect on POMC/beta-endorphin (id:prot-a-2327, id:prot-a-2325), glucagon (id:prot-a-1181), GNRH1/Progonadoliberin-1 (id:prot-a-1233), and total testosterone levels (id:ebi−a−GCST90012112, id:ieu−b−4864) (**Figure 5G**). NPW and CBLN1 were found to be negatively associated with OXT, but the significance of these associations was not found in the reverse MR analysis (Figure 5—figure supplement 2A, B).

In contrast, we could not identify significant associations between OXT and estradiol levels (id:ebi-a-GCST90012105, id:ebi-a-GCST90020092, id:ebi-a-GCST90020091, id:ieu-b-4872, id:ieu-b-4873, id:ukb-e-30800_AFR, id:ukb-e-30800_CSA). Interestingly, QRFP, IGF1, AGRP, TAC4, GRP, CLU, BNF, PCSK7, PACAP, ANP, TAC3, CRH, INSL6, and PRL displayed significant associations with OXT in both MR and reverse MR analysis, indicative of their complex causal effects (Figure 5—figure supplement 2A, B).

The results suggest that elevated Oxt levels induced by 17α-estradiol may have positive associations with endocrine factors governing feeding behavior, glucose metabolism, male reproduction, and sex hormones. Therefore, OXT may serve as a potential mediator of 17α-estradiol.

17α-estradiol activated HPG axis and the elevated Gnrh also took important roles in mediating the effect of 17α-estradiol on other endocrine factors

Since Gnrh and sex hormone expressing neuron subtypes were sensitive to 17α-estradiol treatment (**Figure 3F**, Figure 3—figure supplement 1), we examined their expression profiles alongside representative pathways of two opposing signaling networks related to metabolism and synapses, including c1-up-signature and c4-up-signature (**Figure 6A**). However, neither the c1-



outcome	method	nsnp	OR(95%CI)	pval	Q_pval	pleiotropy.pval
Neurexophilin−1 || id:prot−a−2132	IVW	34	1.327 (1.223 to 1.439)	1e−11	0.12	0.05
Amyloid−like protein 2 || id:prot−a−123	IVW	34	1.264 (1.176 to 1.359)	2.4e−10	0.42	0.2
Pro−opiomelanocortin || id:prot−a−2327	IVW	68	1.147 (1.090 to 1.207)	1.6e−07	0.42	0.46
Agouti−signaling protein || id:prot−a−187	IVW	68	0.882 (0.837 to 0.930)	2.7e−06	0.32	0.11
Glucagon || id:prot−a−1181	IVW	34	1.124 (1.046 to 1.207)	0.0014	0.86	0.35
Insulin−like growth factor−binding protein 2 || id:prot−a−1448	IVW	34	1.124 (1.040 to 1.215)	0.0033	0.21	0.8
Cystatin C levels || id:ebi−a−GCST90025945	IVW	5	1.020 (1.006 to 1.035)	0.0045	0.54	0.88
Cystatin−F || id:prot−a−705	IVW	34	1.108 (1.031 to 1.190)	0.005	0.75	0.43
Cerebellin−1 || id:prot−a−371	IVW	34	0.899 (0.835 to 0.969)	0.0053	0.33	0.87
Neuropeptide W || id:prot−a−2082	IVW	68	0.932 (0.886 to 0.980)	0.006	0.98	0.31
Glial cell line−derived neurotrophic factor || id:prot−a−1198	IVW	34	1.105 (1.023 to 1.194)	0.011	0.24	0.57
Serpin A9 || id:prot−a−2694	IVW	34	1.113 (1.024 to 1.210)	0.012	0.078	0.4
Beta−endorphin || id:prot−a−2325	IVW	34	1.123 (1.025 to 1.229)	0.012	0.015	0.78
Total testosterone levels || id:ebi−a−GCST90012112	IVW	33	1.012 (1.002 to 1.021)	0.015	0.13	0.97
Thyroid Stimulating Hormone || id:prot−a−530	IVW	34	1.092 (1.016 to 1.172)	0.016	0.75	0.58
Progonadoliberin−1 || id:prot−a−1233	IVW	34	1.091 (1.016 to 1.172)	0.017	0.82	0.42
Insulin−like growth factor 1 levels || id:ebi−a−GCST90025989	IVW	5	0.984 (0.970 to 0.998)	0.025	0.71	0.29
Total Testosterone || id:ieu−b−4864	IVW	32	1.011 (1.001 to 1.020)	0.026	0.52	0.92
Thyroid hormone receptor alpha || id:prot−a−2974	IVW	34	1.085 (1.003 to 1.173)	0.041	0.2	0.56
Insulin−like growth factor−binding protein 1 levels || id:ebi−a−GCST90010221	IVW	30	1.148 (1.006 to 1.311)	0.041	0.29	0.92
Insulin−like 3 || id:prot−a−1560	IVW	34	1.106 (1.003 to 1.218)	0.043	0.0022	0.82
Growth hormone receptor || id:prot−a−1211	IVW	68	0.941 (0.887 to 0.999)	0.046	0.019	0.26

Figure 5.

The response of Oxt neurons to 17α-estradiol and the causal effects of Oxt on other endocrine factors.

(A), (B) The top 10 (arrows) and bottom 10 (arrows) types of neurons from 121 subtypes ranked in cell proportions among three groups. **(C)** Dot plots showing the expression profiles of the selected pathways from the two opposing signaling pathways in four types of food uptake-related neurons, which decreased or increased among the top 10 ranks in (A) or (B). Blue arrows: c1.up.signature and c4.up.signature. **(D)** Volcanic plots showing the DEGs between Neuron.O.T and Neuron.O in the pathway synaptic membrane. **(E)** Enzyme immunoassay of the plasma levels of Oxt in three groups. **(F)** Top 25 TF activities in neuron Oxt. **(G)** Significant causal effects ($p < 0.05$, IVW) between exposure OXT (id: prot-a-2159) and 203 endocrine-related outcomes, which were not significant in reverse MR analysis. Significant heterogeneity (Q_pval < 0.05). Significant horizontal pleiotropy (pval < 0.05).

up-signature nor the c4-up-signature was up-regulated in *Gnrh1* neuron in the O.T, similar to the observations in *Esr2* neuron. Only in *Esr1* neuron was the c1-up-signature found to be up-regulated. Meanwhile, both *Ar* and *Esr* neurons displayed high level of c4-up-signature, indicating a relatively healthy status. The data suggest that most of the 4 types of sex hormone-related neurons did not experience severe aging in O.T. However, based on these expression profiles, it's difficult to define the precise physiological status of these subtypes of neurons, particularly regarding neuroendocrine activities. Consequently, we performed enzyme immunoassays of hormones from the serum of O, O.T and Y. The treatment with 17α-estradiol significantly increased the plasma level of Gnrh compared to Y ($p = 0.0099$) and approached significance when compared to O ($p = 0.096$) (**Figure 6B**). More intriguingly, testosterone levels in serum were significantly increased in O.T compared to O ($p = 0.018$) and approached significance when compared to Y ($p = 0.052$).

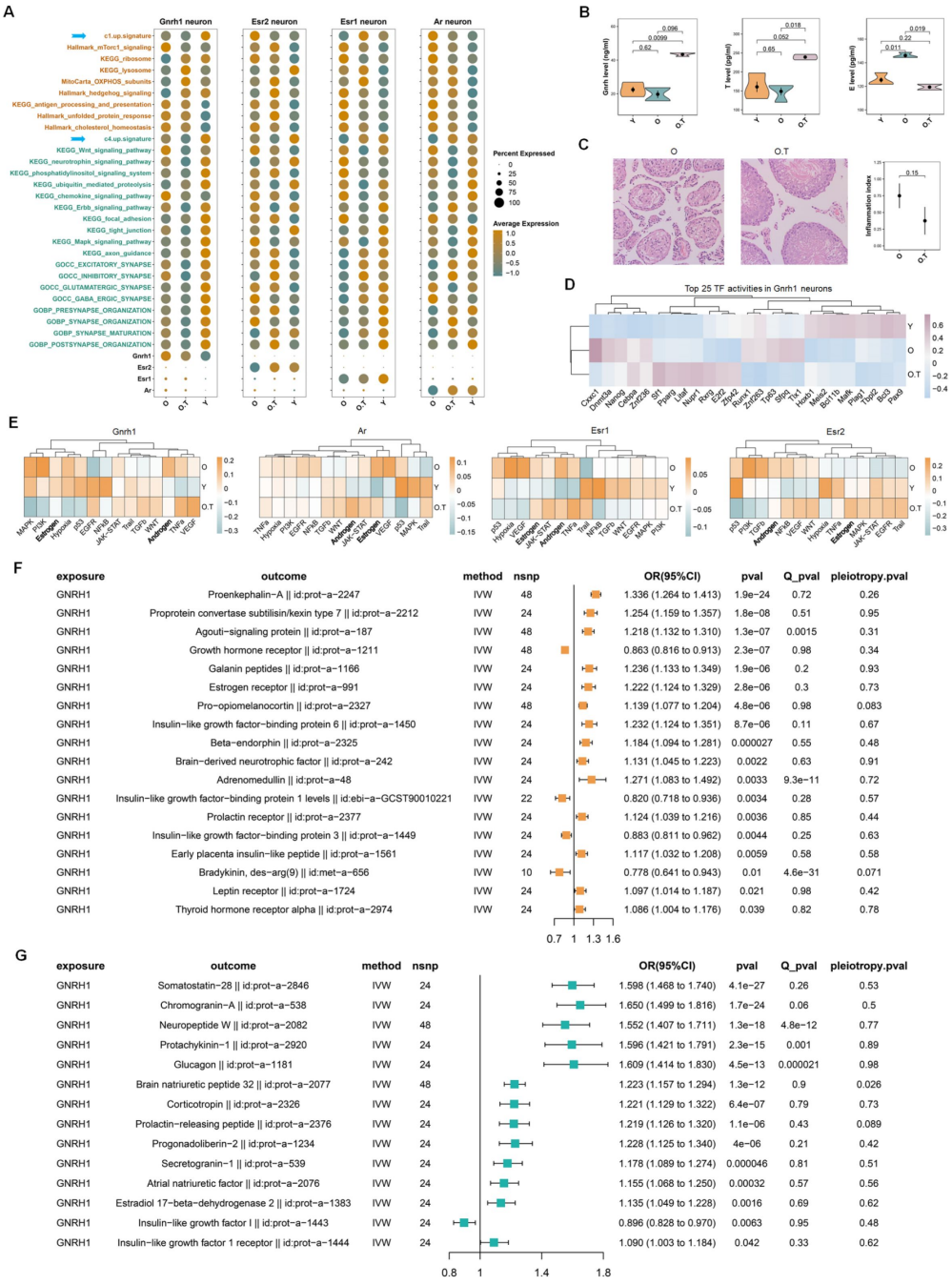
Additionally, the serum estradiol levels were significantly increased in O compared to Y ($p = 0.011$) and significantly decreased in O.T compared to O ($p = 0.019$), suggesting that 17α-estradiol treatment markedly altered the homeostasis of testosterone and estradiol.

Furthermore, most testes from 30-month-old male BN rats exhibited severe age-related inflammation and epithelial collapse of seminiferous tubules (**Figure 6C**). The testes without inflammation in O.T displayed normal morphology. 17α-estradiol treatment slightly decreased the testis inflammation in O.T compared to that in O ($p = 0.15$), indicating a potential positive role of 17α-estradiol treatment in male reproductive system. The elevated TFs such as *Sf1*, *Pparg*, *Litaf*, *Nupr1*, *Rxrg*, *E2f2*, and *Zfp42* may be involved in the transcriptional regulation by 17α-estradiol in O.T (**Figure 6D**). Importantly, the activities of androgen and estrogen pathways were decreased in *Gnrh1* neurons in O.T compared to O, and were distinct from those in *Ar*, *Esr1*, and *Esr2* neurons (**Figure 6E**). These signaling pathways are important for the feedback control of sex hormone secretion in *Gnrh* neurons, and these results may also reflect the strong effect of 17α-estradiol on *Gnrh* neurons.

To decipher the potential effects of elevated serum Gnrh levels on endocrine system, we performed bidirectional MR analysis of the GWAS summary data of human GNRH1 (id: prot-a-1233) and 203 endocrine-related factors with genetic variants SNPs. We found strong causal effects of GNRH1 on GAL/Galanin (id:prot−a−1166), POMC/Beta−endorphin (id:prot−a−2327, id:prot−a−2325), Adrenomedullin (id:prot−a−48), BDNF (id:prot−a−242), and LPR (id:prot−a−1724), which are involved in feeding, energy homeostasis, osmotic regulation, and neuron plasticity (**Figure 6F**). Notably, CRH/Corticotropin (id:prot−a−2326), PRLH/Prolactin−releasing peptide (id:prot−a−2376), NPW/Neuropeptide W (id:prot−a−2082), Glucagon (id:prot−a−1181), Chromogranin−A (id:prot−a−538) displayed bidirectional significance, indicating close and complex causal effects between GNRH1 and these endocrine factors (**Figure 6G**, Figure 6—figure supplement 1A, B). These results also suggest that the role of 17α-estradiol treatment in feeding, energy homeostasis, reproduction, osmotic regulation, stress response, and neuronal plasticity may be mediated, at least in part, by elevated Gnrh secretion.

Discussion

The most striking role of 17α-estradiol treatment revealed in this study showed that HPG axis was substantially improved in the levels of serum Gnrh and testosterone. The underlying molecular mechanism remains unclear; however, prior reports have indicated that 17α-estradiol can bind to ESR1 [9]. In our findings, 17α-estradiol treatment significantly decreased serum estradiol levels while elevating serum testosterone. Based on this evidence, we propose that 17α-estradiol may function similarly to estrogen receptor antagonists or aromatase inhibitors, potentially preventing



exposure	outcome	method	nsnp	OR(95%CI)	pval	Q_pval	pleiotropy.pval
GNRH1	Proenkephalin−A || id:prot−a−2247	IVW	48	1.336 (1.264 to 1.413)	1.9e−24	0.72	0.26
GNRH1	Proprotein convertase subtilisin/kexin type 7 || id:prot−a−2212	IVW	24	1.254 (1.159 to 1.357)	1.8e−08	0.51	0.95
GNRH1	Agouti−signaling protein || id:prot−a−187	IVW	48	1.218 (1.132 to 1.310)	1.3e−07	0.0015	0.31
GNRH1	Growth hormone receptor || id:prot−a−1211	IVW	48	0.863 (0.816 to 0.913)	2.3e−07	0.98	0.34
GNRH1	Galanin peptides || id:prot−a−1166	IVW	24	1.236 (1.133 to 1.349)	1.9e−06	0.2	0.93
GNRH1	Estrogen receptor || id:prot−a−991	IVW	24	1.222 (1.124 to 1.329)	2.8e−06	0.3	0.73
GNRH1	Pro−opiomelanocortin || id:prot−a−2327	IVW	48	1.139 (1.077 to 1.204)	4.8e−06	0.98	0.083
GNRH1	Insulin−like growth factor−binding protein 6 || id:prot−a−1450	IVW	24	1.232 (1.124 to 1.351)	8.7e−06	0.11	0.67
GNRH1	Beta−endorphin || id:prot−a−2325	IVW	24	1.184 (1.094 to 1.281)	0.000027	0.55	0.48
GNRH1	Brain−derived neurotrophic factor || id:prot−a−242	IVW	24	1.131 (1.045 to 1.223)	0.0022	0.63	0.91
GNRH1	Adrenomedullin || id:prot−a−48	IVW	24	1.271 (1.083 to 1.492)	0.0033	9.3e−11	0.72
GNRH1	Insulin−like growth factor−binding protein 1 levels || id:ebi−a−GCST90010221	IVW	22	0.820 (0.718 to 0.936)	0.0034	0.28	0.57
GNRH1	Prolactin receptor || id:prot−a−2377	IVW	24	1.124 (1.039 to 1.216)	0.0036	0.85	0.44
GNRH1	Insulin−like growth factor−binding protein 3 || id:prot−a−1449	IVW	24	0.883 (0.811 to 0.962)	0.0044	0.25	0.63
GNRH1	Early placenta insulin−like peptide || id:prot−a−1561	IVW	24	1.117 (1.032 to 1.208)	0.0059	0.58	0.58
GNRH1	Bradykinin, des−arg(9) || id:met−a−656	IVW	10	0.778 (0.641 to 0.943)	0.01	4.6e−31	0.071
GNRH1	Leptin receptor || id:prot−a−1724	IVW	24	1.097 (1.014 to 1.187)	0.021	0.98	0.42
GNRH1	Thyroid hormone receptor alpha || id:prot−a−2974	IVW	24	1.086 (1.004 to 1.176)	0.039	0.82	0.78

exposure	outcome	method	nsnp	OR(95%CI)	pval	Q_pval	pleiotropy.pval
GNRH1	Somatostatin−28 || id:prot−a−2846	IVW	24	1.598 (1.468 to 1.740)	4.1e−27	0.26	0.53
GNRH1	Chromogranin−A || id:prot−a−538	IVW	24	1.650 (1.499 to 1.816)	1.7e−24	0.06	0.5
GNRH1	Neuropeptide W || id:prot−a−2082	IVW	48	1.552 (1.407 to 1.711)	1.3e−18	4.8e−12	0.77
GNRH1	Protachykinin−1 || id:prot−a−2920	IVW	24	1.596 (1.421 to 1.791)	2.3e−15	0.001	0.89
GNRH1	Glucagon || id:prot−a−1181	IVW	24	1.609 (1.414 to 1.830)	4.5e−13	0.000021	0.98
GNRH1	Brain natriuretic peptide 32 || id:prot−a−2077	IVW	48	1.223 (1.157 to 1.294)	1.3e−12	0.9	0.026
GNRH1	Corticotropin || id:prot−a−2326	IVW	24	1.221 (1.129 to 1.322)	6.4e−07	0.79	0.73
GNRH1	Prolactin−releasing peptide || id:prot−a−2376	IVW	24	1.219 (1.126 to 1.320)	1.1e−06	0.43	0.089
GNRH1	Progonadoliberin−2 || id:prot−a−1234	IVW	24	1.228 (1.125 to 1.340)	4e−06	0.21	0.42
GNRH1	Secretogranin−1 || id:prot−a−539	IVW	24	1.178 (1.089 to 1.274)	0.000046	0.81	0.51
GNRH1	Atrial natriuretic factor || id:prot−a−2076	IVW	24	1.155 (1.068 to 1.250)	0.00032	0.57	0.56
GNRH1	Estradiol 17−beta−dehydrogenase 2 || id:prot−a−1383	IVW	24	1.135 (1.049 to 1.228)	0.0016	0.69	0.62
GNRH1	Insulin−like growth factor I || id:prot−a−1443	IVW	24	0.896 (0.828 to 0.970)	0.0063	0.95	0.48
GNRH1	Insulin−like growth factor 1 receptor || id:prot−a−1444	IVW	24	1.090 (1.003 to 1.184)	0.042	0.33	0.62

Figure 6.

The response of HPG axis in the males to 17α-estradiol and the causal effects of Gnrh on other endocrine factors.

(A) The expression profiles of pathways from the two opposing signaling networks in Gnrh1-, Esr2-, Esr1- or Ar-positive neurons. **(B)** Enzyme immunoassay of the serum levels of Gnrh, total testosterone (T), and estrogen (E) in Y, O and O.T samples. Two-tail unpaired T-test was performed. **(C)** Inflammation of seminiferous tubules in testes of O and O.T. Left two panels: representative HE staining of testis inflammation in O and the normal seminiferous tubules of O.T. Right panel: the mean testis inflammation index of O and O.T. **(D)** The top 25 TF activities in Gnrh1 neurons in three groups. **(E)** The activities of 14 pathways in Gnrh1-, Esr2-, Esr1- or Ar-positive neurons. **(F)** Significant causal effects (IVW, $p < 0.05$) between exposure GNRH1 (id: prot-a-1233) and 203 endocrine-related outcomes, which were not significant in reverse MR analysis. **(G)** Items with significant causal effects (IVW, $p < 0.05$) in both directions of MR analysis between GNRH1 (id: prot-a-1233) and 203 endocrine-related outcomes.

the conversion of androgens to estrogens [28, 29]. These actions could alleviate the feedback inhibition exerted by estrogen on hypothalamus and pituitary, thereby facilitating the secretion of Gnrh, FSH, and LH [30].

The testosterone levels in men gradually decline beginning in the third decade of life [31]. Age-related deterioration of the gonadotropic axis, particularly in older males with low serum testosterone, is often linked to numerous aging symptoms, including loss of skeletal muscle mass, reduced muscle strength and power, low bone mineral density, frailty, impaired physical performance, mobility limitations, increased risk of diabetes, elevated all-cause cardiovascular mortality, cognitive decline, and heightened risk of Alzheimer's disease [32]. Consequently, testosterone supplementation in older men is beneficial. Additionally, Gnrh supplementation may help mitigate age-related declines in neurogenesis and slow aging processes [33]. Importantly, treatment with 17α-estradiol did not result in feminization or adversely affect the sperm parameters and fertility in male animals [6, 14]. Thus, the observed increases in Gnrh and serum testosterone levels due to 17α-estradiol treatment are likely advantageous for older males, particularly those experiencing late-onset hypogonadism.

Postmenopausal women with low estrogen experience aging-related syndromes similar to those of older males with low serum testosterone. Those women also face increased mortality, cardiovascular disease, osteoporosis fracture, urogenital atrophy, and dementia, all of which may benefit from hormone therapy [34]. However, a prior report indicates that 17α-estradiol treatment does not provide positive life extension effects in aged females [13]. The discrepancy may stem from the inhibitory effects of estrogens associated with 17α-estradiol treatment, as evidenced by its inability to enhance female fertility [35]. Nonetheless, due to the lack of parallel data in aged female BN rats treated with 17α-estradiol, further research is needed to definitely address this question in the female subjects in the future.

Another notable effect of 17α-estradiol is its ability to reduce the overall expression levels of energy metabolism in hypothalamic neurons of aged male BN rats. The nutrient-sensing network, mediated by MTORC1 complex, is a central regulator of mRNA and ribosome biogenesis, protein synthesis, glucose metabolism, autophagy, lipid metabolism, mitochondrial biosynthesis, and proteasomal activity [36]. Downregulation of this nutrient-sensing network has been associated with increased lifespan and healthspan [37]. Notably, 17α-estradiol treatment diminished nutrient-sensing network activity in most hypothalamic neurons, which may be a contributing factor in promoting lifespan extension.

In this report, we demonstrated significant changes in neuron populations involved in appetite control, including *Agrp*, *Pomc*, *Oxt*, *Oxtr*, *Gipr*, and *Glp2r* neurons. Among the identified subtypes, the proportion of Oxt neurons saw the most considerable increase due to 17α-estradiol treatment (**Figure 5A**). Oxt plays versatile roles in social behavior, stress response, satiety, energy balance, reproduction, and inflammation [38]. Most *Oxt* neurons originate from the paraventricular nucleus (PVN) and supraoptic nuclei (SON) in the hypothalamus, exhibiting high plasticity during development and intricate circuitry [39, 40]. The PVN, arcuate nucleus (ARC), and ventromedial hypothalamic nucleus together form a neural hub in the hypothalamus that integrates peripheral, nutritional, and metabolic signals to regulate food intake and energy balance [41]. Many effects of Oxt are sex-specific [42]; for instance, females are less sensitive to exogenous Oxt than males regarding social recognition [43]. Interestingly, Oxt injections, facilitated by nanoparticles that enhance blood-brain barrier penetration, reduced body mass while increasing social investigation and the number of Oxt-positive cells in the SON, particularly in male rats [44]. Additionally, intracerebroventricular injections of Oxt in rats showed a reduction in food intake in both sexes, with a more pronounced effect in males [45].Therefore, we propose that Oxt's role in systemic aging and feeding behavior may contribute to the sex-biased effects of 17α-estradiol, warranting further verification.

Furthermore, 17α-estradiol treatment appears to have enhanced stress in HPA axis. One evidence was the increased levels of ferroptosis-signature and UPR in *Crh* neurons. The other evidence was the elevated serum cortisol, which is also a potential hallmark of aging HPA axis [46, 47]. Therefore, more attentions should be paid to the potential side effects of 17α-estradiol especially in its clinical application.

In summary, our findings suggest that 17α-estradiol treatment positively influences the HPG axis and neurons associated with appetite and energy balance. This may be closely linked to the life-extension effects of 17α-estradiol in aged males. Additionally, employing supervised clustering based on neuropeptides, hormones, and their receptors proves to be a valuable strategy for examining pharmacological, pathological, and physiological processes in different neuronal subtypes within the hypothalamus.

Abbreviations

- Ar: androgen receptor
- ARC: arcuate nucleus
- Astro: astrocyte
- AUC: area under the curve
- Cga: glycoprotein hormones, alpha Polypeptide
- Crh: corticotropin releasing hormone
- DEG: differentially-expressed gene
- Endo: endothelial cell
- Esr1: estrogen receptor 1
- Esr2: estrogen receptor 2
- Fibro: fibroblast
- Glp2r: glucagon-like peptide 2 receptor
- GnRH: gonadotropin releasing hormone
- GSEA: gene set enrichment analysis
- HPA: hypothalamic–pituitary–adrenal
- HPG: hypothalamic-pituitary-gonadal
- IEU: MRC Integrative Epidemiology Unit

- IVW: inverse-variance weighting
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- Micro: microglia
- MR: Mendelian randomization
- MTORC1: mechanistic target of rapamycin kinase 1
- Nppa: natriuretic peptide A
- Oligo: oligodendrocyte
- OPC: oligodendrocyte precursor cell
- OR: odds ratio
- OXPHOS: oxidative phosphorylation
- PID: Pathway Interaction Database
- POMC: proopiomelanocortin
- pQTL: protein quantitative trait loci
- PTC: pars tuberalis cell
- PVN: paraventricular nucleus
- ROC: receiver operating characteristics
- snRNA-seq: single-nucleus transcriptomic sequencing
- Tany: tanycyte
- TF: transcription factor
- UPR: unfolded protein response.

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Additional information

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Author contributions

Conceptualization, YL, LL; Methodology, YL, GW, XX; Animal operation: YL, LL; EIA assays: YL, LY; Inflammation test: YL, ZY, LL, YS. Validation, LL, JY, ZY; Formal Analysis, YL, JY; Resources, GW, YS, ZY, ZM, LX; Visualization, YL; Writing – Original Draft, YL, JY; Writing – Review & Editing, LL, YS; Supervision, YS, ZY, YL; Project Administration, YL, ZY, LL; Funding Acquisition, ZY, LL. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors have no conflict of interests to declare.

Availability of data and materials

All data are available at GEO accession number GSE248413.

Competing interests

All authors declare no competing interests.

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Editors

Reviewer #1 (Public review):

Summary:

Previous studies have shown that treatment with 17α-estradiol (a stereoisomer of the 17β-estradiol) extends lifespan in male mice but not in females. The current study by Li et al, aimed to identify cell-specific clusters and populations in the hypothalamus of aged male rats treated with 17α-estradiol (treated for 6 months). This study identifies genes and pathways affected by 17α-estradiol in the aged hypothalamus.

Strengths:

Using single-nucleus transcriptomic sequencing (snRNA-seq) on hypothalamus from aged male rats treated with 17α-estradiol they show that 17α-estradiol significantly attenuated age-related increases in cellular metabolism, stress, and decreased synaptic activity in neurons. Moreover, sc-analysis identified GnRH as one of the key mediators of 17α-estradiol's effects on energy homeostasis. Furthermore, they show that CRH neurons exhibited a senescent phenotype, suggesting a potential side effect of the 17α-estradiol. These conclusions are supported by supervised clustering by neuropeptides, hormones, and their receptors.

Weaknesses:

However, the study has several limitations that reduce the strength of the key claims in the manuscript. In particular:

(1) The study focused only on males and did not include comparisons with females. However, previous studies have shown that 17α-estradiol extends lifespan in a sex-specific manner in mice, affecting males but not females. Without the comparison with the female data, it's difficult to assess its relevance to the lifespan.

(2) It's not known whether 17α-estradiol leads to lifespan extension in male rats similar to male mice. Therefore, it is not possible to conclude that the observed effects in the

hypothalamus, are linked to the lifespan extension.

(3) The effect of 17α-estradiol on non-neuronal cells such as microglia and astrocytes is not well described (Fig.1). Previous studies demonstrated that 17α-estradiol reduces microgliosis and astrogliosis in the hypothalamus of aged male mice. Current data suggest that the proportion of oligo, and microglia were increased by the drug treatment, while the proportions of astrocytes were decreased. These data might suggest possible species differences, differences in the treatment regimen, or differences in drug efficiency. This has to be discussed.

A more detailed analysis of glial cell types within the hypothalamus in response to drug should be provided.

(4) The conclusion that CRH neurons are going into senescence is not clearly supported by the data. A more detailed analysis of the hypothalamus such as histological examination to assess cellular senescence markers in CRH neurons, is needed to support this claim.

Comments on revisions:

Some of the concerns were addressed in this revised version, and the authors responded and addressed study design limitations in both sexes/ages.

However, there are still some concerns that were not sufficiently addressed:

While the term "senescent" was changed to "stressed," some histological/ cellular validation of this phenotype is still needed.

Some discussion on the sex-specific effects of 17α-estradiol in the hypothalamus is still required. Previous studies in mice demonstrated that 17α-estradiol reduced hypothalamic microgliosis and astrogliosis in male but not female UM-HET3 mice.

Additionally, the provided analysis on astrocytes and microglia is superficial.

https://doi.org/10.7554/eLife.100346.2.sa2

Reviewer #2 (Public review):

Summary:

Li et al. investigated the potential anti-ageing role of 17α-Estradiol on the hypothalamus of aged rats. To achieve this, they employed a very sophisticated method for single-cell genomic analysis that allowed them to analyze effects on various groups of neurons and non-neuronal cells. They were able to sub-categorize neurons according to their capacity to produce specific neurotransmitters, receptors, or hormones. They found that 17α-Estradiol treatment led to an improvement in several factors related to metabolism and synaptic transmission by bringing the expression levels of many of the genes of these pathways closer or to the same levels to those of young rats, reversing the ageing effect. Interestingly, among all neuronal groups, the proportion of Oxytocin-expressing neurons seems to be the one most significantly changing after treatment with 17α-Estradiol, suggesting an important role of these neurons on mediating its anti-ageing effects. This was also supported by an increase in circulating levels of oxytocin. It was also found that gene expression of corticotropin-releasing hormone neurons was significantly impacted by 17α-Estradiol even though it was not different between aged and young rats, suggesting that these neurons could be responsible for side effects related to this treatment. This article revealed some potential targets that should be further investigated in future studies regarding the role of 17α-Estradiol treatment in aged males.

Strengths:

• The single nucleus mRNA sequencing is a very powerful method for gene expression analysis and clustering. The supervised clustering of neurons was very helpful in revealing otherwise invisible differences between neuronal groups and helped identify specific neuronal populations as targets.
• There is a variety of functions used that allowed the differential analysis of a very complex type of data. This led to a better comparison between the different groups in many levels.
• There were some physiological parameters measured such as circulating hormone levels that helped the interpretation of the effects of the changes in hypothalamic gene expression.

Weaknesses:

• One main control group is missing from the study, the young males treated with 17α-Estradiol.
• Even though the technical approach is a sophisticated one, analyzing the whole rat hypothalamus instead of specific nuclei or subregions makes the study weaker.
• Although the authors claim to have several findings, the data fail to support these claims.
• The study is about improving ageing but no physiological data from the study demonstrated such claim with the exception of the testes histology which was not properly analyzed and was not even significantly different between the groups.
• Overall, the study remains descriptive with no physiological data to demonstrate that any of the effects on hypothalamic gene expression is related to metabolic, synaptic or other function.

Comments on revisions:

The authors revised part of the manuscript to address some of the reviewers' comments This improved the language and the text flow to a certain extent. They also added an additional analysis including glial cells. However, they failed to address the main weaknesses brought up by the reviewers and did not add any experimental demonstration of their claims on lifespan expansion induced by 17α-estradiol in rats. In addition, they insisted i keeping parts in the discussion that are not directly linked to any of the papers' findings.

https://doi.org/10.7554/eLife.100346.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Previous studies have shown that treatment with 17α-estradiol (a stereoisomer of the 17β-estradiol) extends lifespan in male mice but not in females. The current study by Li et al, aimed to identify cell-specific clusters and populations in the hypothalamus of aged male rats treated with 17α-estradiol (treated for 6 months). This study identifies genes and pathways affected by 17α-estradiol in the aged hypothalamus.*
>
> *Strengths:*
>
> *Using single-nucleus transcriptomic sequencing (snRNA-seq) on the hypothalamus from aged male rats treated with 17α-estradiol they show that 17α-estradiol significantly*

> *attenuated age-related increases in cellular metabolism, stress, and decreased synaptic activity in neurons.*

Thanks.

> *Moreover, sc-analysis identified GnRH as one of the key mediators of 17α-estradiol's effects on energy homeostasis. Furthermore, they show that CRH neurons exhibited a senescent phenotype, suggesting a potential side effect of the 17α-estradiol. These conclusions are supported by supervised clustering by neuropeptides, hormones, and their receptors.*

Thanks.

> *Weaknesses:*
>
> *However, the study has several limitations that reduce the strength of the key claims in the manuscript. In particular:*
>
> *(1) The study focused only on males and did not include comparisons with females. However, previous studies have shown that 17α-estradiol extends lifespan in a sex-specific manner in mice, affecting males but not females. Without the comparison with the female data, it's difficult to assess its relevance to the lifespan.*

This study was originally designed based on previous findings indicating that lifespan extension is only effective in males, leading to the exclusion of females from the analysis. The primary focus of our research was on the transcriptional changes and serum endocrine alterations induced by 17α-estradiol in aged males compared to untreated aged males. We believe that even in the absence of female subjects, the significant effects of 17α-estradiol on metabolism in the hypothalamus, synapses, and endocrine system remain evident, particularly regarding the expression levels of GnRH and testosterone. Notably, lower overall metabolism, increased synaptic activity, and elevated levels of GnRH and testosterone are strong indicators of health and well-being in males, supporting the validity of our primary conclusions. However, including female controls would enhance the depth of our findings. If female controls were incorporated, we propose redesigning the sample groups to include aged male control, aged female control, aged female treated, aged male treated, as well as young male control, young male treated, young female control, and young female treated. We regret that we cannot provide this data in the short term. Nevertheless, we believe this presents a valuable avenue for future research on this topic. In this study, we emphasize the role of 17α-estradiol in overall metabolism, synaptic function, GnRH, and testosterone in aged males and underscore the importance of supervised clustering of neuropeptide-secreting neurons in the hypothalamus.

> *(2) It is not known whether 17α-estradiol leads to lifespan extension in male rats similar to male mice. Therefore, it is not possible to conclude that the observed effects in the hypothalamus, are linked to the lifespan extension.*

Thanks for the reminding. 17α-estradiol was reported to extend lifespan in male rats similar to male mice (PMID: 33289482). We have added the valuable reference to introduction in the new version.

> *(3) The effect of 17α-estradiol on non-neuronal cells such as microglia and astrocytes is not well-described (Figure 1). Previous studies demonstrated that 17α-estradiol reduces microgliosis and astrogliosis in the hypothalamus of aged male mice. Current data suggest that the proportion of oligo, and microglia were increased by the drug treatment, while the proportions of astrocytes were decreased. These data might suggest*

> *possible species differences, differences in the treatment regimen, or differences in drug efficiency. This has to be discussed.*

We have reviewed reports describing changes in cell numbers following 17α-estradiol treatment in the brain, using the keywords "17α-estradiol," "17alpha-estradiol," and "microglia" or "astrocyte." Only a limited amount of data was obtained. We found one article indicating that 17α-estradiol treatment in Tg (AβPP(swe)/PS1(ΔE9)) model mice resulted in a decreased microglial cell number compared to the placebo (AβPP(swe)/PS1(ΔE9) mice), but this change was not significant when compared to the non-transgenic control (PMID: 21157032). The transgenic AβPP(swe)/PS1(ΔE9) mouse model may differ from our wild-type aging rat model in this context.

Moreover, the calculation of cell numbers was based on visual observation under a microscope across several brain tissue slices. This traditional method often yields controversial results. For example, oligodendrocytes in the corpus callosum, fornix, and spinal cord have been reported to be 20-40% more numerous in males than in females based on microscopic observations (PMID: 16452667). In contrast, another study found no significant difference in the number of oligodendrocytes between sexes when using immunohistochemistry staining (PMID: 18709647). Such discrepancies arising from traditional observational methods are inevitable.

We believe the data presented in this article are reliable because the cell number and cell ratio data were derived from high-throughput cell counting of the entire hypothalamus using single-cell suspension and droplet wrapping (10x Genomics).

> *(4) A more detailed analysis of glial cell types within the hypothalamus in response to drugs should be provided.*

We provided more enrichment analysis data of differentially expressed genes between Y, O, and O.T in microglia and astrocytes in Figure 2—figure supplement 3. In this supplemental data, we found unlike that in neurons, Micro displayed lower levels of synapse-related cellular processes in O.T. compared to O.

> *(5) The conclusion that CRH neurons are going into senescence is not clearly supported by the data. A more detailed analysis of the hypothalamus such as histological examination to assess cellular senescence markers in CRH neurons, is needed to support this claim.*

We also noticed the inappropriate claim and we have changed "senescent phenotype" to "stressed phenotype" and "abnormal phenotype" in abstract and in results.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Li et al. investigated the potential anti-ageing role of 17α-Estradiol on the hypothalamus of aged rats. To achieve this, they employed a very sophisticated method for single-cell genomic analysis that allowed them to analyze effects on various groups of neurons and non-neuronal cells. They were able to sub-categorize neurons according to their capacity to produce specific neurotransmitters, receptors, or hormones. They found that 17α-Estradiol treatment led to an improvement in several factors related to metabolism and synaptic transmission by bringing the expression levels of many of the genes of these pathways closer or to the same levels as those of young rats, reversing the ageing effect. Interestingly, among all neuronal groups, the proportion of Oxytocin-expressing neurons seems to be the one most significantly changing after treatment with 17α-Estradiol,*

> *suggesting an important role of these neurons in mediating its anti-ageing effects. This was also supported by an increase in circulating levels of oxytocin. It was also found that gene expression of corticotropin-releasing hormone neurons was significantly impacted by 17α-Estradiol even though it was not different between aged and young rats, suggesting that these neurons could be responsible for side effects related to this treatment. This article revealed some potential targets that should be further investigated in future studies regarding the role of 17α-Estradiol treatment in aged males.*
>
> *Strengths:*
>
> *(1) Single-nucleus mRNA sequencing is a very powerful method for gene expression analysis and clustering. The supervised clustering of neurons was very helpful in revealing otherwise invisible differences between neuronal groups and helped identify specific neuronal populations as targets.*

Thanks.

> *(2) There is a variety of functions used that allow the differential analysis of a very complex type of data. This led to a better comparison between the different groups on many levels.*

Thanks.

> *(3) There were some physiological parameters measured such as circulating hormone levels that helped the interpretation of the effects of the changes in hypothalamic gene expression.*

Thanks.

> *Weaknesses*
>
> *(1) One main control group is missing from the study, the young males treated with 17α-Estradiol.*

Given that the treatment period lasts six months, which extends beyond the young male rats' age range, we aimed to investigate the perturbation of 17α-Estradiol on the normal aging process. Including data from young males could potentially obscure the treatment's effects in aged males due to age effects, though similar effects between young and aged animals may exist. Long-term treatment of hormone may exert more developmental effects on the young than the old. Consequently, we decided to exclude this group from our initial sample design. We apologize for this omission.

> *(2) Even though the technical approach is a sophisticated one, analyzing the whole rat hypothalamus instead of specific nuclei or subregions makes the study weaker.*

The precise targets of 17α-Estradiol within the hypothalamus remain unresolved. Selecting a specific nucleus for study is challenging. The supervised clustering method described in this manuscript allows us to identify the more sensitive neuron subtypes influenced by 17α-Estradiol and aging across the entire hypothalamus, without the need to isolate specific nuclei in a disturbed hypothalamic environment.

> *(3) Although the authors claim to have several findings, the data fail to support these claims. You may mean the claim as the senescent phenotype in Crh neuron induced by 17α-estradiol.*

Thanks. We have changed the "senescent phenotype" to "stressed phenotype" or "abnormal phenotype" in the abstract and results to avoid such claim.

> *(4) The study is about improving ageing but no physiological data from the study demonstrated such a claim with the exception of the testes histology which was not properly analyzed and was not even significantly different between the groups.*

The primary objective of this study is to elucidate the effects of 17α-Estradiol on the endocrine system in the aging hypothalamus; exploring anti-aging effects is not the main focus. From the characteristics of the aging hypothalamus, we know that down-regulated GnRH and testosterone levels, along with elevated mTOR signaling, are indicators of aging in these organs (PMID: 37886966, PMID: 37048056, PMID: 22884327). The contrasting signaling networks related to metabolism and synaptic processes significantly differentiate young and aging hypothalami, and 17α-Estradiol helps rebalance these networks, suggesting its potential anti-aging effects.

> *(5) Overall, the study remains descriptive with no physiological data to demonstrate that any of the effects on hypothalamic gene expression are related to metabolic, synaptic, or other functions.*

The study focuses on investigating cellular responses and endocrine changes in the aging hypothalamus induced by 17α-estradiol, utilizing single-nucleus RNA sequencing (snRNA-seq) and a novel data mining methodology to analyze various neuron subtypes. It is important to note that this study does not mainly aim to explore the anti-aging effects. Consequently, we have revised the claim in the abstract from “the effects of 17α-estradiol in anti-aging in neurons” to “the effects of 17α-estradiol on aging neurons.” We observed that the lower overall metabolism and increased expression levels of cellular processes in the synapses align with findings previously reported regarding 17α-estradiol. To address the lack of physiological data and the challenges in measuring multiple endocrine factors due to their volatile nature, we employed several bidirectional Mendelian analyses of various genome-wide association study (GWAS) data related to these serum endocrine factors to identify their mutual causal effects.

> ***Reviewing Editor Comment:***
>
> *Based on the Public Reviews and Recommendations for Authors, the Reviewers strongly recommend that revisions include an experimental demonstration of the physiological effects of the treatment on ageing in rats as well as the CRH-senescence link. Additional analysis of the glia would greatly strengthen the study, as would inclusion of females and young male controls. The important point was also raised that the work linking 17α-estradiol was performed in mice, and the link with lifespan in rats is not known. Discussion of this point is recommended.*

We acknowledge that 17α-estradiol has been reported to extend lifespan in male rats, similar to findings in male mice (PMID: 33289482), and we have noted this in the Introduction. We apologize for not conducting further experiments to validate this point.

Additionally, we have revised the description of the phenotype of senescent CRH neurons to “stressed phenotype” without carrying out further experiments to confirm the senescent phenotype. To provide more clarity on the performance of glial cells during treatment, we have included additional enrichment analysis data of differentially expressed genes among young (Y), old (O), and old treated (O.T) microglia and astrocytes in Figure 2—figure supplement 3. Notably, the behavior of microglia contrasts with that of total neurons

concerning synapse-related cellular processes. We apologize for being unable to include female and young controls in this study.

> ***Reviewer #2 (Recommendations For The Authors)***
>
> *General comments:*
>
> *(1) The manuscript is very hard to read. Proofreading and editing by software or a professional seems necessary. The words "enhanced", "extensive" etc. are not always used in the right way.*

Thanks for the suggestion. We have revised the proofreading and editing. The words "enhanced" and "extensive" were also revised in most sentences.

> *(2) The numbers of animals and samples are not well explained. Is it 9 rats overall or per group? If there are 8 testes samples per group, should we assume that there were 4 rats per group? The pooling of the hypothalamic how was it done? Were all the hypothalamic from each group pooled together? A small table with the animals per group and the samples would help.*

We appreciate your reminder regarding the initial mistake in our manuscript preparation. In the preliminary submission, we reported 9 rats based solely on sequencing data and data mining. The revised version (v1) now includes additional experimental data, with an effective total of 12 animals (4 per group). Unfortunately, we overlooked updating this information in the v1 submission. We have since added detailed information in the Materials and Methods sections: Animals, Treatment and Tissues, and snRNA-seq Data Processing, Batch Effect Correction, and Cell Subset Annotation.

> *(3) The Clustering is wrong. There are genes in there that do not fall into any of the 3 categories: Neurotransmitters, Receptors, Hormones.*

We have changed the description to “Vast majority of these subtypes were clustered by neuropeptides, hormones, and their receptors within all the neurons”.

> *(4) The coloring of groups in the graphs is inconsistent. It must be more homogeneous to make it easier to identify.*

We have changed the colors of groups in Fig. 1D to make the color of cell clusters consistent in Fig. 1A-D.

> *(5) The groups c1-c4 are not well explained. How did the authors come up with these?*

We have added more descriptions of c1-c4 in materials and methods in the new version.

> *(6) In most cases it's not clear if the authors are talking about cell numbers that express a certain mRNA, the level of expression of a certain mRNA, or both. They need to do a better job using more precise descriptions instead of using general terms such as "signatures", "expression profiles", "affected neurons" etc. It is very hard to understand if the number of neurons is compared between the groups or the gene expression.*

We have changed the "signatures" to "gene signatures" to make it more accurate in meaning. The "affected neurons" were also changed to "sensitive neurons". But sorry that we were not able to find better alternatives to the "expression profiles".

> *(7) Sometimes there are claims made without justification or a reference. For example, the claim about the senescence of CRH neurons due to the upregulation of mitochondrial genes and downregulation of adherence junction genes (lines 326-328) should be supported by a reference or own findings.*

The "senescence" here is not appropriate. We have changed it to "stressed phenotype" or "aberrant changes" in abstract and results.

> *(8) Young males treated with Estradiol as a control group is necessary and it is missing.*

Your suggestion is appreciated; however, the treatment duration for aged mice (O.T) was set at 6 months, while the young mice were only 4 months old. This disparity makes it challenging to align treatment timelines for the young animals. The primary aim of this study is to investigate the perturbation of 17α-estradiol on the aging process, and any distinct effects due to age effect observed in young males might complicate our understanding of its role in aged males, though similar endocrine effects may exist in the young animals. Long-term treatment of hormone may exert more developmental effects on the young than the old. Therefore, we made the decision to exclude the young samples in our initial study design. We apologize for any confusion this may have caused.

> *Specific Comments:*
>
> *Line 28: "elevated stresses and decreased synaptic activity": Please make this clearer. Can't claim changes in synaptic activity by gene expression.*

We have changed it to "the expression level of pathways involved in synapse".

> *Line 32: "increased Oxytocin": serum Oxytocin.*

We have added the “serum”.

> *Line 52 - 54: Any studies from rats?*

Thanks. In rats there is also reported that 17α-estradiol has similar metabolic roles as that in mice (PMID: 33289482) and we have added it to the refences. It’s very useful for this manuscript.

> *Line 62 - 65: It wasn't investigated thoroughly in this paper so why was it suggested in the introduction?*

We have deleted this sentence as being suggested.

> *Line 70: "synaptic activity" Same as line 28.*

We have changed it to "pathways involved in synaptic activity".

> *Line 79: Why were aged rats caged alone and young by two? Could that introduce hypothalamic gene expression effects?*

The young males were bred together in peace. But the aged males will fight and should be kept alone.

> *Lines 78, 99, 109-110: It is not clear how many animals per group were used and how many samples per group were used separately and/or grouped. Please be more specific.*

We have added these information to Materials and methods/Animals, treatment and tissues and Materials and methods/snRNA-seq data processing, batch effect correction, and cell subset annotation.

> *Line 205: "in O" please add "versus young.".*

We have changed accordingly.

> *Line 207: replace "were" with "was" .*

We have alternatively changed the "proportion" to "proportions".

> *Line 208: replace "that" with "compared to" and after "in O.T." add "compared to?"*

We have changed accordingly.

> *Line 223: "O.T." compared to what? Figure?*

We have changed it accordingly.

> *Line 227: Figure?*

We have added (Figure 1E) accordingly.

> *Line 229: "synaptic activity" Same as line 28.*

We have revised it.

> *Line 235: "synaptic activity" and "neuropeptide secretion" Same as line 28.*

We have revised it.

> *Line 256:" interfered" please revise.*

We changed to "exerted".

> *Line 263: "on the contrary" please revise.*

We have changed "on the contrary" to "opposite".

> *Line 270: "conversed" did you mean "conserved"?*

We have changed "conversed" to "inversed".

> *Line 296-298: Please explain. Why would these be side effects?*

It's hard to explain, therefore, we deleted the words "side effects".

> *Line 308: "synaptic activity" Same as line 28.*

We have changed it to "expression levels of synapse-related cellular processes".

> *Line 314: "and sex hormone secretion and signaling"Isn't this expected?*

Yes, it is expected. We have added it to the sentence "and, as expected, sex hormone secretion and signaling".

> *Line 325-328: Why is this senescence? Reference?*

We have added “potent” to it.

> *Line 360-361: This doesn't show elevated synaptic activity.*

"elevated synaptic activity" was changed to "The elevated expression of synapse-related pathways"

> *Line 363-364: "Unfortunately" is not a scientific expression and show bias.*

We have changed it to "Notably".

> *Line 376: Similar as above.*

Yes, we have change it to "in contrast".

> *Lines 382-385: This is speculation. Please move to discussion.*

Sorry for that. We think the causal effects derived from MR result is evidence. As such, we have not changed it.

> *Line 389: Please revise "hormone expressing".*

We have changed it accordingly.

> *Line 401: Isn't this effect expected due to feedback inhibition of the biochemical pathway? Please comment.*

The binding capability of 17alpha-estradiol to estrogen receptors and its role in transcriptional activation remain core questions surrounded by controversy. Earlier studies suggest that 17alpha-estradiol exhibits at least 200 times less activity than 17beta-estradiol (PMID: 2249627, PMID: 16024755). However, recent data indicate that 17alpha-estradiol shows comparable genomic binding and transcriptional activation through estrogen receptor α (Esr1) to that of 17beta-estradiol (PMID: 33289482). Additionally, there is evidence that 17alpha-estradiol has anti-estrogenic effects in rats (PMID: 16042770). These findings imply possible feedback inhibition via estrogen receptors. Furthermore, 17alpha-estradiol likely differs from 17beta-estradiol due to its unique metabolic consequences and its potential to slow aging in males, an effect not attributed to 17beta-estradiol. For instance, neurons are also targets of 17alpha-estradiol, with Esr1 not being the sole target (PMID: 38776045). Nevertheless, the precise effective targets of 17alpha-estradiol are still unresolved.

> *Line 409: This conclusion cannot be made because the effect is not statistically significant. Can say "trend" etc.*

Thanks for the recommendation. We have added "potential" in front of the conclusion.

> *Line 426: "suggesting" please revise.*

sorry, it's a verb.

> *Lines 426-428: This is speculation. Please move to discussion.*

The elevated GnRH levels in O.T., observed through EIA analysis, suggest a deduction regarding the direct causal effects of 17alpha-estradiol on various endocrine factors related to feeding, energy homeostasis, reproduction, osmotic regulation, stress response, and neuronal plasticity through MR analysis. Thus, we have not amended our position. We apologize for any confusion.

> *Lines 431-432: improved compared to what?*

The statement have been revised as " The most striking role of 17α-estradiol treatment revealed in this study showed that HPG axis was substantially improved in the levels of serum Gnrh and testosterone".

> *Line 435: " Estrogen Receptor Antagonists". Please revise.*

Thanks for the recommendation. We have changed it to "estrogen receptor antagonists".

> *Line 438" "Secrete". Please revise.*

Sorry, it is "secret".

> *Lines 439-449: None of this has been demonstrated. Please remove these conclusions.*

These are not conclusions but rather intriguing topics for discussion. Given the role of 17alpha-estradiol in promoting testosterone and reducing estradiol levels in males, we believe it is worthwhile to explore the potential application of 17alpha-estradiol in increasing testosterone levels in aged males, particularly those with hypogonadism.

> *Lines 450-457: No females were included in this study. Why? Also, why is this discussed? It is relevant but doesn't belong in this manuscript since it was not studied here.*

Testosterone levels are crucial for male health, while estradiol levels are essential for the health and fertility of females. Previous studies have demonstrated that 17α-estradiol does not contribute to lifespan extension in females. Given the effects of 17α-estradiol on males—specifically, its role in promoting testosterone and reducing estradiol levels—we believe it is important to discuss the potential sex-biased effects of 17α-estradiol, as this could inform future investigations. Therefore, we have chosen not to make changes to this section.

> *Lines 458-459: This was not demonstrated in this article. Please remove.*

We have restricted the claim to "expression level of energy metabolism in hypothalamic neurons".

> *Line 464: "Promoted lifespan extension" Not demonstrated. Please remove.*

At the end of the sentence it was revised as "which may be a contributing factor in promoting lifespan extension".

> *Line 466: "Showed" No.*

The whole sentence was deleted in the new version.

> *Line 483: "the sex-based effects". Not studied here.*

Since the changes in testosterone levels are significant in this dataset and this hormone has a sex-biased nature, we find it worthwhile to suggest this as a topic for future investigation. We have added "which needs further verification in the future" at the end of this sentence.

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