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# LncRNA-mRNA integrated networks in the neuroendocrine system of bisphenol a-treated mice induce cellular dysfunctions by disrupting transcriptional homeostasis

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## ABSTRACT

Bisphenol A (BPA) is a widely used xenoestrogen that can disrupt neuroendocrine and immune regulation through multiple hormone receptors. This study investigated BPA-induced long non-coding RNA (lncRNA)-mRNA interactions in the cerebral cortex and hypothalamic-pituitary-thyroid (HPT) axis of adult male mice. Transcriptome sequencing and comprehensive lncRNA annotation identified 14,858 novel lncRNA transcripts. Integrated network analysis using weighted gene co-expression network analysis (WGCNA) revealed four distinct tissue-specific modules: neuronal signaling alterations (*Tac1*, *Htr1b*, *Npy*), RNA splicing modifications (*Srsf5*), PI3K/Akt-mediated cellular dysfunction (*Creb5*, *Cdkn1a*), and immune receptor signaling disruptions (*Trbv15*, *Fcrla*). These findings suggest that BPA reprograms transcriptional networks in a tissue-specific manner, potentially disrupting hormone-related neurotransmission, metabolic regulation, and immune signaling via lncRNA-mediated mechanisms. Such systems-level reprogramming of the immune-neuroendocrine network (INEN) provides novel mechanistic insights and biomarker candidates for assessing and mitigating the health impacts of environmental endocrine disruptors.

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Bisphenol A; hypothalamus-pituitary-thyroid (HPT) axis; mRNA-lncRNA interaction network; immune-neuroendocrine network (INEN); transcriptional reprogramming

## 1. Introduction

Endocrine-disrupting chemicals (EDCs) encompass external compounds or combinations of compounds present in the environment that perturb the production, transport, metabolism, regulation, breakdown, and action of hormones (Casals-Casas and Desvergne 2011). EDCs affect hormone targets by acting as agonists or antagonists on receptors, mimicking hormones, or obstructing the normal action of hormones (Wetherill et al. 2007). This interference can lead to developmental, reproductive, and behavioral abnormalities (Schug et al. 2011). Furthermore, recent studies have linked EDC exposure to broader health conditions including obesity, metabolic syndrome, type 2 diabetes, neurological disorders, and immune system dysfunction (Haverinen et al. 2021). Notably, EDCs impact not only nuclear hormone receptors but also non-classical membrane-bound hormone receptors, whose dysregulated activity can have major systemic impacts extending beyond single tissue effects (Guarnotta et al. 2022).

Bisphenol A (BPA) (Rogers et al. 2013), a commercially important and extensively studied EDC, is widely employed for manufacturing epoxy resins and polycarbonate plastics (Tsai 2006). BPA is a xenoestrogen known for binding to and activating estrogen receptors (ER $\alpha$  and ER $\beta$ ) (Shanle and Xu 2011), but also interacts with multiple hormone receptors including glucocorticoid receptor (GR) and the membrane-bound G protein-coupled estrogen receptor (Costa and Cairrao 2024). These multi-receptor interactions have important implications for immune function, as BPA and its analogs significantly impact immune system development and function, particularly during prenatal and early postnatal periods critical for microglial maturation and blood-brain barrier integrity (Santoro et al. 2019; Costa and Cairrao 2024). BPA also influences tumor

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development and progression through interconnections between cancer and immune cells within the tumor microenvironment (Kwon 2022).

The interconnection between immune and neuroendocrine systems forms the immune-neuroendocrine network (INEN), which is particularly relevant for understanding EDC effects (Ogidi et al. 2025). BPA exposure can disrupt this network through GR engagement, leading to alterations in brain-derived neurotrophic factor (BDNF) regulation and hypothalamic–pituitary–adrenal (HPA) axis function (Buoso et al. 2025). Given the shared involvement of the hypothalamus in both hypothalamic–pituitary–thyroid (HPT) and HPA regulation, BPA-induced transcriptional changes may reflect broader neuroendocrine dysregulation beyond the HPT axis alone.

The cerebral cortex, a complex association of neurons covering the outermost part of the brain within the central nervous system (CNS), is a tissue that responds sensitively to EDC (Parent et al. 2011; Javed et al. 2019). In the interplay between the cerebral cortex and hormones, thyroid and sex steroid hormones interfere with neuroendocrine regulation, with estrogen reported to act as a neurotransmitter (Chen et al. 2018). The HPT axis, a key player in hormone regulation and homeostasis, is controlled through intricate feedback processes in the endocrine system (Zoeller et al. 2007). Therefore, investigating the potential disruption of the HPT axis due to BPA exposure would enhance the general understanding of the underlying regulatory mechanisms affecting the body (Ma et al. 2019). However, the regulatory processes accountable for potential harm to the HPT axis due to BPA exposure remain unknown. The knowledge on regulatory mechanisms can be considerably enhanced by studying the interactions between long non-coding RNAs (lncRNAs) and mRNAs, both of which play key regulatory roles.

lncRNAs are longer than 200 nucleotides, lack protein-coding ability, and have open reading frames (ORFs). While lncRNAs have lower expression levels than protein-coding genes (Wu Z et al. 2014), they facilitate rapid effects because they do not undergo translation and have a rapid turnover rate (Zeng et al. 2023). Importantly, some lncRNAs contain cryptic ORFs that can produce proteins with regulatory functions, adding another layer of complexity to their biological roles (Mattick et al. 2023). Furthermore, they have specific structural characteristics and play pivotal functions in various biological processes (Wang C et al. 2017). lncRNAs are located in exonic, intronic, and intergenic regions and often have enhancers situated between protein-coding genes. They can be categorized into sense and complementary antisense regions based on their relative orientation and distribution in the genome (Ransohoff et al. 2018). Together, these features enable lncRNAs to regulate gene expression on multiple levels, operating via both *cis* (neighboring) and *trans* (distal) sequence interactions (Gil and Ulitsky 2020). The regulatory activities of lncRNAs influence various aspects of physiology, such as cell differentiation, development, maintenance of genome integrity, genomic imprinting, and responses to stimuli, playing essential roles in neuronal, muscular, cardiovascular, adipose, and hematopoiesis functions (Fatica and Bozzoni 2014; Zhang et al. 2019; Statello et al. 2021). Moreover, their expression patterns exhibit high specificity concerning tissue, species, and condition, suggesting potential superiority over proteins in targeting-related potential and avoidance of undesirable toxic effects, making them potential therapeutic targets (Schmitz et al. 2016; Statello et al. 2021). These specific features indicate their potential as optimal biomarkers (Chandra Gupta and Nandan Tripathi 2017). However, the incomplete annotation of lncRNAs and the presence of many with unknown functions pose challenges in interpreting their impact on gene expression (Lorenzi et al. 2019).

This study mainly aimed to elucidate the transcriptional mechanisms by which BPA, a xenoestrogen, affects hormone secretion and immune dysregulation in the cerebral cortex and HPT axis. Additionally, it sought to annotate novel lncRNAs in the cerebral cortex and HPT axis of BPA-treated mice and provide a comprehensive understanding of the regulatory mechanisms. This involved identifying lncRNA–mRNA co-expression patterns through integrated network analysis to determine the functions of lncRNAs interacting with mRNAs.

## 2. Materials and methods

### 2.1. Ethical statement

The Institutional Animal Care and Use Committee of Chung-Ang University, Seoul, Republic of Korea, approved all animal experiments (IACUC Number: 202301020018). These experiments were conducted in strict adherence to the standard guidelines for animal studies.

## 2.2. Experimental design

The experimental design is illustrated in Figure S1. This study utilized eight 4-week-old CD-1 (ICR) male mice housed under a 12-h day/12-h night cycle at 20–25°C temperature and 50–60% humidity. The exclusive use of male mice represents a study limitation, as BPA exhibits sex-specific effects due to its xenoestrogenic properties and differences in hormone receptor distribution between sexes. The neuroendocrine and immune impacts of BPA may differ in females due to variations in estrogen receptor expression and regulatory feedback mechanisms. Following a one-week acclimatization period, the mice were divided into two groups ( $n = 4/\text{group}$ ): a control group and a BPA-treated group. The BPA dose of 50 mg/kg bw/day was selected based on U.S. Environmental Protection Agency guidelines as the lowest observed adverse effect level (LOAEL) (Vandenberg et al. 2013). This dose, while higher than typical human exposure levels (which range from ng/kg to  $\mu\text{g/kg}$  bw/day), allows for detection of transcriptomic changes and represents an established reference point for toxicological studies. While high-throughput omics studies commonly employ small sample sizes ( $n = 4$ ), this limitation is acknowledged, though biological replicates and analytical stringency help mitigate reproducibility concerns. The control mice received an oral gavage of vehicle (corn oil) only. The male mice received daily oral administration, with the dosage adjusted according to their daily body weight, from 6 to 11 weeks of age (6 weeks of treatment). A one-week stabilization period was observed before sample collection. Subsequently, the cerebral cortex, hypothalamus, pituitary, and thyroid of each mouse were collected. Tissues were preserved overnight at 4°C in RNAlater (Invitrogen, Carlsbad, CA, USA) and snap-frozen in liquid nitrogen (−196°C) for RNA extraction.

## 2.3. RNA extraction and cDNA library construction

Total RNA was extracted using the QIAzol lysis reagent (Qiagen, Hilden, Germany), following the manufacturer's guidelines. The concentration of total RNA was determined using Quant-IT RiboGreen (Invitrogen). To assess the integrity of the total RNA, samples were analyzed using the TapeStation RNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Only high-quality RNA preparations with an RNA Integrity Number (RIN) greater than 7.0 were used for RNA library construction. For library preparation, 1  $\mu\text{g}$  of total RNA from each sample was independently processed using the Illumina TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA). Initially, rRNA was selectively removed from the total RNA using the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) (Illumina, Inc). Subsequently, the remaining total RNA was fragmented into small pieces using divalent cations under elevated temperatures. The resulting cleaved RNA fragments were reverse-transcribed into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. Subsequently, second-strand cDNA synthesis was performed using DNA Polymerase I, Rnase H, and dUTP. These cDNA fragments were subjected to an end repair process, which included the addition of a single 'A' base and adapter ligation. The products were purified, enriched using PCR, and used to construct a cDNA library. The libraries were quantified using the KAPA Library Quantification Kit for Illumina Sequencing platforms, following the qPCR Quantification Protocol Guide (Kapa Biosystems, Wilmington, MA, USA), and their quality was assessed using the TapeStation D1000 ScreenTape (Agilent Technologies). Indexed libraries were then submitted for sequencing on an Illumina HiSeq4000 platform (Illumina, Inc), and paired-end ( $2 \times 150$  bp) sequencing was performed by Macrogen Incorporation (Seoul, Republic of Korea).

## 2.4. Data processing and creation of novel annotation file

The quality assessment of raw reads was performed using FastQC (v0.11.9) software (Leggett et al. 2013), and the reads were trimmed using Trimmomatic (v0.39) to eliminate low-quality regions and adapters (Bolger et al. 2014). Hisat2 (v2.2.1) was utilized to map the resulting clean reads against the mouse reference genome (*Mus musculus*; GRCm39.104, GenBank assembly accession: GCA\_000001635.9) available on the Ensembl genome browser ([http://www.ensembl.org/Mus\\_musculus/](http://www.ensembl.org/Mus_musculus/), accessed on 16 January 2023), employing the default options of the program (Kim et al. 2019). Samtools (v1.12) was used for sorting the reads according to the specific criteria required for the subsequent application of StringTie (Li H et al. 2009).

The RNA-seq reads were generated for four tissues under control and treatment conditions. From the 32 tissue samples (from four tissues), a total of 7.8 billion sequence reads were produced, averaging 24.0 million

per sample (Table S1). After trimming, the average number of clean reads was 23.0 million sequence reads. Thus, the overall mapping rate was 94.53%.

The transcript assembly for each sample, organized using the StringTie (v2.2.1) program, was generated using the reference annotation file (Mus\_musculus.GRCm39.104.gtf) (Sun H-X and Chua 2019). The GTF files obtained from each sample were merged and compared using the gffcompare program (v0.12.6) and subsequently converted using gffread (v0.12.7). For identifying potential novel lncRNAs, transcripts were screened based on various criteria, such as transcript length exceeding 200 bp and specific class codes, including 'i', 'j', 'o', 'u', and 'x' (Pertea and Pertea 2020). TransDecoder (v5.6.0) was utilized to identify the transcripts with an ORF length of less than 300 bp (Zhao et al. 2020). Furthermore, CPC2 (Kang et al. 2017), PLEK v1.2 (Li A et al. 2014), CPAT v1.2.4 (Wang L et al. 2013), and CNCI (Sun L et al. 2013) were used to predict the coding potential of the remaining transcripts. Transcripts exhibiting a high probability of being non-coding were identified through Pfam (v33.0, The protein families) database analysis using HMMER (v3.3.2) (Finn et al. 2010) to exclude any protein-coding sequences. After applying these filtering criteria, transcripts detected in at least two samples were selected and integrated with the reference genome annotation to generate a unified GTF file for subsequent analyses.

A total of 510,885 transcripts were obtained after assembly and merging, and 507,048 transcripts remained after excluding transcripts that were less than 200 bp in length. After the class codes of the candidate lncRNA were extracted, 276,869 transcripts remained. Further filtering for transcripts with an ORF length of less than 300 bp resulted in 97,747 transcripts. Subsequently, using four coding potential assessment tools and Pfam databases, a set of 82,889 transcripts was derived. Finally, after excluding transcripts identified in only one sample, a total of 14,858 novel lncRNA transcripts were obtained (Figure 1(A)).

## 2.5. Diverse distinctive attributes of lncRNAs

To verify the specific characteristics of the newly identified lncRNA, various structural characteristics of the genome were confirmed. This analysis compared the distribution of exon number, transcript length, and genomic location by gene type with the final integrated GTF file. Given that the specificity of expression represents a notable aspect of lncRNA properties, the specificity index for lncRNA expression was calculated using the Tau index formula, expressed as:

where  $N$  is the number of tissues, and  $x_i$  is the fragment per kilobase of exon per million mapped fragments (FPKM) value of lncRNA/mRNA  $x$  in tissue  $i$  normalized by the maximum value of FPKM (Yanai et al. 2005).

## 2.6. Differential expression analyses of lncRNAs and mRNAs

Differentially expressed (DE) lncRNA and mRNA were analyzed by comparing the control and BPA-treated groups for each tissue, and eight comparisons were made according to the RNA type. The Bioconductor edge R package (v3.36.0) was used to perform all differential expression analyses using the raw counts obtained (Robinson et al. 2010). The Trimmed Mean of M-values (TMM) normalization method was applied to the raw counts (Robinson and Oshlack 2010), and multidimensional scaling (MDS) and volcano plots were visualized using the limma and ggplot2 R packages (Ritchie et al. 2015) to identify the similarities and differences between samples. The cutoff criteria for DE values of RNA types were set as  $P$ -values  $< 0.01$  and absolute  $\log_2$  fold change (FC)  $\geq 1$  (Love et al. 2014).

## 2.7. Construction networks depicting mRNA-lncRNA interactions

To explore the correlation between endocrine tissues and genes exhibiting co-expression patterns among DElncRNAs and DEmRNAs, network analysis was conducted using the Weighted Gene Co-Expression Network Analysis (WGCNA) in R package (v.1.72.1) (Langfelder and Horvath 2008). The correlation was calculated based on co-expression, and the modules (networks) displaying high correlation were established using  $\log_2$ TMM values. The 'pickSoftThreshold' function in the WGCNA R package was used to identify the optimal soft thresholds (power). The relationships between modules were evaluated using Pearson correlation. Subsequently, network reconstruction was performed using 'aracne' function in the bnlearn R

(A)

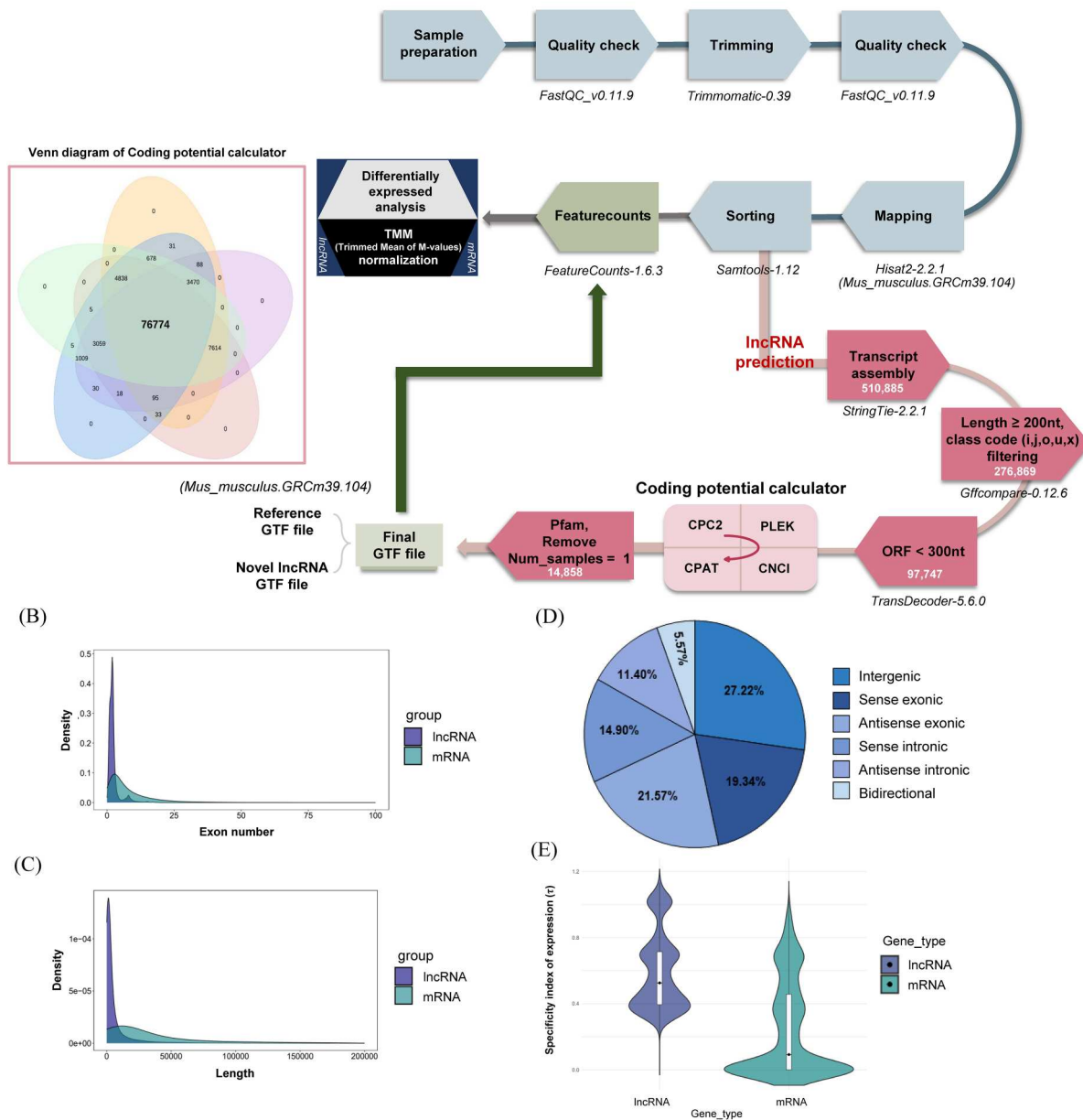


**Figure 1.** Overall data pre-processing and comparison of genomic characterization. (A) Data preprocessing process for mRNAs and lncRNAs. (B) Comparison of exon number distribution between lncRNAs and mRNAs in the generated annotation file. (C) Comparison of transcript length distribution between lncRNAs and mRNAs in the generated annotation file. (D) Visualization of the distribution of the structural characteristics of lncRNAs. (E) Comparison of the specificity index of expression of lncRNAs and mRNAs.

package (v.4.8.1) (Margolin et al. 2006), leading to the identification of four significant networks. Finally, network visualization was performed using the Cytoscape software (v3.9.0) (Shannon et al. 2003).

## 2.8. Identification of the regulatory mechanisms between lncRNAs and mRNAs

The regulatory mechanisms of lncRNAs, whether *cis* or *trans*, were determined based on their proximity to target mRNAs. Specifically, lncRNAs were observed to regulate genomic sequences in their vicinity, and the related results confirmed that such lncRNAs are found within 100 kb of transcriptional regulation-related mRNAs (Gil and Ulitsky 2020). Consequently, mRNAs within a 100 kb range upstream and downstream of the lncRNA were explored because the *cis* regulation of lncRNA is associated with its interaction with

adjacent target mRNAs (Karapetyan et al. 2013). In cases where such proximity-based interactions were not established, the regulation was categorized as *trans* regulation. To ascertain significant lncRNA-mRNA interactions, Pearson correlation analyses were conducted using lncRNA-mRNA pairs within each network based on functional annotation. Following the analysis of the main network, the observations were visualized using the circa software (v1.2.1) to illustrate the regulatory patterns of each module and the relationships between DElncRNAs and DEmRNAs.

## 2.9. Functional analyses

Database for Annotation, Visualization, and Integrated Discovery (DAVID) 2023 was employed to annotate Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the *Mus\_musculus* database, using genes with interactions between DElncRNAs and DEmRNAs in each of the networks. KEGG pathways were visualized as bar plots with  $-\log_{10}$  *P*-values and fold enrichment. The regulatory control of protein-coding genes (DEmRNAs) specifically expressed in significant pathways in each of the four tissues (cerebral cortex, hypothalamus, pituitary, and thyroid) was depicted using Pathview (Ogata et al. 1998; Luo and Brouwer 2013). GO terms were visualized using a bubble plot after filtering for *P*-value < 0.05 (Boyle et al. 2004).

## 3. Results

### 3.1. Overall characteristics of the lncRNAs in endocrine tissues

Various types of information about the predicted lncRNAs were first investigated and then compared with those of the mRNAs to determine the genomic characteristics of the identified lncRNAs. The average exon number of lncRNAs was 2.4, whereas that of protein-coding mRNAs was 11, indicating that lncRNAs tended to have fewer exons than mRNAs (Figure 1(B)). The mean transcript length of the lncRNAs was approximately 8121 bp, whereas that of the protein-coding mRNAs was approximately 49,191 bp, indicating that lncRNAs tended to be shorter than mRNAs (Figure 1(C)), as reported by previous studies (Clark et al. 2012; Derrien et al. 2012). The transcript location of the lncRNA was classified as follows: 27.22% in intergenic regions that did not overlap with the protein-coding gene, 19.34% in sense exonic, 21.57% in antisense exonic, 14.90% in sense intronic, 11.40% in antisense intronic, and 5.57% in bidirectional regions (Figure 1(D)). Finally, the specificity index was calculated to determine the specificity of expression of the lncRNAs and mRNAs. The average specificity index of the lncRNAs and mRNAs was 0.59 and 0.24, respectively, indicating higher specificity for lncRNAs than for mRNAs (Figure 1(E)).

These findings suggest that the newly identified lncRNAs exhibit typical genomic and structural characteristics consistent with previously reported lncRNA features. The lower exon number and shorter transcript length may indicate a streamlined structure enabling rapid regulatory responses without translation. Furthermore, the higher specificity index implies that these lncRNAs may exert more tissue-specific regulatory effects compared to mRNAs, suggesting their potential importance in endocrine tissue-specific transcriptional regulation following BPA exposure.

### 3.2. Tissue-specific expression patterns between lncRNAs and mRNAs

An MDS plot based on transcriptomic types (including lncRNA and mRNA) revealed distinct clusters corresponding to each tissue-specific RNA category. Notably, the cerebral cortex and hypothalamus tissues exhibited closely clustered patterns (Figure 2(A)). The expression levels of lncRNAs and mRNAs across four tissues have been visually represented with color coding for different tissues. The inner layer, lncRNA, is enclosed within the same color-coded tissue, providing a clear visualization of the specific expression patterns in each tissue (Figure 2(B)). The bar graph in Figure 3(B) clearly demonstrates that the expression levels of lncRNAs are significantly lower than those of mRNAs (Figure 2(C)). To investigate the differential expression patterns of lncRNAs and mRNAs in the treatment groups compared to those in the control group, DElncRNAs and DEmRNAs were examined for tissue-specific patterns. As evident from Figure 2(D,E), these differential expression profiles were both tissue-specific and consistent. Furthermore,

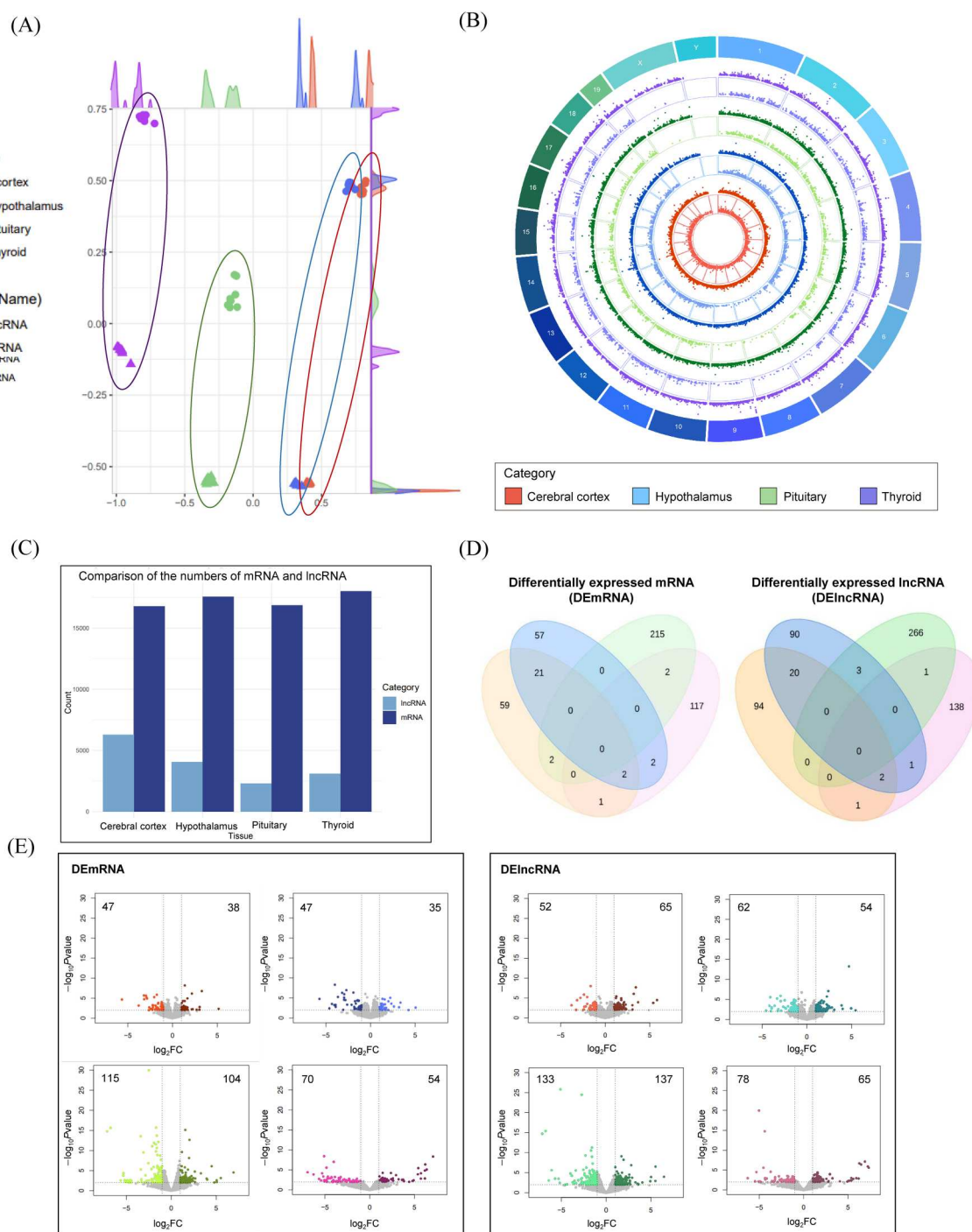


**Figure 2.** Expression patterns of differentially expressed (DE) gene types. (A) Multi-dimensional scaling (MDS) plot illustrating the lncRNA and mRNA data in four endocrine tissues. (B) Circos plot illustrating the lncRNA and mRNA expression patterns for each tissue. (C) Bar plot presenting a direct comparison between lncRNAs and mRNAs. (D) Venn diagram representing the DE mRNAs and DE lncRNAs in the four endocrine tissues. (E) Volcano plot displaying the DE mRNAs and DE lncRNAs in four endocrine tissues.

the DE genes exhibited substantial similarity, particularly in the cerebral cortex and hypothalamus, forming closely related clusters.

These results suggest that BPA exposure induces highly tissue-specific transcriptomic alterations. The similarity between the cerebral cortex and hypothalamus reflects their shared neuroendocrine regulatory functions, whereas the pituitary and thyroid exhibit distinct susceptibility to BPA-driven transcriptional changes.

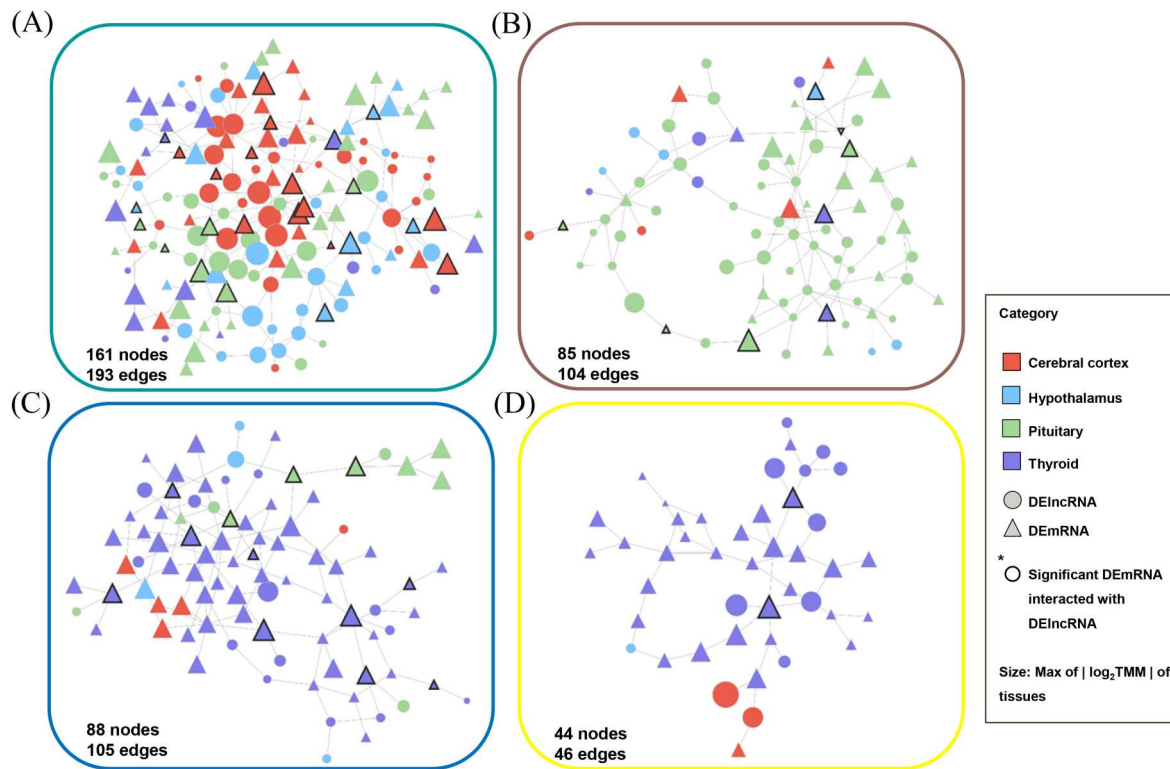


**Figure 3.** Network analysis depicting the interaction between mRNAs and lncRNAs based on the heatmap of four module-trait relationships. The Weighted Gene Co-expression Network Analysis (WGCNA) revealed four distinct modules ([A]; turquoise, [B]; brown, [C]; blue, [D]; yellow), representing groups of co-expressed genes. Node color indicates the type of tissue, node size corresponds to the absolute  $\log_2\text{TMM}$ , and node shape indicates the type of RNA (lncRNA: ellipse; mRNA: triangle). The border of the node indicates the significant DEmRNAs interacting with the DElncRNAs.

### 3.3. Tissue-specific co-expression networks of DElncRNAs and DEmRNAs

To identify the correlations between the co-expressed genes and tissues, four modules (turquoise, brown, blue, and yellow) of DElncRNA and DEmRNA with positive and/or negative correlations (Dai et al. 2018) with the tissues were calculated. While the turquoise network showed a correlation pattern in all tissues, the brown network showed a pituitary-specific negative correlation pattern, and it was anticipated that the effects of BPA treatment would result in important biological changes in the thyroid. Furthermore, two distinct correlation patterns of the thyroid were identified: positive correlation in the blue and negative correlation in the yellow networks (Figure S2). These were set to indicate high significance and correlation to the four tissues. Using WGCNA, co-expression sub-networks were generated based on the modules. Each network consisted of DElncRNAs and DEmRNAs (nodes) and the interactions (edges) between them. The networks were expressed in tissues with high absolute value  $\log_2\text{TMM}$ , and only significant DEmRNAs that interacted with DElncRNAs and were relevant to specific functions were highlighted in bold. The turquoise network (161 nodes and 193 edges) exhibited a high correlation with all tissues (Figure 3(A)). In contrast, the brown network (85 nodes and 104 edges) showed a pituitary-specific negative correlation pattern (Figure 3(B)). Additionally, the blue network (88 nodes and 105 edges) exhibited a thyroid-specific positive correlation pattern (Figure 3(C)), whereas the yellow network (44 nodes and 46 edges) exhibited a thyroid-specific negative correlation pattern (Figure 3(D)). DElncRNAs interacting with DEmRNAs were identified using the co-expression networks. The turquoise network exhibited the highest number, with 74 interactions, followed by the brown network with 49, the blue network with 26, and the yellow network with 12 interactions.

The results of WGCNA revealed that the integrated network was specifically composed of four modules, and the overall regulations between DElncRNAs and DEmRNAs were confirmed by investigating cis/trans acting genes and examining the tendency of gene expression patterns in response to the regulatory mechanisms induced by BPA treatment (Figure S2). The interactions between DElncRNAs and DEmRNAs provided insights into their genomic locations, network associations, expression patterns, and regulatory types for

specific genes. The expression patterns after BPA treatment revealed that the turquoise network included 89 up-regulated and 72 down-regulated genes. The brown network comprised 49 up-regulated and 36 down-regulated genes. The blue network comprised 49 up-regulated and 39 down-regulated genes. The yellow network contained 4 up-regulated and 40 down-regulated genes. In terms of regulatory mechanisms, a total of 161 significant DElncRNAs-DEmRNAs interactions were identified, including 5 cis and 69 trans regulations in the turquoise network, 1 cis and 48 trans regulations in the brown network, 7 cis and 19 trans regulations in the blue network, and 12 trans regulations in the yellow network.

The identification of four distinct co-expression networks suggests that BPA exposure induces complex and tissue-specific transcriptional reprogramming. The turquoise module's broad presence across tissues may represent common pathways affected by BPA, whereas the brown, blue, and yellow modules indicate more specialized alterations, particularly in the pituitary and thyroid. The observed cis and trans regulatory interactions between DElncRNAs and DEmRNAs imply that BPA-mediated lncRNA perturbations could influence gene expression both locally and at distal genomic sites, contributing to systemic endocrine dysregulation.

### 3.4. Functional implications of lncRNA-mRNA interactions

The potential regulatory functions of lncRNAs were evaluated through functional enrichment analysis of coding genes having significant interactions with DElncRNAs in each network (Table S2). All networks confirmed the involvement of functionally consequential genes in the pituitary and thyroid. Even in the turquoise network, which encompassed all tissues, genes with significant expression in the cerebral cortex and hypothalamus, components of the CNS, were involved in neuronal and synaptic activities, as evidenced by the enriched KEGG pathways, such as 'neuroactive ligand-receptor interaction' and 'cAMP signaling pathway', and GO terms, such as 'chemical synaptic transmission' and 'neuropeptide signaling pathway'. Notably, Tachykinin Precursor 1 (*Tac1*), 5-Hydroxytryptamine Receptor 1B (*Htr1b*), and Neuropeptide Y (*Npy*) were the major DEmRNAs identified in functional annotation, and their three interactions (*Tac1* and *Htr1b* with TCONS\_00216561 in the hypothalamus, and *Npy* with TCONS\_00144916 in the cerebral cortex) were confirmed to have robust correlations (Figure 4). In the pituitary-specific brown network, the enrichment of KEGG pathways, such as 'spliceosome', and biological process (BP) terms, such as 'RNA splicing', 'mRNA splicing, via spliceosome', and 'regulation of cell cycle', was observed. Serine and Arginine Rich Splicing Factor 5 (*Srsf5*) emerged as a major DEmRNA implicated in splicing functions, and its two interactions (*Srsf5* with TCONS\_00145985 and ENSMUSG00000087028) were identified to have robust correlations (Figure 5). While the brown network revealed pituitary-specific expression patterns, the interactions identified showed a heightened expression of *Srsf5* in the thyroid. In the blue network, representing thyroid-specific positive correlations, the enrichment of KEGG pathways, such as 'prostate cancer', 'PI3K/Akt signaling pathway', 'parathyroid hormone synthesis, secretion, and action', and 'TNF signaling pathway', and GO terms, such as 'immunoglobulin production' and 'immune response', indicated cellular and immune signaling functions. These functions emphasized enriched cellular responses in immunological and hormonal pathways. cAMP Responsive Element Binding Protein 5 (*Creb5*) and Cyclin-Dependent Kinase Inhibitor 1A (*Cdkn1a*) were identified as DEmRNAs in the functional analysis, and their two interactions (*Creb5* with TCONS\_00360657, and *Cdkn1a* with TCONS\_00135302 and TCONS\_00189363) were authenticated to have robust correlations (Figure 6). In both noteworthy interaction pairs on the network, DElncRNAs exhibited expression patterns associated with the pituitary, while DEmRNAs showed high expression values in the thyroid. Finally, in the yellow network, representing thyroid negative correlations, only one BP term, 'cell surface receptor signaling pathway', was enriched. T Cell Receptor Beta Variable 15 (*Trbv15*) and Fc Receptor-Like A (*Fcrla*) were identified as the DEmRNAs associated with GO terms, and their three interactions (*Trbv15* with ENSMUSG00000086211 and ENSMUSG00000105646 in the thyroid, and *Fcrla* with TCONS\_00054275 in the thyroid) were confirmed to have robust correlations (Figure 7).

These findings suggest that BPA-induced transcriptional changes, mediated by DElncRNAs, drive disruptions in neuronal signaling, RNA splicing regulation, cellular proliferation, and immune responses in a tissue-specific manner. Notably, the strong co-expression relationships observed between DElncRNAs and key DEmRNAs (*Tac1*, *Htr1b*, *Npy*, *Srsf5*, *Creb5*, *Cdkn1a*, *Trbv15*, and *Fcrla*) highlight the potential role of lncRNAs as central regulators orchestrating these biological processes.

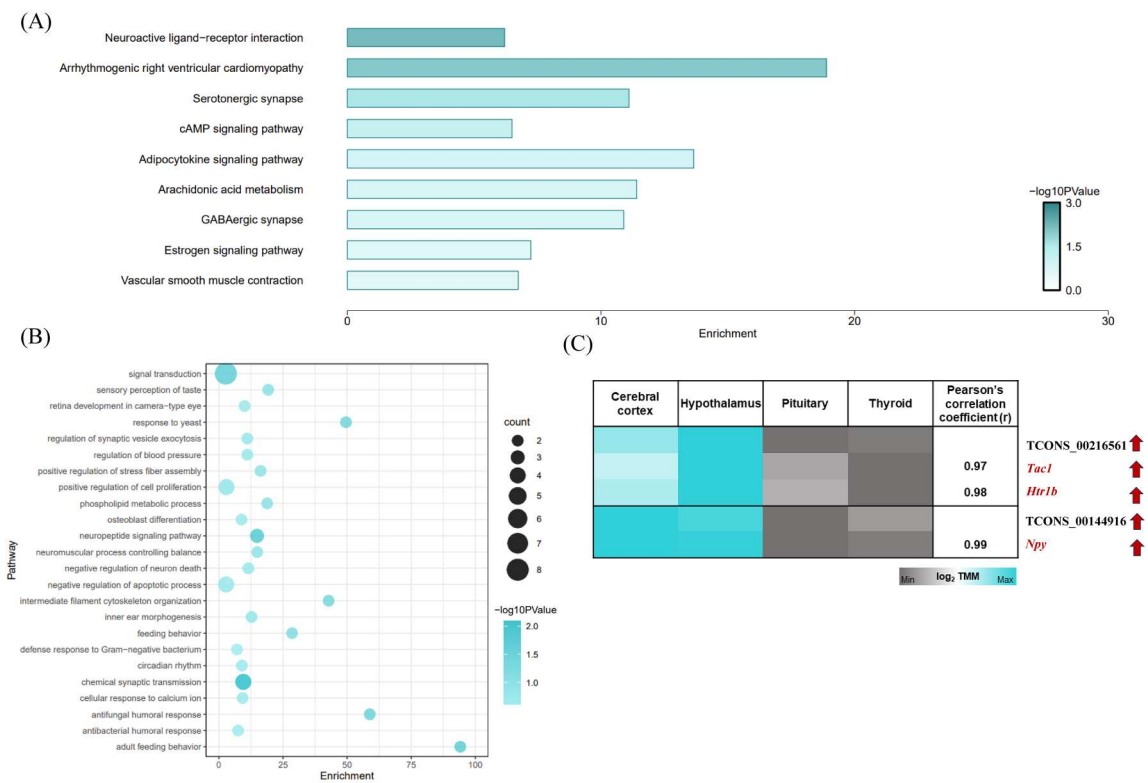


**Figure 4.** Functional enrichment analysis of DEmRNAs interacting with DElncRNAs in the turquoise network. (A) Bar plot based on the KEGG database for functional enrichment of the turquoise network. (B) Bubble plot depicting the functions related to biological processes in Gene Ontology. (C) Heatmap depicting the correlations between significantly selected DElncRNAs and DEmRNAs. Pearson's correlation coefficient ( $r$ ) was calculated using  $\log_2$ TMM values, and the arrow represents  $\log_2$ FC.

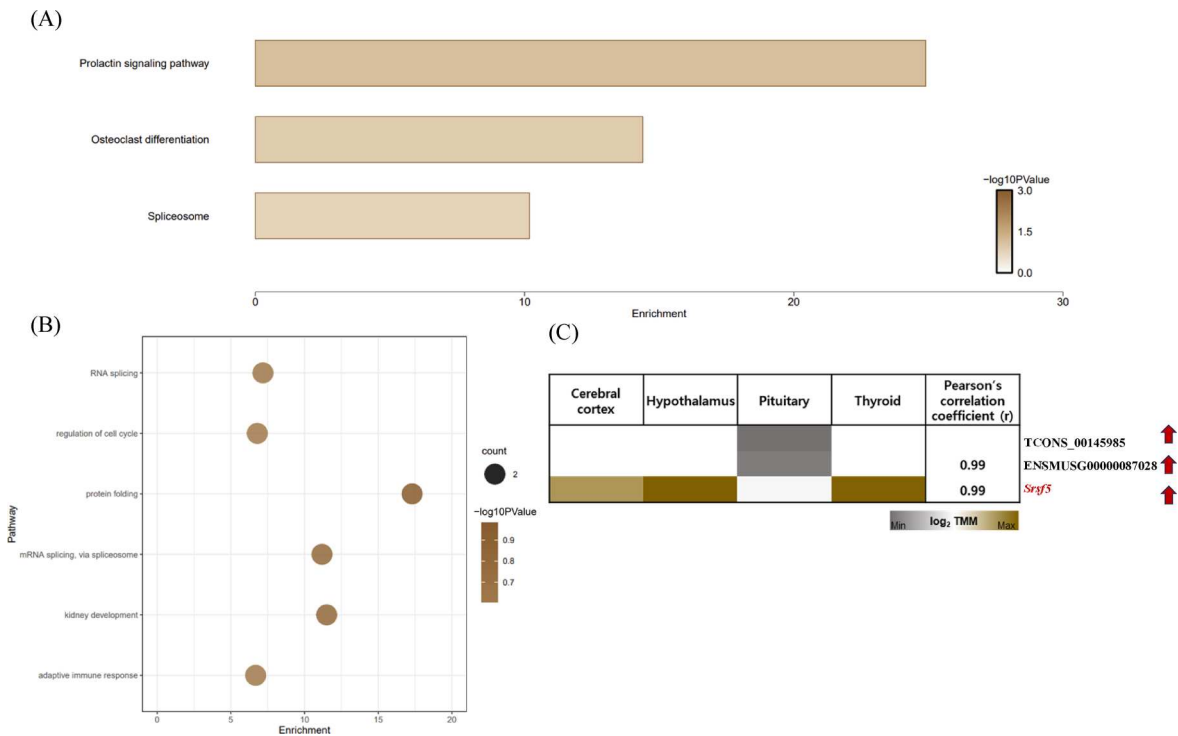


**Figure 5.** Functional enrichment analysis of DEmRNAs interacting with DElncRNAs in the brown network. (A) Bar plot based on the KEGG database for functional enrichment of the brown network. (B) Bubble plot depicting the functions related to biological processes in Gene Ontology. (C) Heatmap depicting the correlations between significantly selected DElncRNAs and DEmRNAs. Pearson's correlation coefficient ( $r$ ) was calculated using  $\log_2$ TMM values, and the arrow represents  $\log_2$ FC.

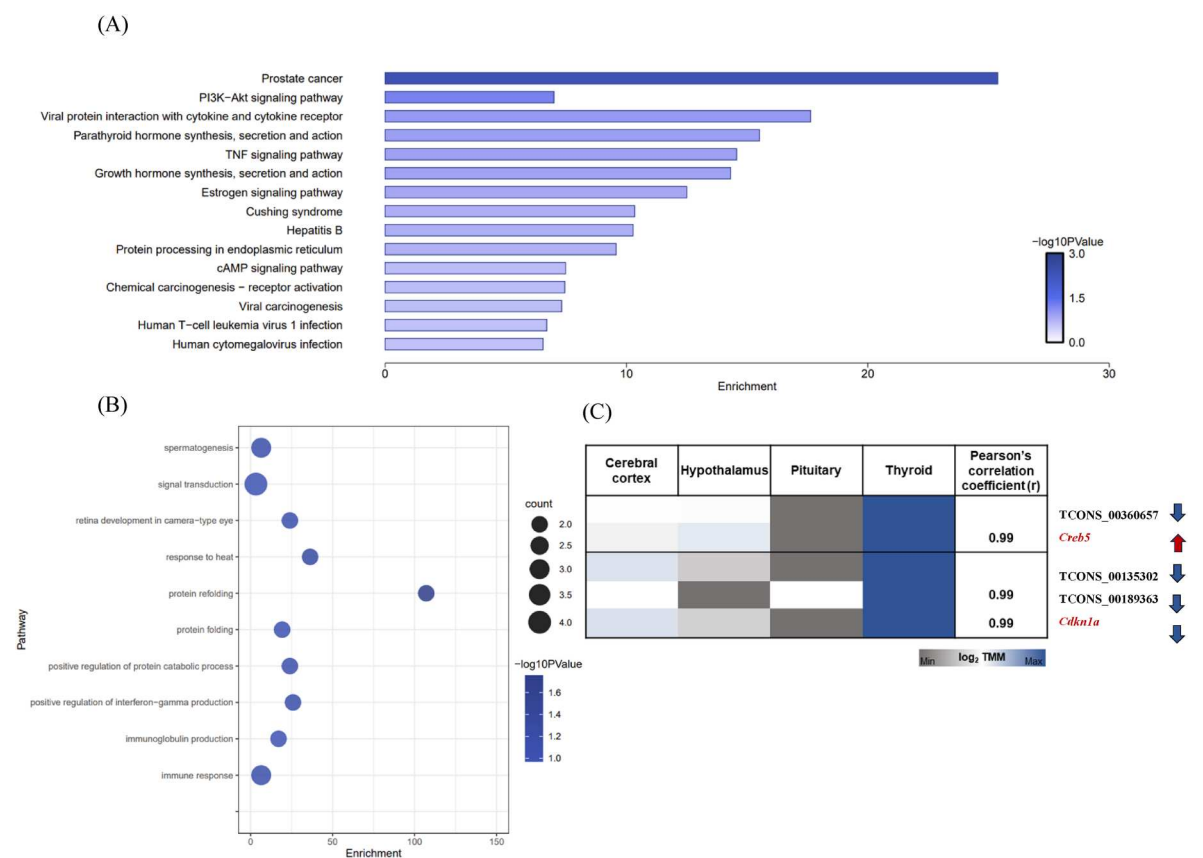


**Figure 6.** Functional enrichment analysis of DEMRNAs interacting with DElncRNAs in the blue network. (A) Bar plot based on the KEGG database for functional enrichment of the blue network. (B) Bubble plot depicting the functions related to biological processes in Gene ontology. (C) Heatmap depicting the correlations between significantly selected DElncRNAs and DEMRNAs. Pearson's correlation coefficient ( $r$ ) was calculated using  $\log_2$ TMM values, and the arrow represents  $\log_2$ FC.

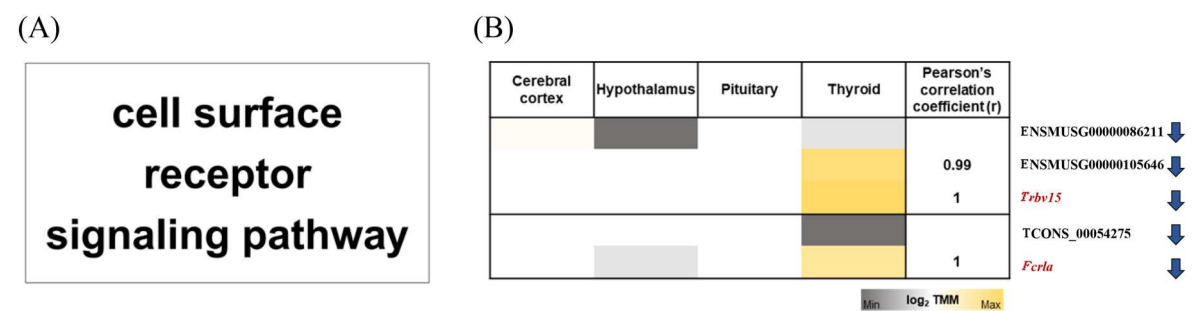


**Figure 7.** Functional enrichment analysis of DEMRNAs interacting with DElncRNAs in the yellow network. (A) Bubble plot depicting the functions related to biological processes in Gene ontology for the functional enrichment of the yellow network. (B) Heatmap depicting the correlations between significantly selected DElncRNAs and DEMRNAs. Pearson's correlation coefficient ( $r$ ) was calculated using  $\log_2$ TMM values, and the arrow represents  $\log_2$ FC.

## 4. Discussion

### 4.1. BPA induces multi-system disruption through lncRNA-mediated networks

The identification of four distinct co-expression modules, each with specific tissue distributions and functional signatures, demonstrates that BPA exposure induces complex, coordinated responses rather than simple receptor-mediated effects. The turquoise module's pan-tissue presence alongside tissue-specific brown, blue, and yellow modules reveals a hierarchical disruption pattern where common pathways are

affected across tissues while specialized functions are disrupted in tissue-specific manners (Patisaul 2021). Critically, our findings reveal that BPA exploits the inherent tissue specificity of lncRNAs (specificity index 0.59 vs 0.24 for mRNAs) to trigger tissue-adapted responses (Figure 1(E)). This explains why BPA exposure produces such diverse health outcomes, from reproductive dysfunction to immune disorders to metabolic disease, as each tissue responds through its own specialized lncRNA-mediated regulatory programs (MacKay and Abizaid 2018). The tissue-specific vulnerability patterns observed reflect the complex interplay between BPA's molecular targets and the unique cellular environments of different neuroendocrine tissues.

#### **4.2. Neuroimmune networks as central targets of BPA toxicity**

Our discovery of extensive immune pathway disruption, particularly in thyroid tissue, provides molecular evidence for the emerging concept of INEN as primary targets of environmental chemical toxicity (Liu et al. 2024). The enrichment of TNF signaling, immunoglobulin production, and immune receptor pathways in the blue and yellow modules demonstrates that BPA simultaneously disrupts immune and endocrine functions through coordinated mechanisms (Figures 6 and 7). This finding has profound implications for understanding BPA's health effects. Rather than viewing immune and endocrine disruption as separate consequences, our results suggest these represent integrated responses within INEN. The tissue-specific nature of immune disruption, concentrated in thyroid tissue, may explain the increased autoimmune thyroid disease risk observed in BPA-exposed populations (Chailurkit et al. 2016) and highlights the thyroid as a particularly vulnerable target organ. The thyroid gland contains resident immune cells and is particularly susceptible to autoimmune disorders, making the observed immune pathway disruption especially concerning for thyroid health and autoimmune disease risk.

#### **4.3. LncRNA-mediated neuronal signaling disruption**

The turquoise network's enrichment in neuronal signaling pathways, particularly 'neuroactive ligand–receptor interaction' and 'cAMP signaling pathway', alongside the robust correlations between key neuropeptide genes (Tac1, Htr1b, Npy) and their interacting lncRNAs, reveals critical insights into BPA's neuroendocrine toxicity mechanisms (Figure 4). These findings suggest that BPA disrupts GnRH neuron function through lncRNA-mediated modulation of neuropeptide expression (Prasad et al. 2015; Fergani and Navarro 2016). The hypothalamus serves as a pivotal element of the HPT axis, maintaining homeostasis through feedback mechanisms that regulate various hormones including GnRH and thyroid-releasing hormone (Patisaul 2021). BPA-targeted GnRH neurons express estrogen receptors and form complex cellular networks dependent on intercellular communication (Constantin et al. 2010). Our identification of lncRNA-mediated regulation of Tac1 (substance P) and Npy, both critical for GnRH neuron function, suggests that BPA exploits epigenetic mechanisms to disrupt the fundamental regulatory circuits controlling reproductive and metabolic homeostasis.

#### **4.4. Splicing dysregulation and cellular metabolic disruption**

The brown network's pituitary-specific enrichment in spliceosome pathways, centered on Srsf5 interactions with regulatory lncRNAs, reveals a previously unrecognized mechanism of BPA toxicity (Figure 5). Splicing dysregulation has emerged as a critical pathway in cancer development, with many cancer-associated mutations linked to spliceosome activity control (Bonnal et al. 2020). The tissue-specific nature of these splicing alterations in the pituitary is particularly concerning given recent evidence linking BPA exposure to increased hormone-dependent cancer risk (Bradley and Anczuków 2023). lncRNAs regulate alternative splicing by interacting with splicing factors or modulating spliceosomal assembly (Romero-Barrios et al. 2018), suggesting that BPA-induced lncRNA alterations could fundamentally reprogram cellular metabolism through altered RNA processing. This mechanism may explain the long-term health consequences of BPA exposure, as splicing changes can persist long after initial exposure and contribute to progressive cellular dysfunction.

#### 4.5. Immune-endocrine network disruption through cellular signaling pathways

The blue and yellow networks' enrichment in immune signaling pathways, particularly involving PI3K/Akt signaling, cell cycle regulation, and immune receptor pathways, demonstrates that BPA simultaneously targets cellular proliferation and immune function (Figures 6 and 7). The robust correlations between Creb5, Cdkn1a, Trbv15, and Fcrla with their regulatory lncRNAs reveal specific molecular mechanisms through which BPA disrupts immune-endocrine crosstalk. The PI3K/Akt pathway serves as a critical integration point for growth factors, cytokines, and hormonal signals, making it an ideal target for endocrine disruption (Evangelisti and Martelli 2016; Hoxhaj and Manning 2020). BPA-induced dysregulation of this pathway through lncRNA-mediated mechanisms may explain the increased cancer risk and metabolic dysfunction observed in exposed populations. The identification of immune receptor disruption (Trbv15, Fcrla) in thyroid tissue provides mechanistic insight into the increased autoimmune disease risk associated with BPA exposure.

#### 4.6. Critical research gap: HPT/HPA axis crosstalk and INEN

A significant limitation of the current study is our focus on the HPT axis without direct investigation of HPA axis interactions, despite mounting evidence that BPA simultaneously disrupts both systems through integrated mechanisms. Recent research demonstrates that BPA binds and activates GR (Wang H et al. 2021), whose activity has been directly correlated with alterations in BDNF regulation (Smith et al. 2021). This GR-BDNF disruption cascade has been causally linked to BPA's detrimental effects on INEN through coordinated HPA axis alterations (Martinez et al. 2024). Given that the hypothalamus serves as the common regulatory center for both HPT and HPA axes, our observation of coordinated transcriptional responses between cerebral cortex and hypothalamic tissues strongly suggests that BPA-induced transcriptional changes reflect broader neuroendocrine dysregulation potentially implicating the HPA axis alongside the HPT system. The tissue-specific lncRNA networks we identified likely represent critical regulatory nodes where GR signaling, BDNF expression, and neuropeptide synthesis converge under environmental chemical stress. Future investigations should prioritize: (1) lncRNA-mediated GR regulation and its cascade effects on HPA axis function; (2) the mechanistic role of lncRNAs in BDNF expression and synaptic plasticity responses to BPA exposure; (3) integrated co-expression network analysis encompassing both HPT and HPA axis components; and (4) functional validation of lncRNA-GR-BDNF regulatory cascades using gain- and loss-of-function approaches.

#### 4.7. Clinical implications, limitations, and future directions

Our findings provide a systems-level perspective on how BPA exposure disrupts the INEN through tissue-specific lncRNA-mRNA co-expression networks. These interactions offer promising directions for the development of biomarkers and therapeutic strategies. For instance, strong correlations such as Tac1-TCONS\_00216561 and Srsf5-associated lncRNAs may serve as indicators of reproductive neuroendocrine dysfunction and cellular stress, respectively, supporting their potential use as exposure biomarkers. From a therapeutic standpoint, key BPA-responsive lncRNAs identified here could represent intervention targets. Antisense oligonucleotides (ASOs) or small molecule inhibitors modulating lncRNA function may offer novel avenues for mitigating the adverse effects of endocrine disruptors (Akanbi et al. 2025). Moreover, the immune-endocrine dysregulation observed suggests the potential benefit of immunomodulatory therapies in BPA-exposed populations.

Nevertheless, we acknowledge several limitations. First, although the lncRNA-mRNA pairs identified through network analysis present compelling hypotheses, they lack experimental validation. These interactions should be interpreted as candidates for future mechanistic studies rather than confirmed regulatory pairs. Second, functional attribution in this study is primarily based on co-expression and genomic proximity, which do not establish causal relationships or physical interactions. Therefore, we emphasize the need for future studies employing techniques such as chromatin interaction mapping, RNA immunoprecipitation to confirm the roles of candidate lncRNAs in transcriptional regulation, particularly in hormone signaling and neuroplasticity.

## 5. Conclusions

BPA, a recognized xenoestrogen, interacts with estrogen, androgen, and thyroid receptors, thereby altering gene expression, metabolism, and immune responses. By integrating lncRNA-mRNA network analyses, this study delineates tissue-specific regulatory perturbations in the cerebral cortex, HPT axis. These include disruptions in neuronal signaling, GnRH regulatory pathways, RNA splicing, and immune receptor-mediated signaling. In particular, splicing and immune pathway alterations in the thyroid suggest potential links to hormone secretion, carcinogenesis, and immune dysregulation. Collectively, these findings indicate that BPA induces systems-level reprogramming of the INEN, extending beyond classical single-receptor mechanisms.

This work represents the first comprehensive characterization of BPA-induced lncRNA-mRNA networks in the brain and HPT axis, providing novel biomarker candidates and therapeutic targets. While the study focuses on acute exposure, further investigation is warranted to assess long-term consequences, elucidate HPT-HPA axis crosstalk, and functionally validate key lncRNA-mediated pathways. Such efforts will enhance risk assessment, inform precision medicine approaches, and support the development of interventions to mitigate the health impacts of environmental endocrine disruptors.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Data availability statement

Data will be made available upon request.

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