Identify the regulatory network of LncRNA HAR1A in neurological development by RNA-Sequencing and bioinformatics analysis.

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Abstract

Background:

LncRNA HAR1A, which is explicitly expressed in Cajal-Retzius neurons (CRs), has been reported to be related to the development of the human brain. As one of the human accelerated regions (HARs) gene, it plays an important role in central neural evolution. Dysregulation of lncRNA HAR1A was associated with many central nervous system diseases.

Methods:

We cloned human gene HAR1A into the EF-1α promoter vector to make the transgenic mice. To observe the changes in memory and cognitive ability of mice, we conducted the Morris water maze (MWM) test and step-down passive avoidance test. RNA-seq analysis was performed to identify the differentially expressed genes (DEGs) of the experiment and control group. Systematic bioinformatic analysis was used to confirm the pathway and function that the DEGs enriched in. 523 human gene expression datasets were downloaded from The Cancer Genome Atlas (TCGA) for Co-expression analysis. Through Co-expression analysis, we obtained the Protein-Protein Interactions Network (PPI-Net) and enrichment pathways of LncRNA HAR1A's co-expression genes in human.

Results:

The memory and cognitive ability of the transgene mice were significantly improved. Results of GO analysis showed that cerebral cortex development is the most significant function related to HAR1A in brain development. DEGs enriched in this function included Lhx2, Emx2, Foxg1, Nr2e1, Emx1. They all play an important role in the regulation of CRs' function. "Cellular response to calcium ion" exhibited the highest rich factor in the Gene Ontology (GO) analysis. Core genes in the PPI-Net were SNAP25, GRIN1, SYN1, DLG4, CAMK2A. SNAP25, SYN1 relate to synaptic function. GRIN1, DLG4 and CAMK2A relate to synapse formation. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs and HAR1A 's Co-expressed genes in 523 human gene expression datasets show that synapse, axon guidance, synaptic signaling and ligand-receptor interaction are significant.

Conclusions:

Overexpress HAR1A will improve the memory and cognitive ability of the transgene mice. The possible mechanism is HAR1A affects brain development by regulating CRs' function. Moreover, HAR1A may be involved in ligand-receptor interaction, Axon guidance and synapse formation, which are important for brain development and evolution. Cellular response to calcium may play an important role in those process.

Introduction

The current research shows that the human acceleration region (HAR) found in highly conserved areas may be responsible for the unique characteristics of our species. It is related to the human brain's size, structure, and complexity evolution(1). HARs are located in intergenic and intronic non-coding regions. These are DNA regions that do not encode for proteins but play a role in gene regulation(2). HAR1A is one of the most different HARs between humans and chimpanzee(3, 4). HAR1A is explicitly expressed in CRs, which are well known for controlling nerve cell migration during brain development suggesting that HAR1A is important in brain development(5, 6). The expression of HAR1A affects the occurrence and development of many brain diseases, including Huntington's disease and glioma(7, 8). However, genes regulated by HAR1A in the developing brain have not been systematically studied, and its association with the development and evolution of the central nervous system remains unclear.

HAR1A belongs to the long non-coding RNA (LncRNA), a non-coding RNA longer than 200 nucleotides. Accumulating evidence demonstrated that lncRNAs affect epigenetics by regulating transcription and post transcription. Moreover, lncRNA has been reported to regulate human brain development, neural stem cells regulation and neuronal axon elongation(9, 10). The involvement of LncRNA in the regulation of neurological growth and development allows the nervous system to grow and differentiate in the regular order of time and space(10).

In the present study, we explored the effects of overexpression of HAR1A on cognition and memory in mice and analyze the related functions and enrichment pathways of DEGs. Furthermore, we analyzed 523 data sets downloaded from TCGA: (https://portal.gdc.cancer.gov/) to depict the PPI-Net of co-expression genes of HAR1A in humans. This study aims to identify the role of LncRNA HAR1A in brain development and deepen the understanding of the molecular regulatory mechanism of LncRNA on neurological development.

Material And Methods

Mouse models

The strain of the mice sample used in the research is C57BL/6. Six transgenic mice were generated and provided by Cyagen Bioscience Inc (Guangzhou, China). Eleven wild-type mice were provided by the animal center of Guangdong medical laboratory (Guangzhou, China). They were analyzed in parallel with transgenic mice as control groups. All mice were placed in a specific pathogen free cage and subjected to a 12 hours light/ dark cycle in an approved facility.

The transgenic mice were produced by cloning human gene HAR1A into an EF-1α promoter vector. The sequence of target HAR1A gene was:

ATGAAACGGAGGAGACGTTACAGCAACGTGTCAGCTGAAATGATGGGCGTAGACGCACGT

CAGCGGCGGAAATGGTTTCTATCAAAATGAAAGTGTTTAGAGATTTTCCTCAAGTTTCA

The combined sequence of the HAR1 gene and EF-1α promoter vector was named H119. After producing transgenic mice, the quality of transgenic mice was screened by PCR assay.

MWM test

The experimental group and the control group were trained in an open round pool (diameter:1.7m, depth:0.3m) at 23℃±1℃(11, 12). The maze was delimited into four equal quadrants (1–4) by specifying two orthogonal axes, and its end was marked as four base points: North (N), South (S), East (E) and West (W). A camera (FDR-AX700, Sony, Japan) was suspended in the middle of the ceiling to record the traces of rats. The space acquisition task was tested four times a day, with an interval of 15 seconds for five consecutive days.

The escape platform (diameter: 0.1 m) was about 1 cm under the surface of the water, located at the centra of quadrant 2 (target quadrant). The mice were gently released from different starting points into the water. They were allowed to seek the hidden platform for 60 seconds. Mice would be manually guided to find the target platform if they failed to find the target platform in 60 seconds. After a six-day delay from the last space acquisition training day, the exploratory trials were conducted to assess the long-time memory of mice (12). The tracking software of the system was used to analyze data results, including the escape latency in the spatial acquisition days and the latency to the target platform (probe time) during the probe trial.

Step-down test

The one-trial step-down test was performed to assess inhibitory avoidance and long-time memory, including 5 minutes of training and 5 minutes of test after 24 hours. The size of the chamber was about 0.18 (height) × 0.12 (width) × 0.12 (depth) m. The floorboard was an electrified grid composed of parallel 1 mm copper bars with a spacing of 0.05 m. There was a high-rising rubber platform (diameter: 0.24 m) in one corner of the chamber. Mice were placed on the platform with their nose directed to the corner of the bottom. In the training session, when the mice stepped on the grid, they would get an immediate shock (36V, AC). They would jump up to the high-rising platform to avoid the electric shock instinctively. We recorded the time for the mice to jump from the high-rising platform into the grid bottom (step-down latency) and how many times mice jumped from the high-rising platform during the training phase (error counts). In the experimental phase, the same procedure was conducted. The apparatus was carefully cleaned after the test session to reduce the possibility of odor interference.

After the step-down test, mice were euthanized by cervical dislocation, and their brain samples were collected respectively(13).

Extracting RNA, Sequencing and Constructing cDNA Library

Trizol method (Invitrogen) was used to extract the total RNA from mice brain tissues. The Agilent 2100 Bioanalyzer was used to acquire the concentration and quality of RNA. TruSeq RNA Sample Preparation Kit (RS-200-0012, Illumina) was used to construct the cDNA library. The sequencing datasets were acquired by using Illumina novaseq 6000. The datasets of the experiment group were named: 4–

6_HL3FVCCXY_L4, 5– 5_HL3FVCCXY_L4, 6 – 4_HL3FVCCXY_L4, 7 – 1_HL3FVCCXY_L4, 8-7N_HL3FVCCXY_L4, 9 – 3_HL3FVCCXY_L4

control group was named: 3-1N_HL3FVCCXY_L8, 3-9N_HL3FVCCXY_L8, 4-3N_HL3FVCCXY_L8, 4–4_HL3FVCCXY_L2, 4-9N_HL3FVCCXY_L8, 5 – 1_HL57YCCXY_L6, 5-4N_HL3FVCCXY_L4, 6–10_HL3FVCCXY_L4, 7-5N_HL3FVCCXY_L8, 7-6N_HL3FVCCXY_L8, 8-5N_HL3FVCCXY_L4

Bioinformatic analysis of RNA-Seq data

After obtaining the raw data, we performed quality control (QC) to remove adapters, N-terminus, and low-quality reads. FastQC (v0.11.5) was used for QC with parameter -a = given adapter sequence, –output = output path and -m = 40 and others using default parameters. Trimmed reads sample data were aligned to reference sequence by using software Bowtie2 with parameters: –read-edit- dist 3, -r 70, –library-type fr-unstranded and others using default parameters. HTSeq-Count (0.6.1p1) was used for the expression quantification with following parameters: –stranded = no, –format = bam, –- mode = intersection-nonempty and others using default parameters. The DEGs among the experiment group and the control group were screened out from the gene-expression results by the DESeq2 package (1.12.4) with parameter: log2 (foldchange) > 1, Padj < 0.05. Here, Padj means the p-adjustment corrected by Benjamin & Hochberg method after multiple test adjustments. The DEGs were analyzed by cluster analysis, pathway significant enrichment analysis (Padj < 0.05) and GO function significant enrichment analysis (Padj < 0.05). GO seq based Wallenius noncentral hypergeometric distribution was implemented for GO enrichment analysis. Gene Ontology and KEGG enrichment analysis by using the website Kobas (http://kobas.cbi.pku.edu.cn). The database used in the research is the mouse full genome (UCSC version mm10).

Download human gene expression datasets from TCGA

523 human RNA-seq datasets were downloaded from the TCGA database according to the TCGA publication guidelines. R package (3.3.1) was used to filter out the top 750 genes positively correlated with HAR1A and related to neural development.

Co-expression analysis for predicting lncRNA function

We used COIN methods from our previous study for co-expression analysis of the top 750 genes related to HAR1A mentioned above(9). The specific steps are as follows:

GO and KEGG pathway enrichment analysis

GO enrichment analysis and KEGG pathway enrichment was performed to analyze Genes mostly related to lncRNA HAR1A using the website Kobas; parameters were listed as follows: (1)The hypergeometric distribution was used to estimate the probability that a set of specific genes are annotated to a pathway; (2)The Bonferroni correction method was used to test and adjust the raw p-value several times(14); (3) Adjusted p-value < 0.05 was the criterion that the pathway was significantly enriched.

PPI-Net for co-expression genes

PPI-Net containing the most related co-expression genes of HAR1A was obtained from the following databases: HPRD (http://www.hprd.org/), IntAct (https://www.ebi.ac.uk/intact/home), BIND (http://binddb.org), MIPS (https://danangwiki.com/), DIP (http://dip.doe-mbi.ucla.edu), PDZBase (http://icb.med.cornell.edu/services/pdz/start), MINT (https://mint.bio.uniroma2.it/), and Reactome, parameter: default. All relevant interactomes regarding HAR1A found in at least one of these databases will be taken into our analysis. The interactive network of 103 cerebral neural genes co-expression with HAR1A was constructed by STRING database (https://string-db.org). The visualization of the result was performed by Cytoscape software (3.7.1).

Verify the experimental results in human gene expression datasets

Tbr1, Kcnv1 and Phactr1 were three genes expressed very differently between the experimental and control groups. TBR1, KCNV1 and PHACTR1 are orthologous genes of Tbr1, Kcnv1 and Phactr1 in humans. To verify the experiment's reliability, we performed linear correlation analysis to analyze the correlation between the TBR1, KCNV1 and PHACTR1 with HAR1A in 523 human gene expression datasets downloaded from TCGA.

Results

Validation of transgenic mice

After producing transgenic mice, the quality of transgenic mice was screened by PCR assay. We got six strong positive results, 1–4 and 8–9 (Fig. 1). It means that the six mice were successfully overexpressed HAR1A. They were selected for the follow-up experiment.

Results of the MWM test and the step-down passive avoidance test

Our study used the MWM test to estimate spatial learning and long-time working memory. During the space acquisition days, how long it takes the mice to find the hidden target platform (the escape latency) was measured. (Fig. 2a). The escape latency was reduced during five days of training in the experimental group; the fifth day (36.0 ± 13.2 s) was 60% of the value on the first day (60 ± 5.6 s). When on the fifth day, the escape latency of the control group (43.1 ± 5.6 s) still accounted for 79% of the value on the first day (54 ± 3.6 s). After six days delay from the training course, a probe test was conducted to assess the long-time memory of animals. About the probe time, it took 24.0 ± 5.4 seconds for the experimental group to reach the platform position for the first time, while it took 66.1 ± 14 seconds for the control group, $p < 0.001$ (Fig. 2b). The probe time of the experimental group was significantly reduced compared with the control group.

In this study, step-down latency and error count was measured to assess memory retention (Fig. 2c-f). During the training period, the latencies of the experiment group and control group were 24s and 22s. The difference between groups was not statistically significant (Fig. 2c). In the test stage, the step-down latency of the experimental group increased by 63 ± 22 s compared with the control group by 25.5 ± 24 s ($P < 0.01$). The mice in the experiment group showed smarter performance, with fewer errors in the training period (5.2 ± 2.3 vs.8.2 ± 2.8, $P < 0.01$, Fig. 2d). In the test period, the error counts of the experimental group (2.4 ± 1.1) reduced more compared with the error count of the control group (6.2 ± 1.4, $P < 0.01$).

The results of the MWM test and step-down passive avoidance test show that overexpression of HAR1A had significantly improved performance in both short-term and long-term memory, spatial learning and cognitive ability of mice.

A review of RNA-SEQ datasets

Results of Quality Control showed that the base composition was satisfied in all 17 samples. After excluding the adaptor sequences and the low-quality reads, the lowest clean reading obtained from the 17 groups was 72,540,330. The number of mapped reads for each sample is more than 65 million, which is sufficient to give valid data for further analysis. The map rates (mapped reads/ raw reads) were all above 88% (Table 1). It suggested that all constructed libraries had excellent quality.

Table 1
Sequencing reads number of different groups of mice

sample_id	group	raw_reads	clean_reads	mapped_reads
3-1N_HL3FVCCXY_L8	control	79312928	79253222	70219241
3-9N_HL3FVCCXY_L8	control	85405460	85316740	76309652
4-3N_HL3FVCCXY_L8	control	72612004	72540330	65026158
4–4_HL3FVCCXY_L2	control	80117816	80026942	71216056
4–6_HL3FVCCXY_L4	case	80192986	80124336	72195909
4-9N_HL3FVCCXY_L8	control	80282660	80207448	71801854
5 – 1_HL57YCCXY_L6	control	91129066	91083962	81399419
5-4N_HL3FVCCXY_L4	control	91384118	91307294	81740179
5–5_HL3FVCCXY_L4	case	89480536	89402044	80713062
6–10_HL3FVCCXY_L4	control	83319872	83242190	75149506
6 – 4_HL3FVCCXY_L4	case	82980490	82898530	74828041
7 – 1_HL3FVCCXY_L4	case	93213296	93125238	83373952
7-5N_HL3FVCCXY_L8	control	87108060	87026102	76998314
7-6N_HL3FVCCXY_L8	control	87314292	87234908	77238419
8-5N_HL3FVCCXY_L4	control	78169916	78111572	70444218
8-7N_HL3FVCCXY_L4	case	90977174	90899290	81926184
9 – 3_HL3FVCCXY_L4	case	77339916	77272796	68799994

GO function analysis of DEGs

GO enrichment analysis was performed to identify the primary functions of DEGs. We ultimately obtained 1361 important GO terms, and we selected the top20 of functional enrichment for the generation of bubble chart (Fig. 3a). " Cellular response to calcium ion" of "biological process" exhibited the highest Rich factor value while "membrane" as a "cellular component" showed the highest significance with the lowest p-value. Also, DEGs were enriched in Glutamatergic synapse, synapse, memory, positive regulation of long-term synaptic potentiation and neuron differentiation which are associated with neural development.

Table 2
Results of GO analysis related to brain development

ID	Description	pvalue	geneID
GO:0098978	glutamatergic synapse	1.74692E-12	Stx1a, Akap5, Cnih3, Lrrc7, Synpo, Nrgn, Kalrn, Chrm4, Mal2, Camk2a, Chrm1, Drd1, Grin2b, Ptk2b, Arc, Cacng3, Camkv, Ngef, Homer2, Shisa7
GO:0045202	synapse	5.27484E-11	Cnih3, Synpo, Kcnj4, Camk2a, Ddn, Pdzrn3, Stx1a, Nrgn, Lrrc7, Syt5, Drd1, Grin2b, Shisa7, Homer2, Igfn1, Akap5, Kctd16, Chrm4, Chrm1, Dlgap2, Arc, Grm2
GO:0007613	memory	4.47961E-09	Kalrn, Plk2, Rin1, Drd1, Grin2b, Adrb1, Shisa7, Pak6, Csmd1
GO:1900273	positive regulation of long-term synaptic potentiation	1.46644E-08	Akap5, Nrgn, Kalrn, Drd1, Adrb1, Shisa7
GO:0030182	neuron differentiation	1.57162E-08	Mef2c, Lhx2, Lhx6, Wnt10a, Wnt1, Emx2, Tbr1, Emx1, Wnt9a
GO:0014069	postsynaptic density	1.66394E-08	Stx1a, Akap5, Lrrc7, Nrgn, Kalrn, Chrm4, Bcl11a, Camk2a, Chrm1, Grin2b, Ptk2b, Shisa7, Homer2
GO:0043197	dendritic spine	4.20E-08	Grin2b, Synpo, Akap5, Lrrc7, Camk2a, Drd1, Dlgap2, Ptk2b, Arc, Arhgap33
GO:0021542	dentate gyrus development	8.08E-08	Emx2, Neurod6, Nr2e1, Fezf2, Drd1
GO:0045211	postsynaptic membrane	2.0757E-07	Cnih3, Kctd16, Kcnj4, Chrm4, Chrm1, Grin2b, Nrgn, Arc, Ddn, Shisa7
GO:0043025	neuronal cell body	5.06105E-07	Camk2a, Akap5, Kcnj4, Kalrn, Pde1a, Chrm4, Rin1, Syt5, Cpne5, Drd1, Grin2b, Nrgn, Ptk2b, Enc1, Homer2
GO:0007399	nervous system development	1.86339E-06	Mef2c, Lhx2, Neurod6, Lhx6, Fezf2, Kalrn, Robo3, Ntn5, Enc1, Ngef, Islr2
GO:0021895	cerebral cortex neuron differentiation	3.03972E-06	Lhx6, Nr2e1, Fezf2, Emx1
GO:0021987	cerebral cortex development	1.13404E-05	Lhx2, Emx2, Foxg1, Nr2e1, Emx1
GO:0021902	commitment of neuronal cell to specific neuron type in forebrain	1.32497E-05	Satb2, Fezf2, Tbr1
GO:0048664	neuron fate determination	1.32497E-05	Foxg1, Wnt1, Fezf2

ID	Description	pvalue	geneID
GO:0048168	regulation of neuronal synaptic plasticity	1.35E-05	Grin2b, Kalrn, Arc, Camk2a
GO:0000976	transcription regulatory region sequence-specific DNA binding	2.21E-05	Sp9, Grhl1, Fezf2, Egr2, Egr3, Egr1, Mef2c, Ovol2, Dlx5
GO:0048169	regulation of long-term neuronal synaptic plasticity	2.52E-05	Grin2b, Egr1, Synpo, Drd1
GO:0022008	neurogenesis	2.96E-05	Wnt1, Lhx2, Foxg1, Ntn5
GO:0099061	integral component of postsynaptic density membrane	3.60E-05	Grin2b, Chrm4, Lrrc7, Chrm1, Cacng3

Besides, we listed GO items related to the brain development of DEGs; cerebral cortex development had the lowest p-value. It is mainly related to the directional differentiation of neural stem cells and the formation of spatial brain structure (Table 2).

Table 3
GO, REAC, HPA enrichment analysis results of HAR1A (Long non-coding RNA)

	Enrichment analysis	GO Enrichment pathway	p-value
1	GO:0097458	Neuron Part	5.1E-81
2	GO:0045202	Synapse	1.8E-75
3	GO:0099536	Synaptic Signaling	2.4E-75
4	GO:0099537	Trans-synaptic Signaling	2.4E-75
5	GO:0098916	Anterograde Trans-synaptic Signaling	2.4E-75
6	GO:0007268	Chemical Synaptic Transmission	2.4E-75
7	GO:0044456	Synapse Part	8.4E-73
8	REAC:112316	Neuronal System	5.4E-70
9	GO:0043005	Neuron Projection	2.7E-59
10	REAC:112315	Transmission Across Chemical Synapses	3.8E-45
11	HPA:007040_02	Cerebral Cortex; Neuropil	1.2E-43
12	GO:0097060	Synaptic Membrane	2.5E-43
13	GO:0098793	Presynapse	4.7E-43

As multiple databases can provide more credible results, we performed a series of analyses on DEGs with GO, REAC (https://reactome.org/) and HPA databases (https://www.atlasantibodies.com/). According to Table 3, the p-values of the top 13 enrichment pathways are all below 4.7E-43, which are highly significant. The Cerebral Cortex pathway has the lowest p-value, besides the neuron part pathway, the Synaptic signaling pathway, the Neuronal System pathway, and the presynapse pathway are very significant.

KEGG pathway analysis of DEG

Table 4
Results of KEGG analysis related to brain development

ID	Description	pvalue	geneID
mmu04080	Neuroactive ligand-receptor interaction	1.70E-06	Cort, Mas1, Rxfp1, Chrm4, Chrm1, Drd1, Grin2b, Adrb1, Sstr4, Glp2r, Grm2
mmu04360	Axon guidance	3.90E-05	Ngef, Robo3, Camk2a, Trpc6, Trpc4, Pak6, Nov
mmu04020	Calcium signaling pathway	6.16E-05	Camk2a, Pde1a, Mylk3, Chrm1, Drd1, Adrb1, Ptk2b
mmu04725	Cholinergic synapse	2.62E-03	Chrm4, Camk2a, Chrm1, Kcnj4

KEGG pathway analysis was performed to investigate the pathways in which DEGs were significantly enriched. Apart from GO terms, 124 KEGG pathways were found to be related to DEGs. The top 20 significantly enriched pathways of the DEGs are shown in Fig. 3b. The neuroactive ligand-receptor interaction pathway showed the greatest significance with the lowest p-value, while melanogenesis showed the highest Rich factor value. Besides, DEGs notably enriched in neuroactive ligand-receptor interaction, axon guidance, calcium signaling and cholinergic synapse, which are related to neural development (Table 4).(15)

KEGG and GO analysis of HAR1A 's co-expression genes in 523 datasets downloaded from TCGA

The top 750 brain neural gene clusters with the highest positive correlation of HAR1A were listed in Supplementary Table 1.

GO analysis and KEGG analysis of the top 750 co-expressed genes

GO analysis and KEGG analysis were performed on the top 750 co-expressed genes with HAR1A to analyze the regulation network of HAR1A in humans. The results of GO (Fig. 4a) analysis showed that modulation of chemical synaptic transmission-, regulation of trans-synaptic signaling- exhibited the

highest significance with the lowest p-value. The results of the KEGG (Fig. 4b) analysis showed that the top two significant pathways were neurodegeneration - multiple diseases and neuroactive ligand-receptor interaction-.

PPI-Net for co-expression genes

We conducted PPI analysis to assess the interaction of co-expressed genes of HAR1A by Cytoscape (Fig. 4c). For further mining the data of this network, we counted each core node's interaction weight (numbers of neighbors) (Fig. 4d). It depicted the gene regulation network related to HAR1A, in which SNAP25, GR1N1, SYN1, DLG4 and CAMKZA were in the center (Fig. 4c), suggesting these genes play crucial roles.

Verify the experimental results in human gene expression datasets

The Correlation coefficient of TBR1, KCNV1 and PHACTR1 with HAR1A were respectively 0.5053, 0.5498 and 0.6132, and the p-values were 3e-35, 1.2e-42 and 2.44e-55. The P-values of correlation were all less than 0.05 and Pearson's r values were all greater than 0.5 (Fig. 5). It means that the correlations of TBR1, KCNV1 and PHACTR1 with HAR1A are all significant. The results showed that the DEGs identified in our experiment were highly correlated with HAR1A in the human gene expression database downloaded from TCGA. This verifies the reliability of our experimental data.

Discussion

In the existing studies, HAR1A has been confirmed to be related to the development and evolution of the human brain(16). The existed studies mainly explored the possible action pathway through bioinformatics analysis of human gene transcriptome(17). However, confirming the regulatory network of HAR1A by directly overexpressing HAR1A and observing the DEGs has not yet been reported. This study confirms that overexpressing HAR1A led to an obvious improvement in mice's cognition and memory. The possible pathways and mechanisms were explored through bioinformatics analysis.

Potential pathways of HAR1A affecting cortical development through CRs

HAR1A is specifically expressed in CRs, which existed in the developing human neocortex during gestational weeks 7–19 (18). CRs is crucial in the specification and migration of cortical neuron, suggesting that HAR1A plays an impotent role in neurogenesis(19). The results of GO analysis of DEGs showed that cerebral cortex development had the lowest p-value in all GO items related to brain development. Five differential genes were enriched in this biological process: Lhx2, Emx2, Foxg1, Nr2e1, Emx1.

FOXG1 encodes a transcription repression factor which is essential of the regional subdivision in the formation of the telencephalon and brain development(20). When FOXG1 is expressed in cortical

progenitor cells, it stops the production of CRs by directly inhibiting a default transcriptional network(21). FOXG1-LHX2 interactions instruct the cessation of the CRs' production(22). CRs originate from the cortical hem[21]. Neurons in ventral pallium and cortical hem, which is in the medial pallium become subplate and CRs respectively after migrating and developing towards the neuroepithelium(23, 24). EMX1 and EMX2 are required to establish the Wnt-rich cortical hem domain(25, 26). EMX1 and EXM2 respectively encode a homeobox-containing transcription factor. They cooperate to promote the generation of CRs. NR2E1 participates in a feedback loop with the brain-specific microRNA, microRNA-9(miR-9), and Mouse miR-9 targets Foxg1 to control the generation of CRs in the medial pallium properly(27)، (28)، (29).

All DEGs enriched in Biological Process "cerebral cortex development" were involved in regulating CRs' function. CRs generated early in the cortex's marginal zone synthesize glycoprotein Reelin and secrete it into the peripheral intercellular stroma to regulate the morphology and biochemical maturation of radial glia cells (RGCs) (30)، (31). RGCs' fibers act as scaffolds when newborn neurons migrate to their final destinations. Reelin is a stop signal of neurons migration, decides the neurons' orientation and positioning in their layers; abnormal function of CRs can lead to various neurological diseases, including Alzheimer's disease, Schizophrenia, Lissencephaly, Temporal lobe epilepsy(32).

HAR1A might affect CRs' function by regulating the expression of Lhx2, Emx2, Foxg1, Nr2e1, Emx1. HAR1A may affect cerebral cortex development in this way. The specific mechanism needs to be further studied.

HAR1A may affect synaptic function and formation and finally affects brain development and evolution.

The result of KEGG pathways showed that the neuroactive ligand-receptor interaction pathway showed the greatest significance, besides DEGs were notably enriched in neuroactive ligand-receptor interaction, axon guidance and cholinergic synapse. Results of GO analysis showed that DEGs were enriched in Glutamatergic synapse, synapse, memory, positive regulation of long-term synaptic potentiation. Multiple database analyses (GO, REAC, HPA) of DEGs showed the Synaptic signaling pathway and the presynapse pathway are very significant. Go analysis of 750 genes co-expressed with HAR1A in the datasets downloaded from TCGA showed that modulation of chemical synaptic transmission and regulation of trans-synaptic signaling were the top two significant enrichment items.

Based on the above analysis, it can be concluded that HAR1A take an important role in neuroactive ligand-receptor, synaptic and axon guidance. Those are all critical processes in the early development of the brain(33). Neurons migrate to proper locations to make connections by emitting axons during the development of the brain(34). Multiple environmental signals control these neurons to migrate and axons to grow. After axons get the appropriate position, synapses will be formatted for neurons' contact with each other(35). Axonal branches are over-formatted during the early development of the brain. Then specific neuronal connections are created by pruning the redundant synapses. (36),(37)Several ligand-receptor pairs are involved in each of these cellular events(38).

PPI analysis showed the co-expressed genes of HAR1A in humans made a regulation network, SNAP25, GRIN1, SYN1, DLG4, CAMK2A were the core genes of this network. SNAP25 produces a membrane protein on presynaptic plays an important role in neurotransmitter release. The protein encoded by SNAP25 is important in the docking and fusion of the vesicle membrane(39). The protein encoded by GRIN1 is an essential part of N-methyl-D-aspartate receptors, which belong to the glutamate receptor channel superfamily. They are heteromeric protein complexes with several subunits, which arrange into a certain spatial structure to form a ligand-gated ion channel. These subunits are crucial to the plasticity of synapses, which is the foundation of learning and memory(40). SYN1 belongs to the synapsin gene family, which encode neuronal phosphoproteins. It relates to the formation of the synaptic vesicles located on the cytoplasmic surface(41). DLG4 encodes a protein that belongs to the membrane-associated guanylate kinase (MAGUK) family. It is heteromultimeric with DLG2 (another MAGUK protein). DLG4 and DLG2 located on postsynaptic sites cooperate to form clustering receptors and ion channels by making a multimeric scaffold(42). CAMK2A encodes an alpha chain, which is essential for spatial learning and long-term hippocampal potentiation (LTP). It contains CaMK2A, NMDARs, and AMPARs which belong to the glutamatergic signaling family(43). They are essential for appropriate synaptic development and plasticity.

Briefly speaking, SNAP25 and SYN1 affect the transmission function of synapse; GRIN1, DLG4, CAMK2A encode proteins that makeup synapses; they involve in synapses' formation and plasticity. Existing evidence showed that HARs regulate neurodevelopmental processes that have diverged between humans and chimpanzees, such as synapse development(44). The human prefrontal cortex (PFC) is particularly enlarged more than other portions of the brain. It is related to the increasing of neocortical volume in primates. Changes in the synaptic distribution in the primate PFC may cause by different expression and transcription patterns of specific genes(45). Based on our experimental results and the conclusions of the existing studies, it can be speculated that HAR1A may affect the evolution of the human brain by regulating the expression of genes related to synaptic generation.

HAR1A regulates the crucial second signal calcium ion during development

Results of GO analysis of the DEGs showed that "Cellular response to calcium ion" of "biological process" exhibited the highest Rich factor value.

The calcium ion (Ca21) plays a crucial role in neurogenesis as an essential second messenger (46). Neural stem cells stay in the neocortex's ventricular zone to produce progenitor cells, glial cells and subsequently neurons, which build up the entire adult brain(47). Proliferation, migration, and differentiation are strictly regulated by multiple signals during establishing the accurate structures of the cerebral cortex, including cytosolic calcium ion (Ca21). The regulation of Ca2 + is essential in the delicate process of cortical development.

Conclusion

Our study confirmed that overexpressed HAR1A would improve the memory and cognitive ability of mice. We identified the regulatory network of LncRNA HAR1A in the mouse by RNA sequencing. Furthermore, co-expression analysis was performed to predict the function of HAR1A in humans. The results showed that HAR1A might affect brain development through CRs; HAR1A relates to ligand-receptor interaction, Axon guidance, synapse formation, which are important processes for brain development and evolution; HAR1A plays an important role in the regulation of the second signal calcium ion. These findings provide valuable insights into the molecular mechanisms of how HAR1A affects brain development and find the possible role of HARs in the evolution of the brain.

Abbreviations

CRs: Cajal-Retzius neurons

RGCs: radial glia cells

DEGs: differentially expressed genes

Lnc RNA: Long non-coding RNA

MWM test: Morris water maze test

PPI-Net: Protein-Protein Interactions Network

TCGA: The Cancer Genome Atlas

GO analysis: Gene Ontology (GO) analysis

KEGG: Kyoto Encyclopedia of Genes and Genomes

COIN: Correlation-interaction-network; DSCR: Down

Declarations

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Authors' contributions

Min Chen, Nan Li and Shengmou Lin conceived and designed the study. Kailing Huang, Luting Zhang, Shengmou Lin and Allen Chen analyzed the data. Kailing Huang, Xiaoshun Shi and Zhouxia Zheng performed the molecular and animal experiments and prepared figures 1-5. Luting Zhang, ShuHan Shen,

Jimei Sun and JingYin Kong wrote the main manuscript text and edited figures 1-5. All authors reviewed the manuscript.

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Availability of data and materials

The datasets supporting the results of this article are publicly available at NCBI (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE197554).

Ethics approval and consent to participate

All animal experiments were carried out according to the guideline of Experimental Animal Center of Guangzhou Medical University and approved by the ethics committee of Guangzhou Medical University (approval number: 2020-121). Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

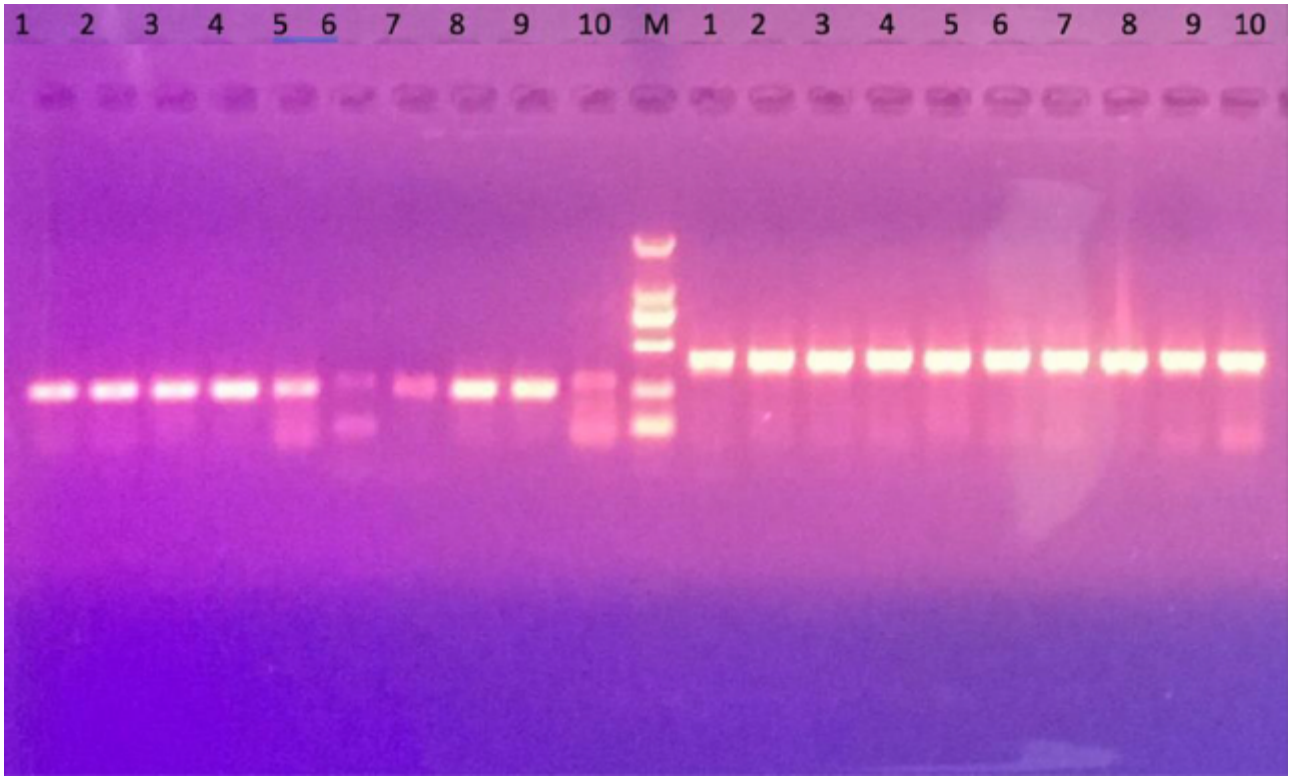

Figure 1

Results of PCR assay of transgenic mice.

HAR1A gene is on the left of marker (M): transgene PCR products size: 252bp; 7 strong positive: 1-4 and 8-9; 5-7 are general / weak positive; No.10 on the left is the control and it is negative. Control gene is on the right side of marker (M): internal control PCR products size: 413bp, No.10 is the control group, its PCR products size: 413bp

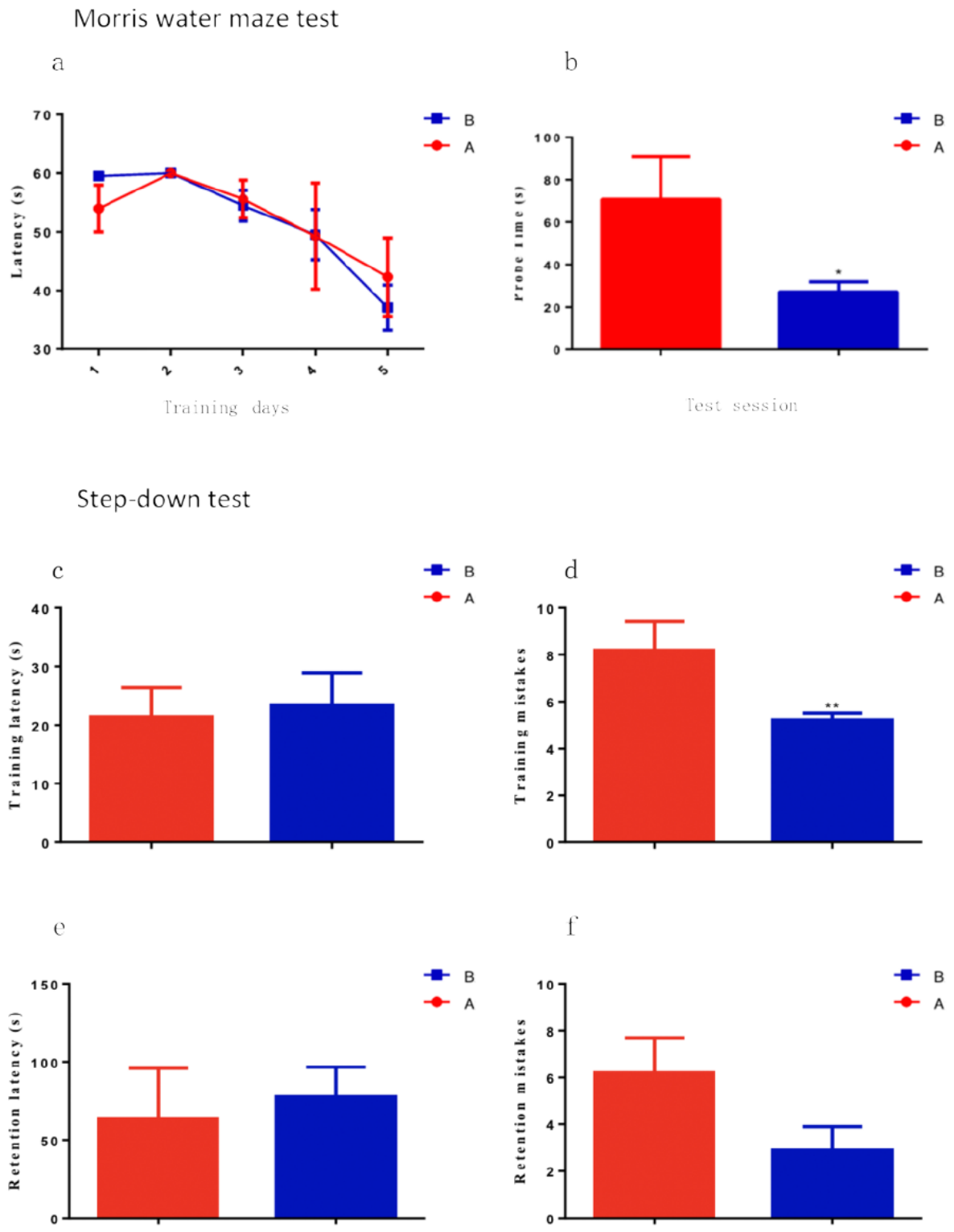

Figure 2

Group A represents the control group and group B represents the experimental group. The escape latencies of the two groups over five consecutive training days (a). The escape latencies of the two groups in the test session (b). The step-down latency of the animals in the two groups during the training (c) and the test session (e). Error counts in the experiment and control groups during the training (d) and the test session(f).

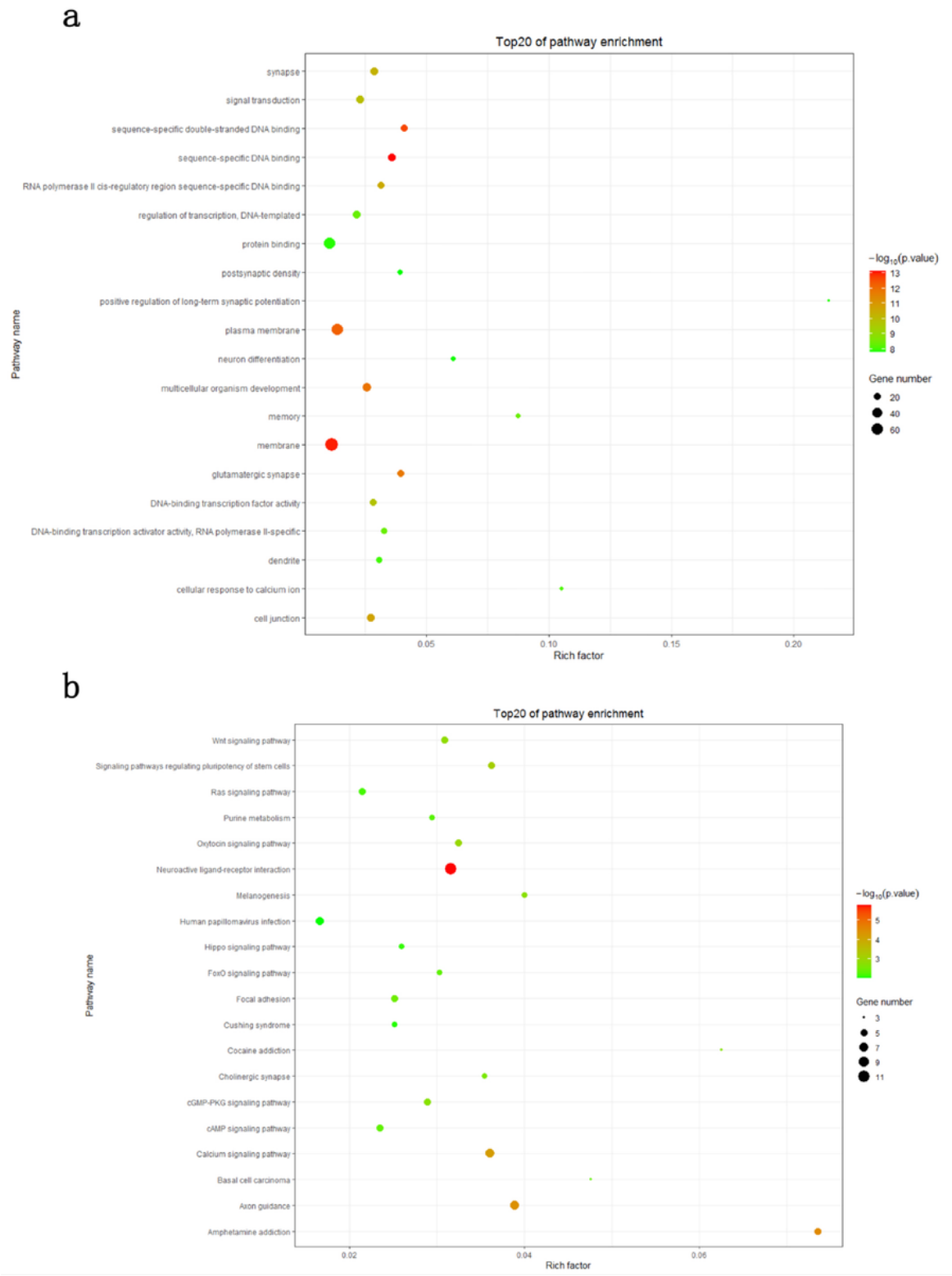

Figure 3

Bulb map of Top 20 of function enrichment in GO analysis（a）and pathway enrichment in KEGG analysis （b）. Rich factor represented the enrichment degree of functions. Y axis showed the name of enriched functions. The area of node represented the number of genes. The p-value is represented by a color scale. The statistical significance increased from green (relatively lower significance) to red (relatively higher significance).

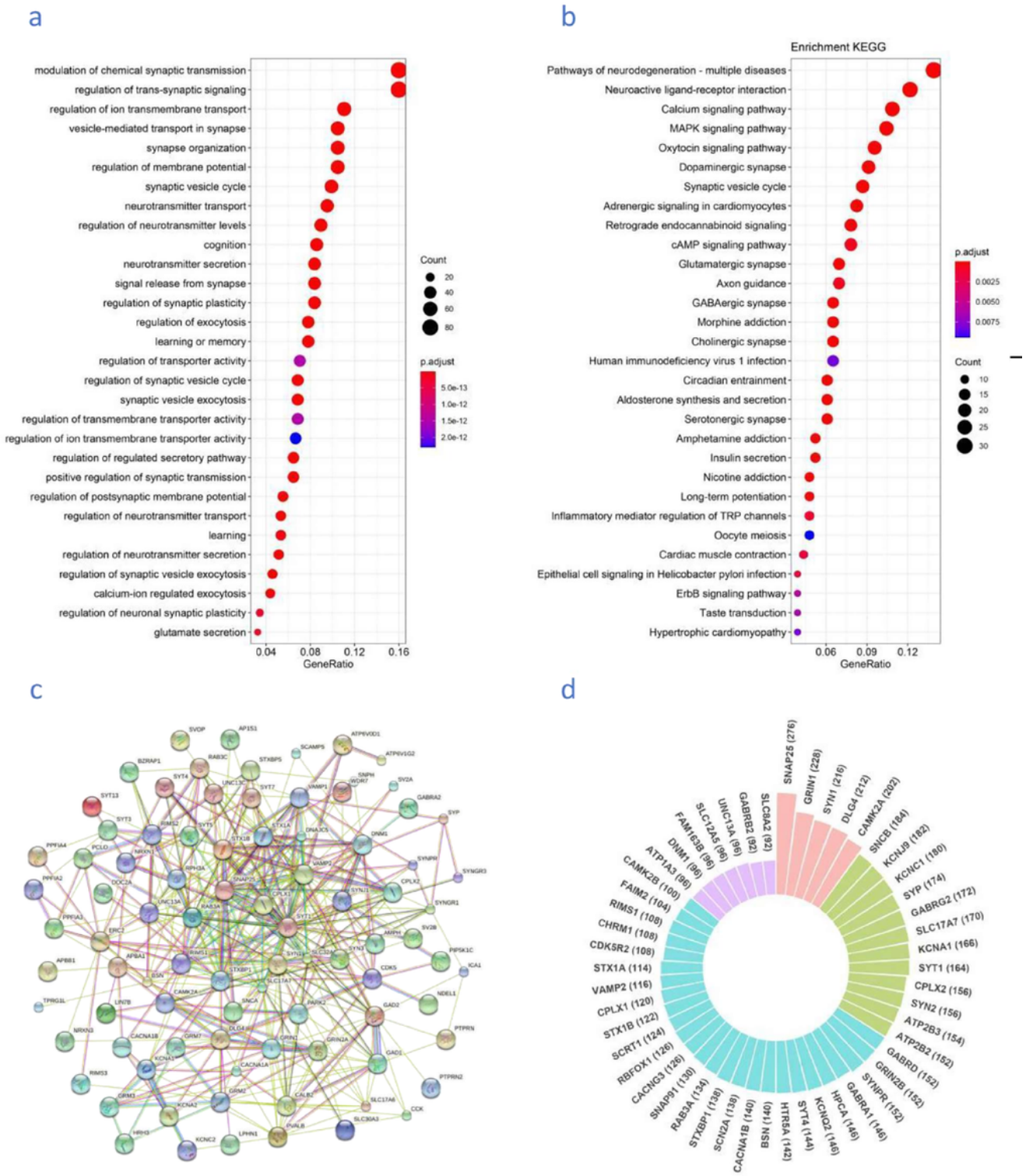

Figure 4

a: Bulb map of Top 20 of function enrichment in GO analysis. b: Bulb map of Top 20 of pathway enrichment in KEGG analysis. Rich factor represented the enrichment degree of pathways. c: Protein interaction network of 103 co-expressed genes associated with HAR1A. d: The 50 genes that are most closely related to other genes in the HAR1A related PPI-Net, the numbers of genes associated with the listed terms are shown in parentheses.

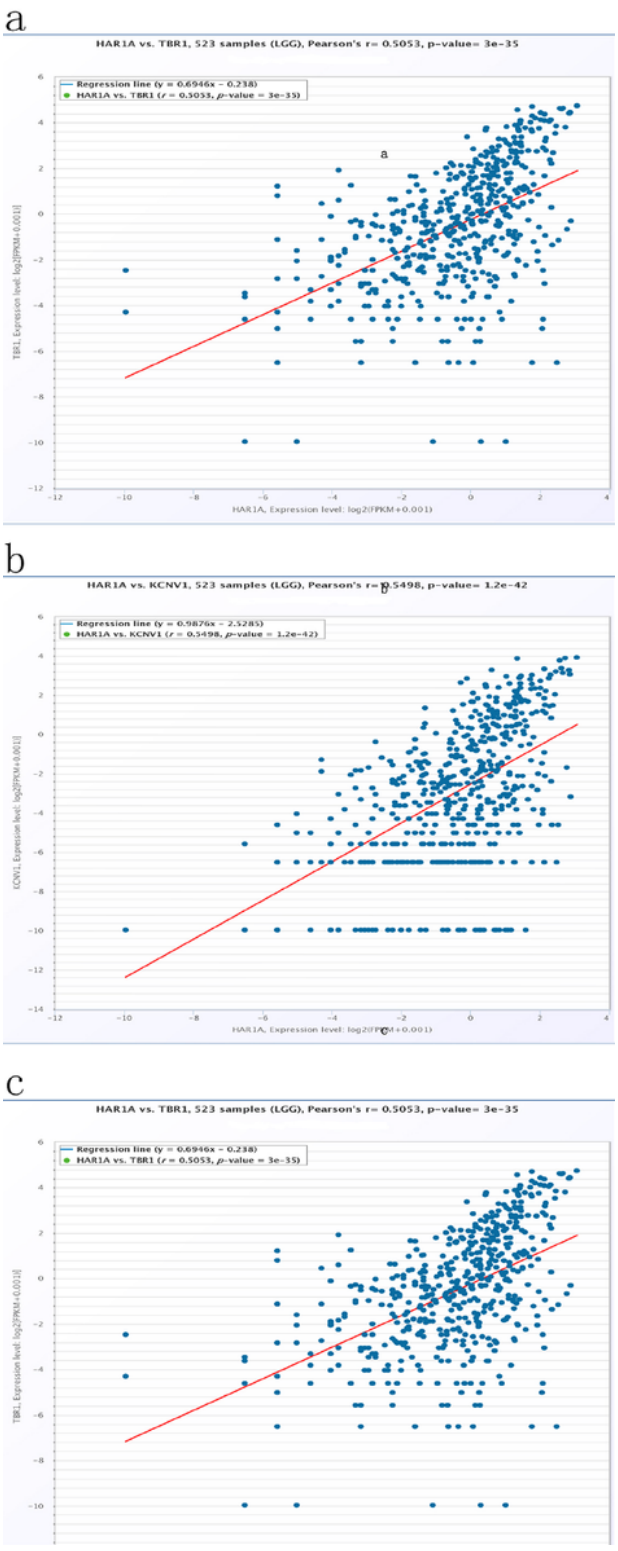

Figure 5

Co-expression diagram of lncRNA-HAR1A with three different genes (significantly differently expressed in 17 animal samples) in the 523 datasets downloaded from the TCGA, a presents the correlation coefficient of lncRNA-HAR1A with TBR1, b presents The correlation coefficient of lncRNA-HAR1A with KCNV1, c presents The correlation coefficient of lncRNA-HAR1A with PHACTR1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementTable1.pdf