

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

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Abstract

In this study, we investigated the role of 17 α -estradiol in lifespan extension and its potential side effects for long-term administration. Pooled hypothalami from aged male Norway brown rats treated with 17 α -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 17 α -estradiol in anti-aging in neurons, supervised clustering on neurons by neuropeptides and their receptors were used to evaluate the responses of each neuron subtype during aging and after 17 α -estradiol treatment. The elevated cellular metabolism, stresses and decreased synaptic activity in neurons initiated by aging were remarkably attenuated by 17 α -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 17 α -estradiol treatment. 17 α -estradiol treatment not only increased Oxytocin (Oxt), but also increased the activity of hypothalamic-pituitary-gonadal (HPG) axis, evidenced by significantly elevated levels of plasma GnRh, bioavailable testosterone, and decreased estradiol. Elevated GnRh1 was verified to be one of the causal effects mediating the role of 17 α -estradiol in energy homeostasis, neural synapse, and stress response. Notably, *Crh* neurons displayed most prominently senescent phenotype among all checked neuron subtypes in O.T, which may be a potential side effect of 17 α -estradiol treatment. Therefore, HPG axis and energy metabolism may be key targets of 17 α -estradiol in male lifespan extension. Additionally, supervised clustering of neurons was shown to be a useful method to assess the responses to treatment among different neuron subtypes in the hypothalamus.

eLife assessment

This study demonstrates the potential role of 17 α -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 17 α -estradiol lifespan extension in rats, demonstration of physiological effects of the treatment, and insufficient analysis of glial cells and cellular senescence in CRH neurons.

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Background

The hypothalamus is the central hub controlling energy homeostasis, stress response, temperature, learning, feeding, sleeping, social behavior, sexual behavior, hormone secretion, reproduction, osmoregulation, blood pressure, visceral activities, emotion, and circadian rhythms [1]. The hypothalamic energy-sensing system, especially circuits regulating food intake, is critical for life span extension [2]. Elevated metabolic activity involving many regulatory signaling pathways has been reported in aged hypothalamus, including enhanced mTor signaling [3, 4]. Decreases in gonadotropin-releasing hormone (GnRH), Ghrh, Trh, monoamine neurotransmitters, and blood supply are also hallmarks of aging hypothalamus [5].

Previous studies have shown that 17 α -estradiol extends the lifespan of male mice, has beneficial effects on metabolism and inflammation with a similar mechanism as that of rapamycin and acarbose [6–8]. Further investigations revealed certain unique features of 17 α -estradiol in life extension distinct to rapamycin and acarbose [9, 10]. Further, 17 α -estradiol was shown to target hypothalamic *POMC* neurons to reduce metabolism by decreasing feeding behavior via anorexigenic pathways [11]. Interestingly, the lifespan extension effect is only found in male animals [12]. The safety of 17 α -estradiol is key for translation into clinical treatment, and the side effects on reproduction and potential feminization by 17 α -estradiol treatment need to be considered. However, contradictory results have been reported regarding its side effects on reproduction and feminization [6, 13, 14]. A previous study showed that 17 α -estradiol elicits similar genomic binding and transcriptional activation through *Esr1* (estrogen receptor α , ER α) to that of 17 β -estradiol, suggesting a potential side effect on reproduction and feminization [15]. Therefore, the underlying mechanism of lifespan extension and the safety of 17 α -estradiol still need further investigation and verification.

In this report, we used single-nucleus transcriptomic sequencing and performed supervised clustering of neurons by neuropeptides, hormones, and their receptors. Supervised clustering provides better resolution in cell cluster screening than is achieved by traditional unsupervised clustering. The effects of 17 α -estradiol on metabolism, stress responses, ferroptosis, senescence, inflammation, and synaptic activity were assessed in each neuron subtype, and the most sensitive neurons were ranked. The effects of 17 α -estradiol on reversing measures of aging were evaluated by two opposing regulatory networks involved in hypermetabolism, stress, inflammation, and synaptic activity. Several key endocrine factors from serum were checked. Additionally, the potential side effects of 17 α -estradiol on certain neurons were also evaluated.

Materials and methods

Animals, treatment and tissues

Nine Norway brown male rats were acquired from Charles River (Beijing). Aged rats were housed one per cage and young rats (1 month old) were housed two per cage. Aged rats were randomly allocated into control and 17 α -estradiol-treated groups. Aged rats treated with 17 α -estradiol (Catalog #: E834897, Macklin Biochemical, Shanghai, China) were fed freely with regular diet mixed with 17 α -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 17 α -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 CO₂, hypothalami, testes and blood serum were collected for subsequent experiment 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 gonadoliberein-1 (Catalog #: EIAR-GNRH) were obtained from Raybiotech (GA, USA). Enzyme immunoassay kits for rat serum bioavailable 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 μ m 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, and two to three pooled intact hypothalami from each group were homogenized in 500 μ L ice-cold homogenization buffer (0.25 M sucrose, 5 mM CaCl₂, 3 mM MgAc₂, 10 mM Tris-HCl [pH 8.0], 1 mM DTT, 0.1 mM EDTA, 1 $\times$ protease inhibitor, and 1 U/ μ L RiboLock RNase inhibitor) with Dounce homogenizer. Then, the homogenizer was washed with 700 μ L ice-cold nuclei washing buffer (0.04% bovine serum albumin, 0.2 U/ μ L RiboLock RNase Inhibitor, 500 mM mannitol, 0.1 mM phenylmethanesulfonyl fluoride protease inhibitor in 1 $\times$ phosphate buffer saline). Next, the homogenates were filtered through a 70- μ m 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 $\times$ g at 4 $^{\circ}$ 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 $\times$ g at 4 $^{\circ}$ C. The nuclei were filtered through a 40- μ m 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/ μ L, 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 ($\geq 15\%$) were filtered out. Batch effect correction was performed by SCTransform function built in Seurat V4.4.0.

Pathways, 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 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 wilcoxau function in R package presto. The minimal cell number in either one of the comparing pairs should be no less than 15. Ranks of 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 99 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 signatures

The quarters of the mixed cell populations from O, O.T and Y hypothalamic neurons were equally divided with R function fivenum from R package stats according to pathway expression levels. The total neurons were then divided into 4 clusters (c1–c4) accordingly. The cell proportion from O.T, O and Y neurons in each cluster were weighted against the total neurons in the 3 groups. The unique markers of each cluster were calculated with FindAllMarkers function of Seurat package. The intersection of the unique markers from 6 pathways were obtained for heatmap plotting. 19 genes highly expressed in c1 were calculated as c1.up.signature via the PercentageFeatureSet function in R package Seurat. 12 genes were highly expressed in c4 and thus were calculated as c4.up.signature. There were no intersected unique markers in cluster c2 and c3 of the 6 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

Subtypes of neurons were clustered by neuropeptides and receptors for neuropeptides or hormones within 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 neuron 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 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 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 ($r^2 < 0.001$) 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 ($P < 1 \times 10^{-5}$, $r^2 < 0.001$) 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 17 α -estradiol treatment via snRNA-seq profiling

To investigate the hypothalamus as a potential key target of 17 α -estradiol's effects on life extension, we performed snRNA-seq on the entire hypothalamus of aged and 17 α -estradiol-treated aged Norway brown rats, and used the hypothalamus from young adult male rats as a control. We obtained 10 major cell types according to cell markers of the hypothalamus (Figure 1A-B [↗](#)).

Notably, the proportions of non-neural cells were changed extensively in O (**Figure 1C**). For example, Oligo, OPC, and Micro were increased, and Astro, Tany, Fibro, PTC, and Endo were decreased in O compared with Y. The proportion of Oligo, OPC, and Micro were increased in O.T than those in Y. Endo was also increased in O.T. The proportions of Astro, Tany, Epen, and PTC were decreased more in O.T than those in O in comparison with those in Y. The results indicated that 17 α -estradiol treatment had extensive effects on the proportions of non-neural cells in hypothalamus.

Cell communication analysis indicated that the ligand–receptor pairs between neurons and other cell types also changed remarkably, such as those in Endo, Fibro, Tany, and Atro (**Figure 1D**). The significant ligand–receptor pairs among Neurons were changed in O.T and O, especially in O (**Figure 1E**). Notably, of 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 Neuron.O, in comparison with neurons of O.T and Y, which suggested 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. These ligand–receptor pairs were related to or belonged to synaptic activity, cellular adhesion, opioid system, and vasodilation, which indicated unique roles of 17 α -estradiol in restoring certain physiological functions in aging hypothalamus. The enhanced *Pomc* signal in O.T neurons is in line with prior report that 17 α -estradiol treatment decreased food uptake in mice is potentially correlated with *Pomc* neurons [11].

Gene set enrichment analysis (GSEA) based on DEGs also verified the expression profiles in stress responses and synaptic activity in neurons (**Figure 1F**). ROC analysis of significantly differently expressed pathways related to neural synapse manually selected from Gene Ontology databases showed that most top-ranked pathways related to synaptic activity according to area under the curve (AUC) values were downregulated in aged neurons, and that 17 α -estradiol treatment reversed this pattern (**Figure 1G**, **Table S1**).

Together, the findings suggested that 17 α -estradiol extensively reshaped the cell populations, cellular communication, neuropeptide secretion, and neural synapse activity in aged hypothalamus, which was distinct from that of the young hypothalamus and aged hypothalamus.

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

In order to monitor the metabolism and neural status interfered by 17 α -estradiol, energy metabolism pathway MitoCarta OXPHOS subunits was used to calculate the positively-or negatively-correlated pathways in hypothalamic neurons (**Figure 2—figure supplement 1**). Energy metabolism and synapse activity was thus revealed to be two opposing regulatory signaling networks in hypothalamic neurons and 17 α -estradiol strongly balanced these two opposing signaling networks (**Figure 2A**). Underlying these opposing signaling pathways were two category opposing transcriptional factors (TFs) (**Figure 2B**). For example, *Calr*, *Clu*, *Peg3*, *Prnp*, *Ndufa13*, *Actb*, *Ywhab*, *Nfe2l1*, *Mtdh*, *Npm1*, *Bex2*, *Aft4*, and *Maged1* were positively correlated with pathways in OXPHOS subunits, lysosome, protein export, mTorc1 signaling, unfolded protein response (UPR) in O, O.T and Y neurons, and were negatively correlated with pathways in ubiquitin mediated proteolysis, endocytosis, tight junction, focal adhesion, axon guidance, and MAPK signaling. Additionally, TFs *Myt1l*, *Ctnnd2*, *Tenm4*, *Camta1*, *Med12l*, *Rere*,

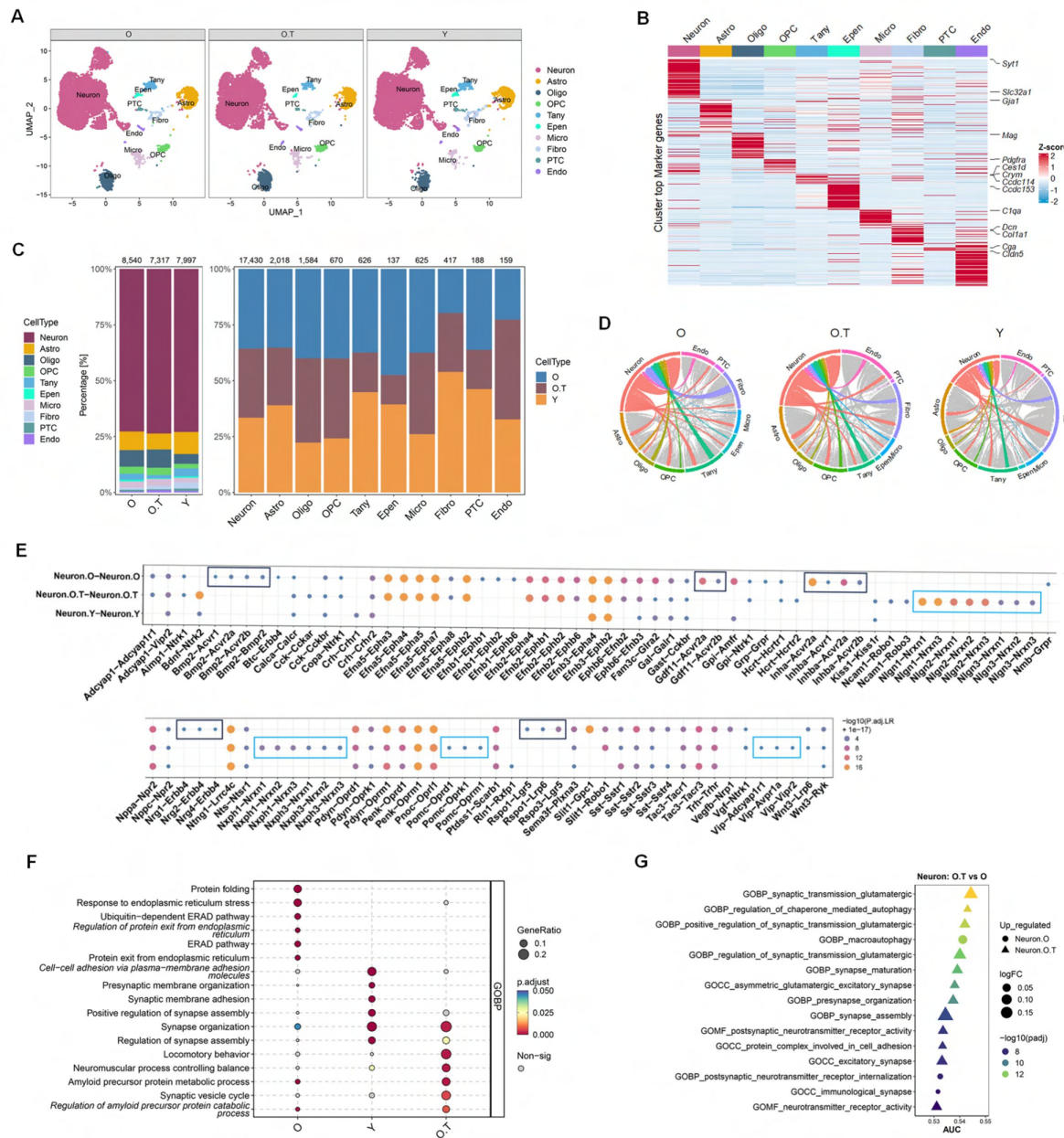


Figure 1.

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), 17 α -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 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.

Csrnp3, *ErbB4*, *Jazf1*, *Dscam*, *Klf12*, and *Kdm4c* had 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 neurons of the hypothalamus.

Then, we tried to establish gene signatures to represent these two opposing signaling networks to display the cell status of aging and evaluate the effect interfered by 17 α -estradiol. To accomplish this, 4 quarters (c1-c4) according to the expression level of each of the 6 selected pathways from the two opposing signaling networks were evenly divided in quantity among the mixed neurons from O, O.T and Y and the shared unique markers in each quarter were calculated (**Figure 2C**, **D**). From the distribution patterns we revealed that the neuron proportion decreased in O from c1 to c4 in metabolism pathways (MitoCarta OXPHOS subunits and Hallmark mTorc1 signaling), and this trend was reversed in the opposing signaling pathways (GOBP synapse organization and KEGG Mapk signaling pathway) (**Figure 2C**). But in Y this trend was on the contrary, implying the expression level of 4 quarters (c1-c4) from the two opposing signaling networks can be used to monitor the aging status. 17 α -estradiol treatment alleviated this trend or even reversed the trend in O. We then screened the shared unique markers of each quarter from the 6 selected pathways to try to establish the signatures representing the two opposing signaling networks. Unique markers in c1 (19 genes, c1.up.signature) and c4 (12 genes, c4.up.signature) were obtained, but c2 and c3 didn't have unique markers shared by the 6 pathways (**Figure 2D**). Accordingly, the 19 genes in c1.up.signature displayed conversed correlation pattern with the 12 genes in c4.up.signature, indicating the two opposing signatures are capable of reflecting the two opposing signaling networks in hypothalamic neurons (**Figure 2E**). But the two opposing signaling networks balanced by 17 α -estradiol in non-neural cell types were not so striking as that in neurons, displaying variable effects on non-neurons (**Figure 2—figure supplement 2**). Therefore, in this report we mainly focused on hypothalamic neurons and its response to aging and 17 α -estradiol.

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

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

To further determine the most affected neurons in response to aging and 17 α -estradiol, we calculated the frequency of each neuron subtype by combining the top 10 neurons in each of the 16 pathways and signatures (**Figure 3C**, **Table S2**). Serpine1-secreting neurons were enriched exclusively in aged hypothalamus, suggesting this type of neuron was an effective target of 17 α -estradiol. Additionally, *Mlnr*, *Nmb*, *Pomc*, *Ednra*, *Serpine3*, *Gast*, and *Pcsk6* neurons were all affected by 17 α -estradiol (**Figure 3D**). *Galp*, *Glp1r*, *Serpig1*, *Sstr2*, *Sstr3*, and *Vip* neurons were enriched in O, O.T and Y hypothalamus, suggesting a lack of effect of 17 α -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 the consequence of side effects or compensatory effects by 17 α -estradiol treatment.

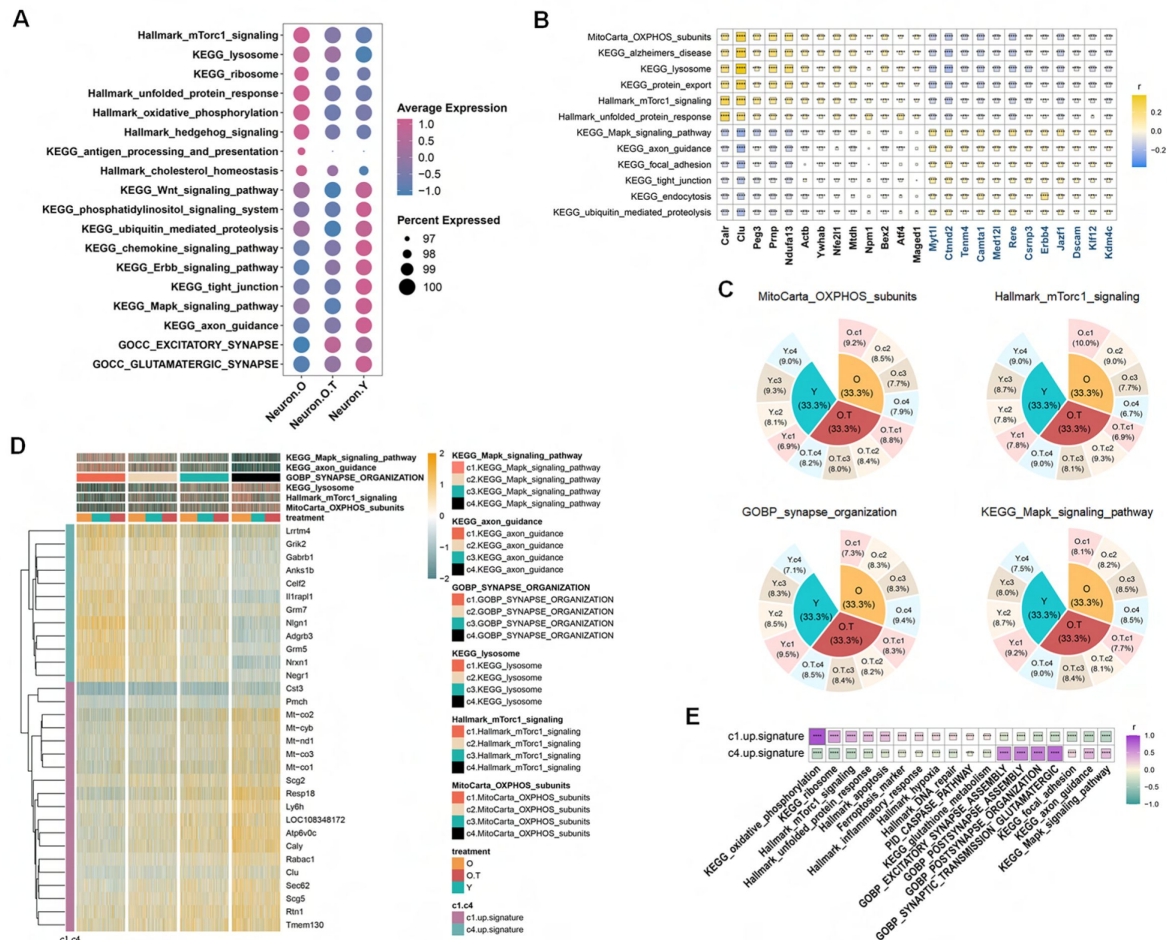


Figure 2.

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.

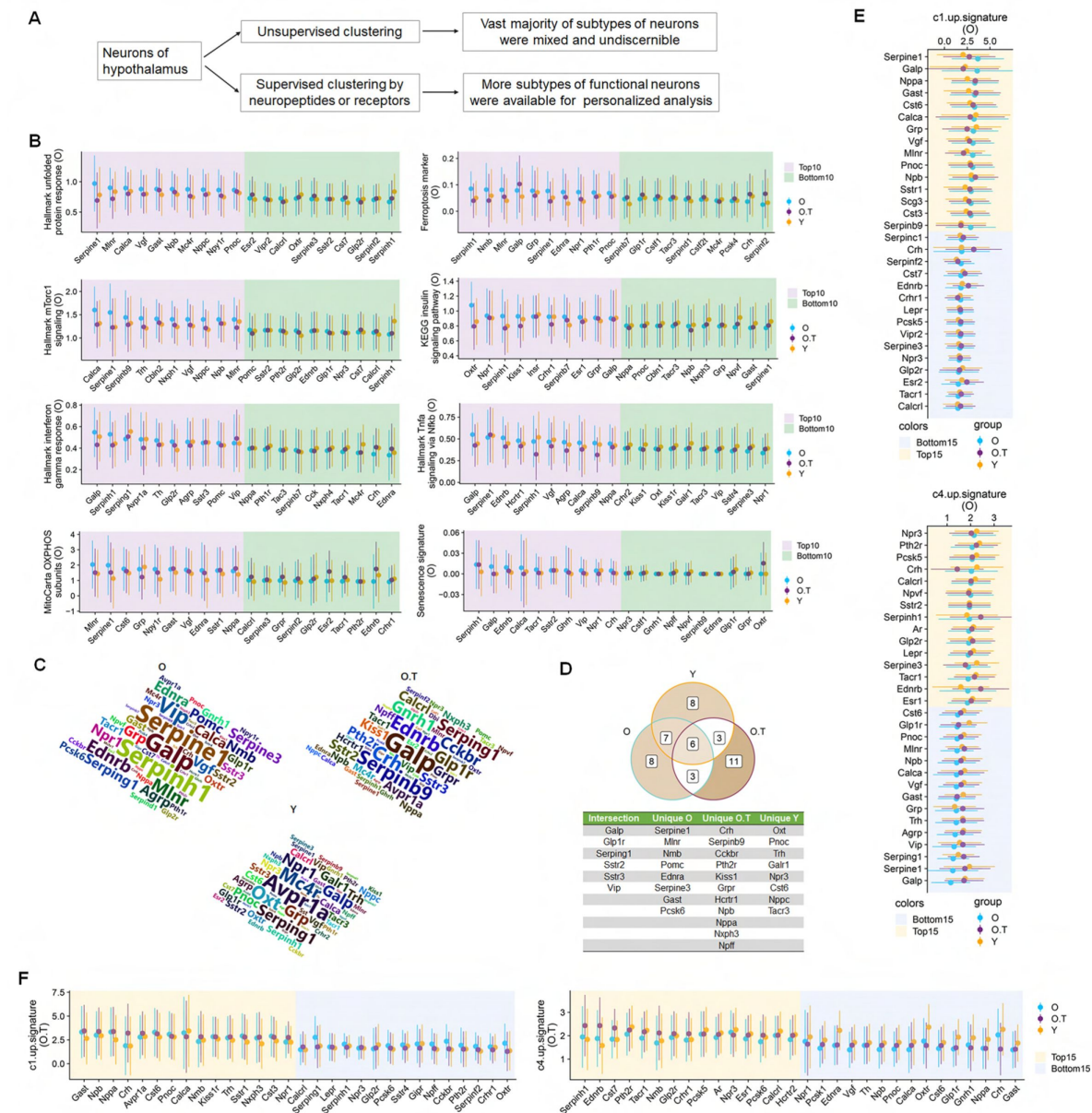


Figure 3.

Screening of neuron subtypes by supervised clustering that responded distinctly to aging and 17 α -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 the least affected neurons by aging via 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 neuron subtypes in aged hypothalamus which was similar to the combined ranks of several pathways (**Figure 3—figure supplement 1**). *Npr3*, *Crh*, *Ar*, *Esr1*, and *Esr2* neurons were among the least affected neuron subtypes in aged hypothalamus (**Figure 3E**). The neuron subtypes ranked by c1.up.signature and c4.up.signature in O.T indicated that neurons *Gast*, *Npb*, *Nppa*, and *Crh* were affected most by the treatment of 17 α -estradiol (**Figure 3F**). And neurons *Oxtr*, *Glp1r*, *Gnrh1*, and *Crh* also showed lowest levels of c4.up.signature, indicative of aging phenotype. Meanwhile, neurons *Glp2r*, *Ar* and *Esr1* were among the top ranked neurons by the level of c4.up.signature indicating higher synapse activities and relatively less stress status in these types of neurons in O.T.

These results indicated that supervised clustering of each subtype of neurons facilitated the visualization of different subtypes of neurons in response to aging and medical treatments. Additionally, it also strongly suggested that *Crh*, *Oxtr*, *Glp1r*, *Gnrh1*, *Glp2r*, *Ar*, and *Esr1* neuron subtypes were most sensitive to long-term 17 α -estradiol treatment, which were associated with appetite, glucose metabolism, stress, and sex hormone secretion and signaling.

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

To further investigate the potential side effect or compensatory effect of 17 α -estradiol treatment, we performed stricter screening by intersection of the top 10 ranks in UPR, OXPHOS subunits, and ferroptosis signature (**Figure 4A**). *Crh* and *Nppa* were the only two intersected neuron subtypes with this strict screening. 17 α -estradiol elevated several key metabolic pathways in *Crh* neurons comparing with that in Y and O (**Figure 4B**). 17 α -estradiol treatment also increased c1-up-signature, and at the same time lowered many pathways associated with synapse activity and c4-up-signature in *Crh* neurons of O.T, indicative of senescent phenotype in *Crh* neuron. But in *Nppa* and *Nppc* neurons, decreased c1-up-signature in O.T implied lesser extent of senescent phenotype in these neurons than *Crh* neurons. The senescence of *Crh* neurons was also verified by the increased number of DEGs of mitochondria-expressed genes in O.T and lowered the number of DEGs in pathway adherens junction (**Figure 4C**). The status of *Crh* neurons in O.T may be associated with the elevated TF activities of *Esr2*, *Usf2*, *Hdac5*, *Creb3l1*, *Tfam*, *Preb*, *Pou3f2*, and *Hoxb5* (**Figure 4D**).

Notably, the HPA axis was altered by 17 α -estradiol treatment as evidenced by the elevated cortisol in O.T comparing with O ($p = 0.078$) (**Figure 4E**). The correlation of the elevated cortisol production and the increased senescence in *Crh* neurons by 17 α -estradiol treatment needs further investigation. Additionally, as a key knot of renin-angiotensin-aldosterone system, the significantly increased serum aldosterone in O.T and its potential role in sodium reabsorption and cardiovascular fitness also needs further detailed investigation (**Figure 4E**).

In summary, 17 α -estradiol treatment changed the activity of HPA axis in male BN rats and also brought increased potential side effects to *Crh* neuron.

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

Aging and 17 α -estradiol treatment also changed the proportions of various neuron subtypes among O, O.T and Y (**Figure 5A**, **B**, **Table S3**). The proportions of *Grp*, *Pmch*, *Npb*, *Serpinh9*, *Sstr2*, *Agrp*, *Sstr3*, *Mlnr*, and *Hcrt* neurons ranked in the top 10 in O, and *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*, *Cck*, *Crh*, *Tacr1*, and *Kiss1* neurons were the bottom 10 neurons in O, and *Oxtr*, *Galp*, *Agrp*,

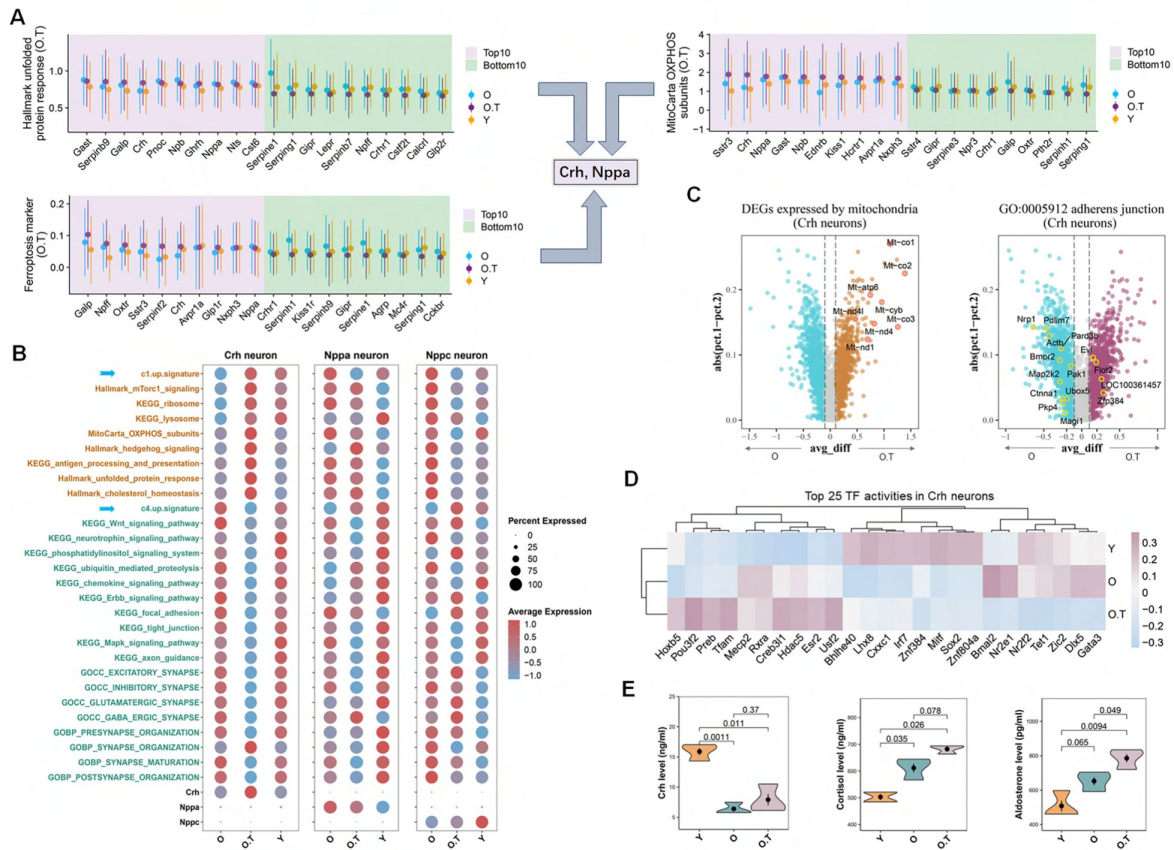


Figure 4.

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.

Serpinb9, *Npvf*, *Serpinh1*, *Ednrb*, *Agt*, *Gipr*, and *Pomc* neurons were 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 upon hunger and *Pomc* neurons are activated by satiety in the ARC of the hypothalamus. 17 α -estradiol treatment effectively elevated the expression profiles of the c4.up.signature and synapse activities in neuron subtypes *Agrp*, *Pomc*, *Oxt* and *Glp2r* in O.T in comparison with O, which may relieve the adverse effects of reduced cell populations in *Pomc* and *Agrp* neurons in aging hypothalamus (Figure 5C), which indicated a potential role of 17 α -estradiol in appetite control, as has been previously reported [11]. Additionally, 17 α -estradiol treatment resulted in elevated proportions of *Vip*, *Avp*, *Npff*, *Calca*, and *Tacr1* neurons, which ranked in the top 10, and decreased proportions of *Agt* neurons, all of which are associated with blood pressure regulation (Figure 5—figure supplement 1). Notably, the proportions of *Oxt* neurons and *Glp2r* neurons, two types of neurons with anorexigenic effects [26, 27], were increased in O.T. In addition to the increased number of *Oxt*-positive neurons, the expression level of *Oxt* was also increased in O.T. The elevated synapse activities were also evidenced by the increased DEGs in enriched pathway synaptic membrane in *Oxt* neurons (Figure 5D). More importantly, serum level of *Oxt* was significantly elevated in O.T in comparison with O ($p=0.04$), though its level was still lower than that of Y (Figure 5E). Unfortunately, the top TF activities in O.T and O were much different from those in Y (Figure 5F). The elevated *Hopx* and *Xbp1* may be associated with the response to 17 α -estradiol treatment.

Due to the intricate regulatory networks between various endocrine factors, the elucidation of the causal effect of *Oxt* on other endocrine factors is thus very complicate through traditional methods. MR analysis with variant SNPs as genetic tools has much advantageous in such task. We thus performed 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 IEU (Table S4). As an exposure, OXT was revealed to be the 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. The significance of these significant associations was not found in the reverse MR analysis (Figure 5—figure supplement 2A, B). Unfortunately, we were unable to screen 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, indicative of their complicate causal effects (Figure 5—figure supplement 2A, B). The results suggested that elevated *Oxt* levels induced by 17 α -estradiol may have positive associations with endocrine factors governing feeding behavior, glucose metabolism, male reproduction, and sex hormones. Therefore, OXT was a potential mediator of 17 α -estradiol.

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

Since *Gnrh* and sex hormone expressed neuron subtypes were sensitive to 17 α -estradiol treatment (Figure 3F, Figure 3—figure supplement 1), we then checked their expression profile with representative pathways of two opposing signaling networks of metabolism and synapse, including c1-up-signature and c4-up-signature (Figure 6A). But both c1-up-signature and c4-up-signature were not up-regulated in *Gnrh1* neuron in O.T, which was similar to *Esr2* neuron. Only in *Esr1* neuron, the c1-up-signature was up-regulated. Meanwhile, both *Ar* and *Esr* neurons also displayed high level of c4-up-signature, indicative of relatively healthy status. The data implied that most of the 4 types of sex hormone-related neurons didn't experience severe aging in O.T. However, from these expression profile, it's hard to define the exact physiological status of these subtype of neurons, especially neural endocrine activities. Therefore, we further performed

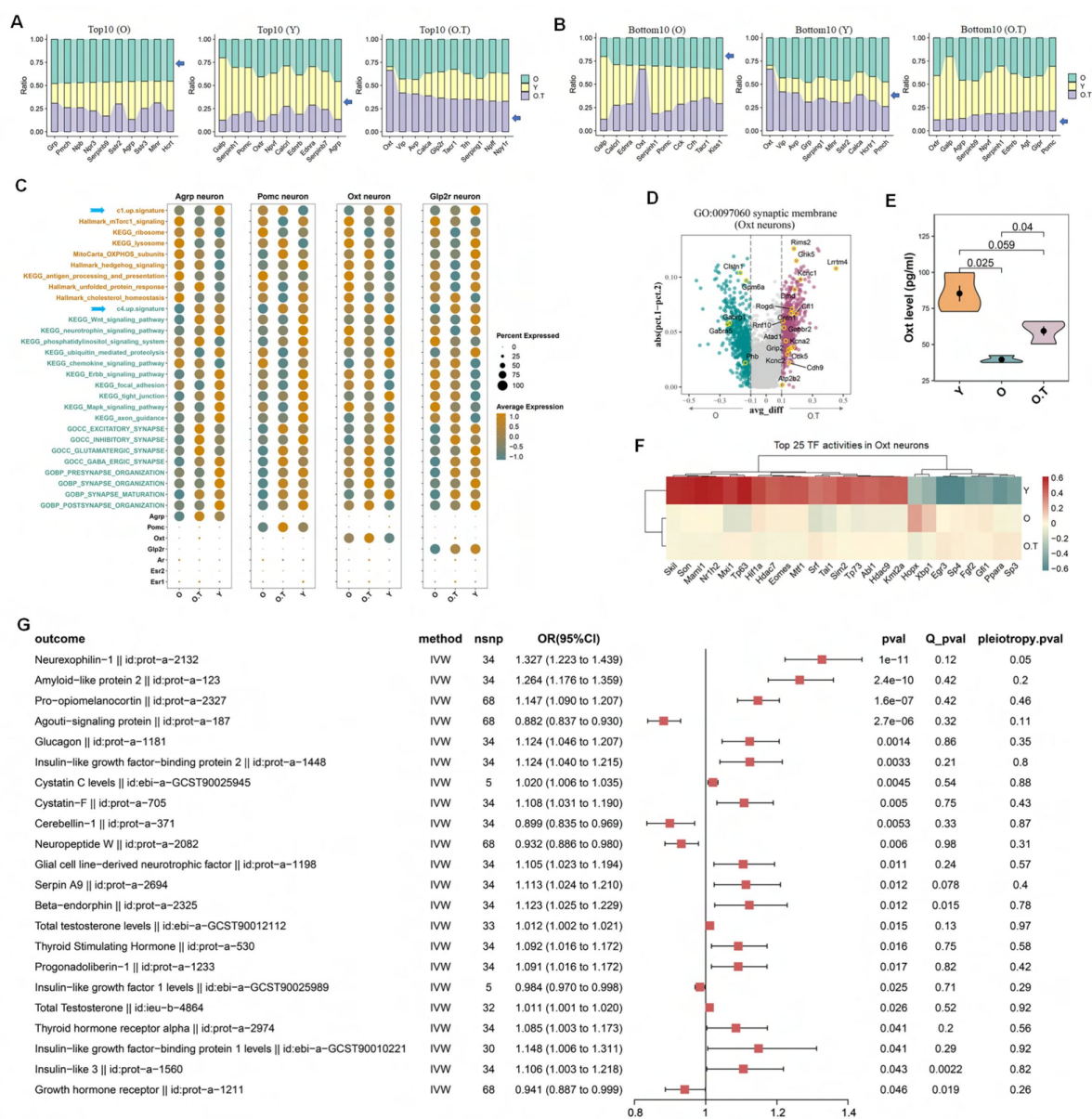


Figure 5.

The response of Oxt neurons to 17 α -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 ($pval < 0.05$).

enzyme immunoassays of hormones from the serum of O, O.T and Y. 17 α -estradiol treatment significantly increased the plasma level of GnRh in comparison with Y ($p = 0.0099$) and O ($p = 0.096$) (**Figure 6B**). More interestingly, testosterone level in serum was significantly increased in O.T in comparison with O ($p = 0.018$) and Y ($p = 0.052$). Meanwhile, the serum level of estradiol was significantly increased in O comparing with Y ($p = 0.011$) and significantly decreased in O.T in comparison with O ($p = 0.019$), suggesting 17 α -estradiol treatment strongly altered the homeostasis of testosterone and estradiol. Additionally, most testes from 30-month-old male BN rats experienced severe age-related inflammation and epithelial collapse of seminiferous tubules (**Figure 6C**). The testes without inflammation in O.T displayed normal morphology. 17 α -estradiol treatment slightly decreased the testis inflammation in O.T in comparison with that in O ($p = 0.15$), indicating a positive role of 17 α -estradiol treatment in male reproductive system. Additionally, the elevated transcriptional factors as *Sf1*, *Pparg*, *Litaf*, *Nupr1*, *Rxrg*, *E2f2*, and *Zfp42* may be involved in the transcriptional regulation by 17 α -estradiol in O.T (**Figure 6D**). More importantly, both the activities of androgen and estrogen pathways were decreased in *Gnrh1* neurons in O.T comparing with O and distinct to those in *Ar*, *Esr1*, and *Esr2* neurons (**Figure 6E**). These signaling is important for the feedback controlling of sex hormone secretion in *Gnrh* neurons. This result may also reflect the strong effect of 17 α -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. The 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 complicated causal effects between GNRH1 and these endocrine factors (**Figure 6G**, **Figure 6—figure supplement 1A, B**). These results also suggested that the role of 17 α -estradiol treatment in feeding, energy homeostasis, reproduction, osmotic regulation, stress response, and neuron plasticity may be mediated at least by elevated GnRh secretion.

Discussion

The most striking role of 17 α -estradiol treatment revealed in this study showed that HPG axis was substantially improved. The underlying molecular mechanism is still unknown. But prior report indicated that 17 α -estradiol is able to bind ESR1 [15]. In this report, 17 α -estradiol treatment significantly decreased serum estradiol and elevated serum testosterone. Combining these information, we propose 17 α -estradiol may have similar roles of estrogen antagonists or the roles of aromatase inhibitors, which may prevent the conversion of androgens to estrogens [28, 29]. These roles can remove the feedback inhibition by estrogen on hypothalamus and pituitary to secrete GnRh, FSH, and LH [30].

The testosterone levels declined gradually from the third decade of life in men [31]. Ageing of the gonadotropic axis, especially aged males with low serum testosterone, is usually associated with many aging symptoms as the age-related loss of skeletal muscle mass, muscle strength, muscle power, low bone mineral density, frailty, impaired physical performance, mobility limitation, increased risk of diabetes, higher all-cause mortality and cardiovascular mortality, cognitive decline, and increased risk of Alzheimer's disease [32]. Therefore, testosterone supplementation to older men is benefit. Additionally, GnRh supplement alleviates aging-impaired neurogenesis and thus decelerates aging [33]. 17 α -estradiol treatment didn't lead to

feminization and didn't affect the sperm parameter, fertility in male animals [6, 13]. Therefore, the increased GnRH levels and serum testosterone by 17 α -estradiol treatment is likely benefit to older males especially to those with syndrome of late-onset hypogonadism.

Similar aging syndrome to aged males with low serum testosterone, postmenopausal women with low estrogen also experience increased mortality, cardiovascular disease, osteoporosis fracture, urogenital atrophy and dementia, which are also benefit from hormone therapy [34]. But why 17 α -estradiol treatment doesn't display positive life extension roles in aged females according to prior report [12]? The underlying key point may be the inhibition of estrogens by 17 α -estradiol treatment. This is also evidenced by the inability of 17 α -estradiol to improve female fertility [35]. However, due to lacking parallel data in aged female BN rats treated with 17 α -estradiol, the exact answer to this question needs more investigation in the females in the future.

Another striking effect of 17 α -estradiol to hypothalamus neurons was to decrease the overall energy metabolism in aged male BN rats. The nutrient-sensing network, mediated by mTORC1 complex, is a central regulator of mRNA and ribosome biogenesis, protein synthesis, glucose, autophagy, lipid metabolism, mitochondrial biosynthesis, and proteasomal activity [36]. Downregulation of the nutrient-sensing network can increase lifespan and healthspan [37]. 17 α -estradiol treatment attenuated nutrient-sensing network activity in most hypothalamic neurons, which promoted lifespan extension. Additionally, synaptic aging leads to a decline of synaptic activity and estrogen improves the performance of aging synapses [38]. In line with this, our data showed that the benefit of improved synaptic activity by 17 α -estradiol treatment was prominent in aging male hypothalamus.

In this report, we showed that neuron populations in appetite control, specifically *Agrp*, *Pomc*, *Oxt*, *Oxtr*, *Gipr*, and *Glp2r* neurons, were extensively changed. The *Oxt* neuron proportion was elevated the most by 17 α -estradiol in all detected neuron subtypes (Figure 5A). *Oxt* plays versatile roles in social behavior, stress response, satiety, energy balance, reproduction, and inflammation [39]. Most *Oxt* neurons are produced by the PVN and supraoptic nuclei (SON) in hypothalamus with very high plasticity during development and intricate circuits [40, 41]. PVN, ARC, and ventromedial hypothalamic nucleus compose the neural hub in the hypothalamus that integrates peripheral, nutritional, and metabolic signals to control food intake behavior and energy balance [42]. Many effects of *Oxt* are sex-specific [43]. For example, females are less sensitive to exogenous *Oxt* than males in social recognition [44]. More interestingly, *Oxt* injection, which was delivered via improved blood-brain barrier penetration nanoparticles, decreased body mass, and increased social investigation and *Oxt*-positive cell number in the SON of hypothalamus preferentially in male rats [45]. Intracerebroventricular *Oxt* injection in rats showed that food intake was reduced in both sexes with a more pronounced effect in males [46]. Therefore, we propose that the role of *Oxt* in systemic aging and feeding behavior is another potential contributing factor in mediating the sex-biased effects of 17 α -estradiol.

17 α -estradiol treatment apparently induced elevated aging of HPA axis. One evidence was the increased senescence of *Crh* neurons. The other evidence was the elevated serum cortisol, which is a hallmark of aging HPA axis [47, 48]. Therefore, more attentions should be paid to the potential side effects of 17 α -estradiol especially in its clinical application.

Together, the findings suggest that HPG axis and neurons involved in appetite and energy balance were improved by 17 α -estradiol treatment, which may be closely related to the life-extension roles of 17 α -estradiol in aged males. Additionally, supervised clustering by neuropeptides, hormones and their receptors is proved to be a useful strategy to investigate the pharmacological, pathological and physiological processes in each subtype of neurons in 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: tanyocyte; TF: transcription factor; UPR: unfolded protein response.

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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

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Reviewer #1 (Public Review):

Summary:

Previous studies have shown that treatment with 17 α -estradiol (a stereoisomer of the 17 β -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 17 α -estradiol (treated for 6 months). This study identifies genes and pathways affected by 17 α -estradiol in the aged hypothalamus.

Strengths:

Using single-nucleus transcriptomic sequencing (snRNA-seq) on the hypothalamus from aged male rats treated with 17 α -estradiol they show that 17 α -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 17 α -estradiol's effects on energy homeostasis. Furthermore, they show that CRH neurons exhibited a senescent phenotype, suggesting a potential side effect of the 17 α -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 17 α -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 is not known whether 17 α -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 17 α -estradiol on non-neuronal cells such as microglia and astrocytes is not well-described (Figure 1). Previous studies demonstrated that 17 α -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.

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

(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.

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Reviewer #2 (Public Review):

Summary:

Li et al. investigated the potential anti-ageing role of 17 α -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 17 α -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 17 α -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 17 α -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 17 α -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.

(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.

(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.

Weaknesses:

- (1) One main control group is missing from the study, the young males treated with 17 α -Estradiol.
- (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.
- (3) Although the authors claim to have several findings, the data fail to support these claims.
- (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.
- (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.

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