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Original Article

A Comparative Transcriptome and Proteome Analysis in Rat Models Reveals Effects of Aging and Diabetes on Expression of Neuronal Genes☆

Qian Xu [1 †], Jing Cai [1 †], Yi-Bo Cong [1], Shao-Jian Xiao [2], Yun Liu [1], Wei Qin [1], Shi-Ya Chen [1], Hong Shi [1 *]

[1] *Institute of Integrated Chinese and Western Medicine, Fujian University of Traditional Chinese Medicine*, [2] *The Third People's Hospital of Fujian Province, Fuzhou, Fujian 350122, PR China*

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SUMMARY

Background: To understand neuronal molecular changes in senile diabetes we established a rat senile diabetes model and analyzed transcriptome and proteome changes.
Methods: Wistar rats were fed a high sugar, high fat diet for 16 months to induce diabetes. Non-diabetic aged rats and young rats were used as controls. Transcript and protein levels in the liver were then analyzed by microarray and antibody arrays, respectively.
Results: Neuronal genes that were differentially expressed between senile diabetic rats, non-diabetic aged rats, and young rats were distributed across 12 pathways and 23 Gene Ontology (GO) clusters. Among them, 2267 genes were aging-related, 1230 genes were diabetes-associated, and 9 proteins might be associated with neurological disorders.
Conclusion: In this study, we investigated transcriptome and proteome changes in animal models, analyzed the impact of aging and diabetes on neuronal molecules, and confirmed the correlations. Our study provides support for further studies on mechanisms of neuronal diseases.

1. Introduction

Growth in personal income and an increase in average life expectancy have contributed to a dramatic increase in the number of older people with diabetes mellitus (DM) and associated complications, and the mortality rate in this group is much higher than in the general population. Hyperglycemia causes significant damage to the body through the accumulation of metabolic toxins such as superoxides and advanced glycation end products, resulting in ischemia, hypoxia, oxidative stress, and inflammation. It can also cause apoptosis of neurons and gliosis, which may lead to cognitive impairment and even central nervous system disorders such as Alzheimer's disease (AD)[1–3]. Current studies have demonstrated that multiple genes or proteins play important roles in diabetic neuropathy, but there is a lack of data on transcriptome and proteome changes in animal models used in research to investigate the effects of on multiple genetic loci involved in nervous system pathways.

2. Materials and methods

2.1. Animal models

Eighty specific-pathogen-free (SPF) healthy female Wistar rats (6 months of age, weighing 250 ± 20 g) were obtained commercially. Animal experiments were approved by the Ethics

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* Correspondence to: Prof. Hong Shi, Institute of Integrated Chinese and Western Medicine, Fujian University of Traditional Chinese Medicine, No. 1, Qiuyang Road, Minhou, Fuzhou 350122, PR China.
E-mail address: shihong3327@sina.com (H. Shi).

† The first two authors contributed equally to this study. I certify that all my affiliations with or financial involvement in, within the past 5 years and foreseeable future, any organization or entity with a financial interest in or financial conflict with the participant matter or materials discussed in the manuscript are completely disclosed (e.g., employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, royalties).

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Committee and were performed in accordance with international guidelines[4]. After feeding the rats for 7 days, animals were randomly divided into 3 groups: 10 in the young group, 20 in the aged group, and 50 in the senile diabetes group. Samples from the rats in the young group were immediately prepared as described in the next section. The aged group rats were fed a basic diet (flour 19%, corn starch 23%, sorghum flour 6%, bran 10%, skimmed milk powder 20%, fish powder 10%, starch 7%, glycine 3%, and beer yeast 2%). The senile diabetes group rats were fed a high fat, high sugar diet (sugar 15%, lard 10%, cholesterol 4%, bile salt 0.3%, egg yolk powder 10%, and basic diet 60.7%). The animals were fed continuously for 16 months and the level of blood glucose was monitored. If an animal did not become diabetic, i.e., did not have a blood glucose level $\geq$16.7 mmoL/L characteristic of rats with senile diabetes, a low dose of streptozotocin (STZ) (20 mg/kg) was injected intraperitoneally. Three days later, the blood glucose level was monitored again and animals with a high blood glucose level were selected. The remaining animals received another intraperitoneal injection of STZ and qualified animals were selected after a further 3 days.

2.2. Sample preparation

Rats in each group were weighed and anesthetized with 1% urethane. The abdomen of each rat was then opened, and liver tissues were collected and placed in liquid nitrogen for storage. To obtain RNA for transcriptome analysis, 2 g of liver per animal was obtained from 5 animals chosen at random in each group, and total RNA was extracted from the liver samples using Trizol. For RNA quality control, the value of the RNA integrity number (RIN) measured by the Agilent 2100 Bioanalyzer had to be $\geq$7.0 and the value of 28 S/18 S had to be $\geq$0.7.

2.3. Microarray

Rat RNA and tissue samples were sent to the Shanghai Biotechnology Corporation and analyzed using the Agilent Rat Gene Expression 4 $\times$ 44 K Microarray Kit and the Full Moon Explorer Antibody Array.

2.4. Bioinformatics

SAS software was used for bioinformatics analysis. Differences between means of the 3 groups were analyzed by one-way ANOVA. Differences between pairs of groups were analyzed by Dunnett's test. A P value less than 0.05 was considered statistically significant. Fold change (FC) was calculated, and genes were clustered by Gene Ontology (GO). The effects of aging and diabetes on neuronal genes were analyzed by Pearson's χ^2 test. The *P* values of genes in different signaling pathways were calculated by Fisher's exact test. The genes in the resulted file were then cross-checked and annotated with databases from NCBI Entrez Gene, KEGG, and Biocarta.

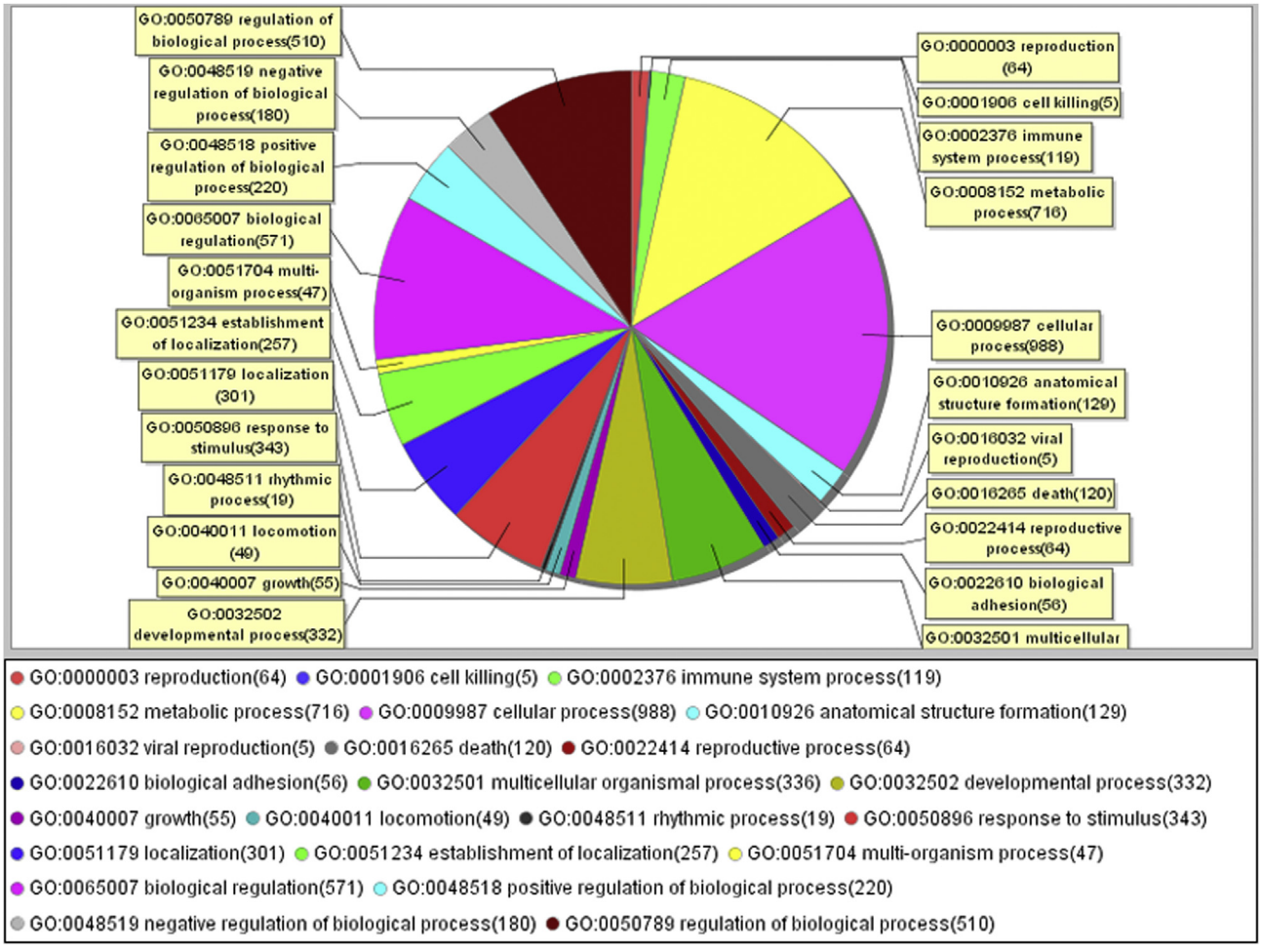

Fig. 1. Pie chart of biological processes of the mRNA.

Table 1
The Gene Ontology of differentially expressed neuronal molecules.

Pathway name	Hits	Total	Percent	*P*
Alzheimer's disease	37	214	17.29%	0.0076
Amyotrophic lateral sclerosis (ALS)	22	66	33.33%	0.0343
Axon guidance	14	127	11.02%	0.0994
Cholinergic synapse	10	112	8.93%	0.3174
Huntington's disease	28	221	12.67%	0.0063
Long-term depression	8	71	11.27%	0.1679
Neuroactive ligand-receptor interaction	17	284	5.99%	0.8179
Neurotrophin signaling pathway	9	129	6.98%	0.6003
Olfactory transduction	140	1035	13.53%	0.0512
Parkinson's disease	21	167	12.57%	0.0174
Regulation of actin cytoskeleton	22	210	10.48%	0.0723
Taste transduction	4	50	8.00%	0.5051

We used the SAS system (SBC Analysis System) to do the annotation of the different genes (http://www.ebioservice.com/sas.html) and all the pathway names came from the KEGG database (http://www.kegg.jp/). *P* value was calculated by Fisher's exact test.

Table 2
Statistical analysis of the effects of aging and diabetes on neuronal genes.

	Neuronal genes	Other genes	Total	χ^2	*P*
Aging	189	2078	2267	12.326	4.47 E-04
Diabetes	63	1167	1230		
Total	252	3245	3497		

3. Results

3.1. Differentially expressed mRNA

ANOVA analysis of the mRNAs revealed 5486 differentially expressed genes ($P < 0.05$) that were clustered in 23 biological processes (Fig. 1). The differentially expressed neuronal genes were clustered to 12 pathways as shown in Table 1. As shown in Table 2, 2267 genes were associated with aging (aged group vs. young group), and 1230 genes were associated with diabetes (senile diabetic group vs. aged group) (Tables 2–4).

3.2. Differentially expressed proteins

In all, 656 proteins were analyzed by the Explorer Antibody Array, and each sample was repeated twice. Nine proteins associated with the nervous system were correlated with age or diabetes (Fig. 2). All the differentially expressed proteins and their interactive proteins are shown in Fig. 3.

4. Discussion

Many researchers have recognized that obesity, hyperglycemia, and hypercholesterolemia are risk factors for senile neuropathy, especially neurodegenerative diseases such as AD and Parkinson's disease (PD)[5–9]. Chronic high blood glucose can cause neuropathy and microvascular disease, and induce vascular PD. Acute complications of diabetes such as ketoacidosis and nervous system damage caused by hyperosmolar coma can lead to secondary Parkinson's disease[9]. Results of epidemiological research are controversial. Studies have shown that the risk of PD in patients with DM was 40% higher than in non-DM patients, especially for people who had had diabetes for more than 10 years (Odds Ratio = 1.75)[10]. However, other studies showed that neither weight nor waist circumference, both indicators of obesity associated with risk for developing DM, was correlated with PD[11].

Of the differentially expressed proteins that we identified, the cadherin family of proteins plays an important role in development of the nervous system. E-cadherin can inhibit differentiation and promote migration of neural epithelial cells. E-cadherin expression is low in multiple tumor tissues, including human squamous cell esophageal cancer, nasopharyngeal carcinoma, thyroid carcinoma, squamous cell head and neck cancers, malignant melanoma, breast cancer, and retinoblastoma[12–17]. This study confirmed that expression of cadherin and E-cadherin was low in the aged and senile diabetes groups, similar to results found in cancer research studies.

Collagen IV (C-IV) is one of the main components of the basement membrane. C-IV can promote cell adhesion, growth, and differentiation[18,19]. Lein et al. found that C-IV could promote axon growth, as well as increase the number and length of nerve fibers[20]. It is believed that C-IV functions through receptors in the membrane of nerve cells to promote axon growth[21]. In this study, expression levels of many cytoskeleton genes changed. In particular, expression levels of C-IV significantly decreased in the aged and senile diabetes groups compared to young rats.

Increased expression of ferritin (Fer) proteins is considered an important and independent predictor of the development of DM. Epidemiological studies indicate that an increased level of Fer in serum is positively correlated with an increased incidence of DM[22–24]. Upregulation or downregulation of Fer or the transferrin receptor (Tfr) disrupts iron metabolism and leads to degenerative diseases of the nervous system. In brain tumors, Tfr-induced oxidation inhibits tyrosine phosphatase, activates mitogen-activated protein kinase (MAPK) and Akt, inhibits cell cycle regulators p21 and cyclin-dependent protein kinases (CDK), and

Table 3
Diabetes-related and differentially expressed rat neuronal genes.

Description	Symbol	Gene Id	Chromosome	*P*	FC
Cytoskeleton associated protein 2	Ckap2	306575	16	0.0032	0.2282
Cell division cycle 2, G1 to S and G2 to M	Cdc2	54237	20	0.0379	0.2318
Cyclin B1	Ccnb1	25203	2	0.0278	0.3403
Cyclin D1	Ccnd1	58919	1	0.0455	0.3479
Neuronal pentraxin 2	Nptx2	288475	12	0.0253	0.3823
Butyrylcholinesterase	Bche	65036	2	0.0267	0.3922
Olfactory receptor 428	Olr428	296689	3	0.0285	0.3956
Neuropeptide FF receptor 1	Npffr1	64107	20	0.0044	0.4092
Olfactory receptor 1583	Olr1583	289241	13	0.0455	0.4514
Olfactory receptor 1096	Olr1096	302027	7	0.0310	0.4540
Olfactory receptor 789	Olr789	296042	3	0.0396	0.4944
GLIS family zinc finger 2	Glis2	302946	10	0.0378	2.1169
Deleted in malignant brain tumors 1	Dmbt1	170568	1	2.00 E-04	2.7358
Triggering receptor expressed on Myeloid cells 2	Trem2	301227	9	0.0120	3.6642

Gene Id denotes the ID number of the corresponding NCBI Entrez gene. FC = expression of the senile diabetes group/expression of the aged group. This table lists neuronal genes with FC > 2 or <0.5 between the senile diabetes and aged groups ($P < 0.05$).

Table 4
Aging-related and differentially expressed rat neuronal genes.

Description	Symbol	Gene Id	Chromosome	*P*	FC
Collagen, type IV, alpha 5	Col4a5	363457	X	5.00 E-04	0.1515
Transferrin receptor	Tfr	64678	11	0.0016	0.1928
Collagen, type III, alpha 1	Col3a1	84032	9	0.0020	0.2332
Collagen, type I, alpha 2	Col1a2	84352	4	0.0061	0.3543
Odorant binding protein I f	Obp1f	192267	X	0.0021	0.3859
Tubulin, alpha 4A	Tuba4a	316531	9	0.0078	0.4764
Brain expressed myelocytomatosis oncogene	Bmyc	311807	3	0.0046	2.0929
Deleted in malignant brain tumors 1	Dmbt1	170568	1	2.00 E-04	2.9055
Cytoskeleton associated protein 2	Ckap2	306575	16	0.0061	4.9598
Cholinergic receptor, nicotinic, alpha 7	Chrna7	25302	1	3.00 E-04	5.3270

Gene Id denotes the ID number of the corresponding NCBI Entrez gene. FC = expression of the aged group/expression of the young group. This table lists neuronal genes with FC > 2 or <0.5 between the aged groups and young groups ($P < 0.05$).

promotes proliferation of tumor cells[25]. Restless legs syndrome (RLS) may be caused by destabilization of Tfr1 mRNA, which may decrease cellular uptake of iron[26]. When Tfr1 expression increases, expression of amyloid precursor protein decreases, which slows the progression of AD[27]. Our study found that expression of Tfr protein in senile DM was significantly increased, and confirmed the correlation between DM and the level of Fer protein. Compared with the young group, Tfr mRNA expression in the aged group was significantly reduced, but the protein level was not obviously different between the 2 groups, which suggests that translational regulation controls Tfr protein levels during the aging process.

The neurotrophic factor insulin can stimulate axonal growth and promote regeneration of the peripheral nervous system, and is required for survival of the sympathetic nervous system. In diabetic peripheral neuropathy, a lack of nervous system glucose uptake caused by decreased insulin plays a major role[28]. Our research suggested a similar injury; expression of insulin-like growth factor-1 (Igf-1) protein was not significantly different between the young and aged groups, but was significantly reduced in the senile diabetes group. However, Igf-1 mRNA transcripts were not obviously different, which suggest that regulation is at the translation level.

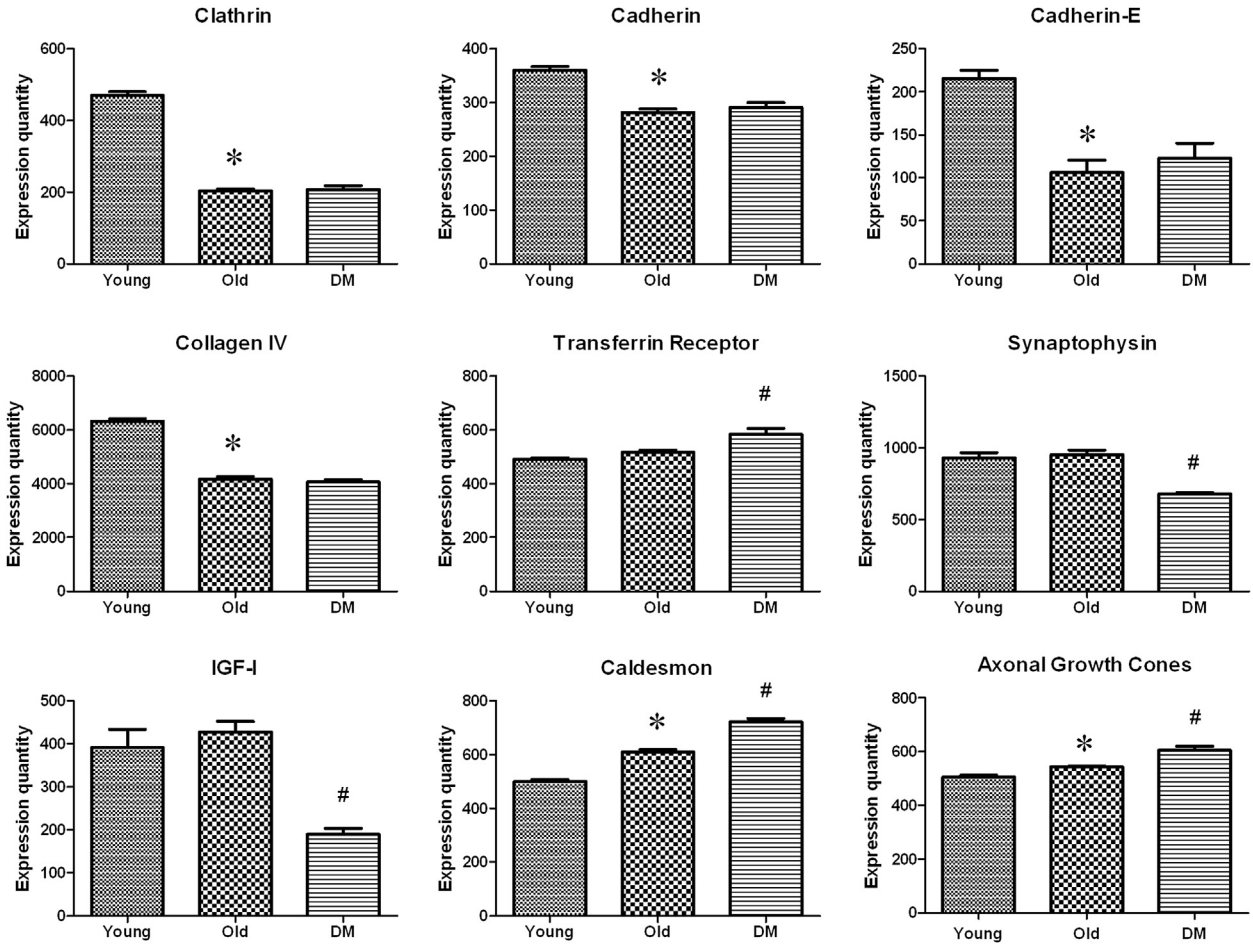

Fig. 2. Expression levels of differentially expressed proteins. The expression level is the relative expression of proteins normalized to the control β-actin. *Indicates a significant difference between the aged and young groups ($P < 0.05$). #Indicates a significant difference between the senile diabetes and aged groups ($P < 0.05$).

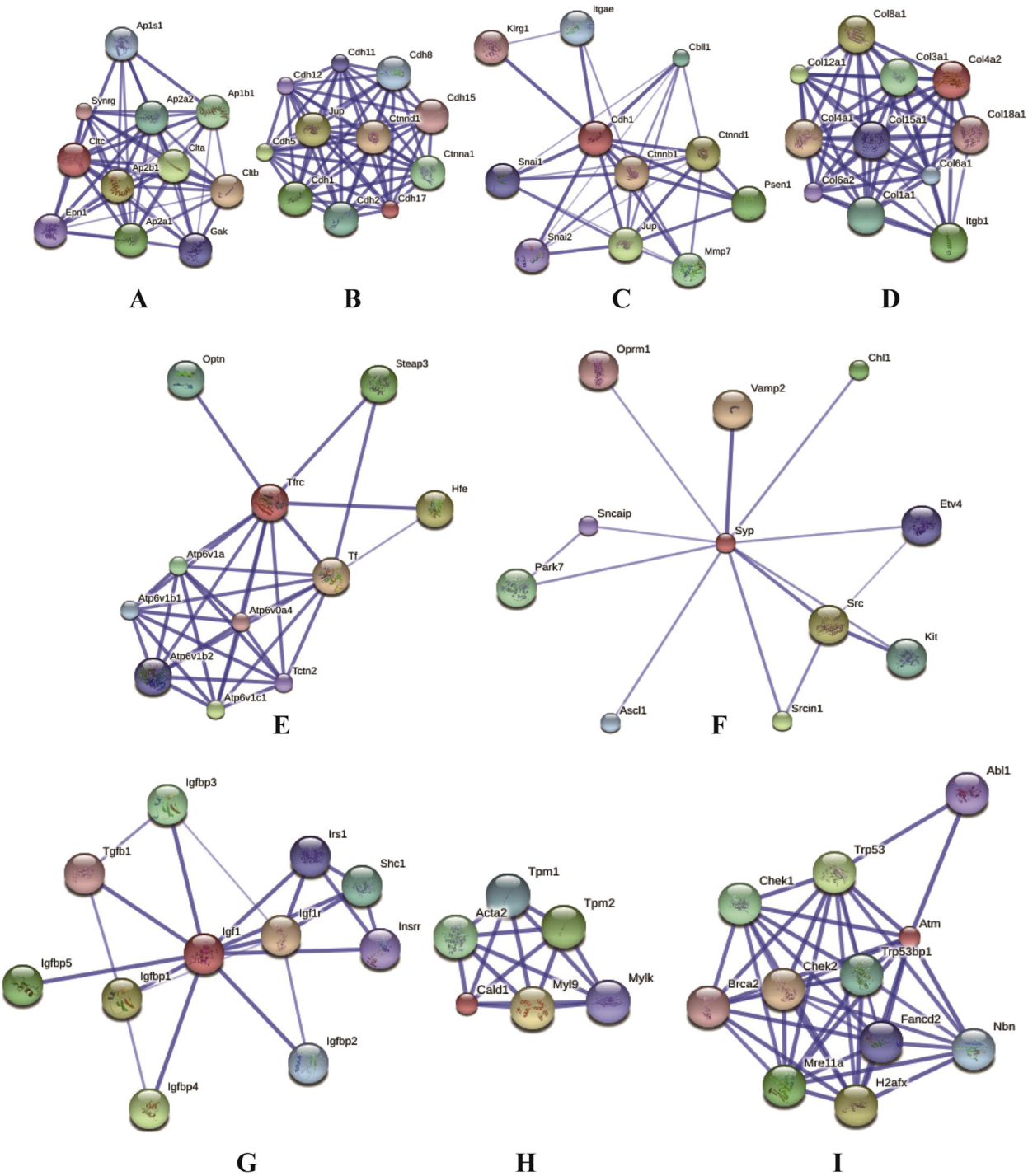

Fig. 3. Interactions of differentially expressed proteins. STRING is a database of predicted functional associations among genes/proteins (http://www.bork.embl-heidelberg.de/STRING/). This is the confidence view. Stronger associations are represented by thicker lines. The red spheres in the pictures represent the differentially expressed proteins. A. Cltc-clathrin. B. Cdh-cadherin C. CDH1-cadherin 1, cadherin-E. D. Col4a2 – collagen, type IV, alpha 2. E. Tfrc-transferrin receptor protein 1. F. Syp – Synaptophysin. G. Igfr1-Insulin-like growth factor I. H. Cald1-caldesmon 1. I. Atm-ataxia telangiectasia mutated.

The physiological role of synaptophysin (Syn) is not completely understood, but it is thought to play roles in synaptic vesicle exocytosis and Ca^{2+}-dependent neurotransmitter release, synaptic vesicle recycling, and synapse formation[29,30]. In senile diabetic rats, the expression of Syn was significantly reduced, which suggests a direct role in diabetic neuropathy.

The mRNA levels of many olfactory receptors including olfactory receptor 789 and olfactory receptor 1096 were downregulated in senile diabetic rats, but protein levels were not obviously changed. Olfactory receptors belong to a large family in the genome, and many receptors in this family were affected. Impaired olfaction reflects initial nervous system damage, but the mechanism is not clear. This study also found that levels of mRNA and protein expression were not correlated, which suggests that they are regulated at transcriptional and translational levels.

5. Conclusions

The pathogenesis of diseases begins with molecular changes at the micro level and progresses to phenotypic changes at the macro level. We propose that the mechanism by which traditional Chinese medicine improves the health of the nervous system in senile diabetes acts via regulation of multiple targets and multiple pathways, as suggested both by animal experiments and by clinical observations in patients. This study provides a molecular basis for the prevention and early diagnosis of DM and senile neurological diseases. The roles of the genes, proteins, and signaling pathways that are affected require further verification and study.

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