


Research Article

Autophagic Network Analysis of the Dual Effect of Sevoflurane on Neurons Associated with GABARAPL1 and 2

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Background. Sevoflurane is commonly used as a general anesthetic in neonates to aged patients. Preconditioning or postconditioning with sevoflurane protects neurons from excitotoxic injury. Conversely, sevoflurane exposure induces neurotoxicity during early or late life. However, little is known about the underlying mechanism of the dual effect of sevoflurane on neurons. Autophagy is believed to control neuronal homeostasis. We hypothesized that autophagy determined the dual effect of sevoflurane on neurons. **Methods.** DTome was used to identify the direct protein target (DPT) of sevoflurane. The STRING database was employed to investigate the proteins associated with the DPTs. Protein-protein interaction was assessed using Cytoscape. WebGestalt was used to analyze gene set enrichment. The linkage between candidate genes and autophagy was identified using GeneCards. **Results.** This study found that 23 essential DPTs of sevoflurane interacted with 77 proteins from the STRING database. GABARAPL1 and 2, both of which are DPT- and autophagy-associated proteins, were significantly expressed in the brain and enriched in GABAergic synapses. **Conclusions.** Taken together, our findings showed that the network of sevoflurane-DPT-GABARAPL1 and 2 is related to the dual effect of sevoflurane on neurons.

1. Background

Sevoflurane is commonly used as a sedative and general anesthesia in neonates to elderly patients. A growing number of preclinical studies have demonstrated that sevoflurane exposure during early life leads to neurodegeneration in rodents and nonhuman primates [1]. Moreover, accumulating evidence has shown that sevoflurane exposure is associated with postoperative cognitive dysfunction in aged patients [2, 3]. Conversely, either preconditioning or postconditioning with sevoflurane has been shown to exert a neuroprotective effect on cerebral ischemia injury in adult rodents [4–6]. Thus, sevoflurane has a dual effect on neurons in the brain in different states. However, the mechanism underlying the dual effect of sevoflurane on neurons remains unclear.

Autophagy plays a crucial role in neuron survival and neurodegenerative diseases, maintaining homeostasis within cells [7, 8]. In response to excitotoxicity, which is characterized by an abrupt and prolonged increase in cytoplasmic Ca^{2+} levels and neurotoxicity [9], which is caused by neurotoxins, emerging evidence strongly suggests that autophagy is triggered to reduce the detrimental effects of excitotoxicity and neurotoxicity [10]. Autophagy induction contributed to protecting against sevoflurane-induced neurotoxicity in elderly patients [11]. Conversely, autophagy blockade ameliorated the cognitive impairment induced by sevoflurane in young mice [12]. In a rat model of focal cerebral ischemia, sevoflurane preconditioning plays a neuroprotective role in reducing Akt signaling activity to activate autophagy [4, 13]. Interestingly, after transient global cerebral ischemia in

rats, sevoflurane postconditioning reduces cell apoptosis via activating the JAK-STAT pathway [14]. Recent observations have suggested that STAT3 plays a role in regulating the autophagic process by functioning as an enhancer or suppressor in the nucleus, cytoplasm, and mitochondria [15].

In summary, autophagic activation promotes neurotoxicity in neonatal animals, opposite to exerting a protective effect on neurotoxicity in aged animals. Autophagy induced by sevoflurane preconditioning or postconditioning contributes to protecting against brain injury caused by ischemia and reperfusion. However, no previous study has revealed how autophagy determines the dual effect of sevoflurane on neurons in the brain under different conditions. In recent decades, an increasing number of interesting gene hits have been found using microarray and proteomics and other high-throughput screening assays [16]. Data obtained from humans have contributed to building a network linking a drug and its targets to interacting proteins. DTome, a web-based tool, is used to construct a drug-target interactome [17]. We hypothesized that the dual effect of sevoflurane on neurons in the brain is determined by the autophagic network. In this study, we used DTome to investigate the direct protein targets (DPTs) of sevoflurane and we utilized the STRING database to query protein-protein interactions (PPIs). Furthermore, bioinformatics analysis was used to identify the properties of genes related to the dual effect of sevoflurane.

2. Materials and Methods

2.1. Sevoflurane Target Query. DTome is composed of such three open-source datasets, namely, DrugBank, PharmGSK, and PINA [17]. Sevoflurane was searched in DrugBank (website: <https://www.drugbank.ca/>) to find information about sevoflurane and identify its DPTs.

STRING version 10.5 (website: <https://string-db.org/cgi/input.pl>) was used to generate a PPI network. In the STRING database, the DPTs identified from DTome were searched using a single protein name. The organism was searched as *Homo sapiens*. In the basic settings of the STRINGdb browser, the meaning of network edges was set as evidence (line color indicates the type of interaction evidence). Active interaction sources were set as text mining, experiments, databases, coexpression, neighborhood, gene fusion, and cooccurrence. The minimum required interaction score was set as the medium confidence (0.400), and the score was set as the highest confidence (0.900) for searching gamma-aminobutyric acid receptor-associated protein-like (GABARAPL)1 and 2. The maximum number of interactors was set as 50 in the 1st shell, and none was selected in the 2nd shell. Data regarding the proteins that interacted with the DPTs were exported as simple tabular text from the STRING database. The overall proteins that interacted with the DPTs were verified by deleting duplicated proteins, and the remaining proteins were used to compile a dataset.

2.2. Gene Set Enrichment Analysis. WebGestalt 2019 (website: <http://www.webgestalt.org/>) [18], a web-based gene set analysis toolkit, was used to analyze the enrichment of sevoflurane-associated genes. In basic parameters, the organ-

ism of interest was selected as “*hsapiens*,” which was evaluated using KEGG pathway enrichment analysis. In the gene list, the gene ID type was selected as a gene symbol. The DPTs and DPT-associated genes were uploaded as a gene list. The reference used for enrichment analysis was set as the genome, which was used to determine the Gene Ontology and KEGG enrichment.

2.3. Network Visualization. Cytoscape version 3.8.0 (website: <http://www.cytoscape.org/>), an open-source software platform, was used to visualize the sevoflurane-mediated network. In a text file, sevoflurane-mediated DPTs were compiled as node 1, and DPT-associated genes were compiled as node 2. After the text file was input into Cytoscape, node 1 was set as the source node, and node 2 was set as the target node. DPT-associated genes enriched in the top 10 pathways were selected for investigating the first neighbor outgoing nodes. The significantly targeted genes were further identified as candidate genes.

2.4. Identification of Autophagy-Associated Genes. The Human Gene Database (website: <http://www.genecards.org/>), GeneCards, was used to search for the genomic, transcriptomic, proteomic, genetic, clinical, and functional information of the candidate genes from approximately 125 web sources. The symbol of the candidate gene was searched, and GeneCards provided a great deal of information on the candidate genes, including aliases, expression information, functional genomics, location, pathways, transcripts, domains, and proteins. Based on the functions and pathways of the genes, more information was gathered on the candidate genes associated with autophagy, including their genomics, location, domains, proteins, and expression. Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to map the autophagy pathway. UniProtKB/Swiss-Prot was used to investigate the protein sequences and functional information of the candidate genes. STRING version 10.5 was used to investigate the PPIs of the proteins expressed by the candidate genes. The PPI data were downloaded from the STRING database and uploaded to Cytoscape V3.8.0 for network analysis. The map node size and color of the map were defined in terms of indegree; similarly, the map edge size and color of the map were defined according to the combined score.

3. Results

3.1. Characterization of the DPTs and DPT-Associated Proteins of Sevoflurane. As described in Table 1, sevoflurane was queried as DB01236 in DrugBank and categorized as an anesthetic agent, a hematologic agent, and a platelet aggregation inhibitor. Sevoflurane is generally applied to induce and maintain general anesthesia, especially in pediatric patients. As shown in Table 2, 23 essential DPTs of sevoflurane were identified using DrugBank.

To determine the PPIs of these DPTs in *Homo sapiens*, a single DPT name was used to search STRING version 10.5. After mapping in the STRING database, 77 proteins were identified as DPT-associated genes related to sevoflurane (Figure 1).

TABLE 1: Characteristics of sevoflurane using DTome.

DB_ID	Name	Groups	Categories	Indication
DB01236	Sevoflurane	Approved, vet approved	Anesthetics	Used for induction and maintenance of general anesthesia in adult and pediatric patients for inpatient and outpatient surgery
			Anesthetics, general	
			Anesthetics, inhalation	
			Central nervous system agents	
			Central nervous system depressants	
			Cytochrome P-450 CYP2A6 substrates	
			Cytochrome P-450 CYP2B6 substrates	
			Cytochrome P-450 CYP2E1 substrates	
			Cytochrome P-450 CYP3A4 substrates	
			Ethers	
			Hematologic agents	
			Hydrocarbons, halogenated	
			Nervous system	
Platelet aggregation inhibitors				
QTc-prolonging agents				
(indeterminate risk and risk modifying)				

TABLE 2: Identification of direct protein targets of sevoflurane using DTome.

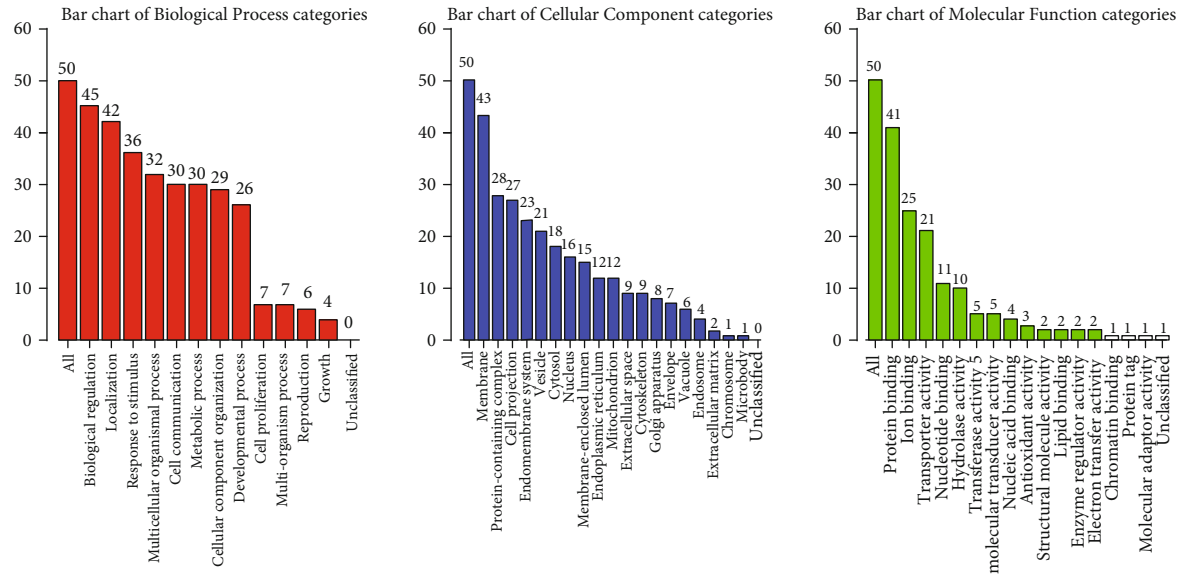
Searched_Drug (1/1)		Target (8)				Action
Number	DB_ID	Name	Target_Symbol	UniProt ID		
1	DB01236	Sevoflurane	GABRA1	P14867		Agonist
2	DB01236	Sevoflurane	GLRA1	P23415		Agonist
3	DB01236	Sevoflurane	GRIA1	P42261		Antagonist
4	DB01236	Sevoflurane	KCNA1	Q09470		Inducer
5	DB01236	Sevoflurane	ATP2C1	P98194		Inhibitor
6	DB01236	Sevoflurane	ATP5D	P30049		Other/unknown
7	DB01236	Sevoflurane	MT-ND1	P03886		Unknown
8	DB01236	Sevoflurane	GABRA1/2/3/4/5/6*	P14867/P47869/P34903/P48169/P31644/Q16445		PAM
8	DB01236	Sevoflurane	GABRB1/2/3, GABRD, GABRE*	P18505/P47870/28472, Q14764, P78334		PAM
8	DB01236	Sevoflurane	GABRG1/2/3, GABRP, GABRQ*	Q8N1C3/P18507/Q99928, O00591, Q9UN88		PAM

GABRA1: gamma-aminobutyric acid receptor subunit alpha-1; glycine receptor subunit alpha-1; glutamate receptor 1; potassium voltage-gate channel subfamily A member 1; calcium-transporting ATPase type 2C member 1; ATP synthase subunit delta, mitochondrial; NADH-ubiquinone oxidoreductase chain 1; *GABA-A receptor (anion channel); PAM: positive allosteric modulator.

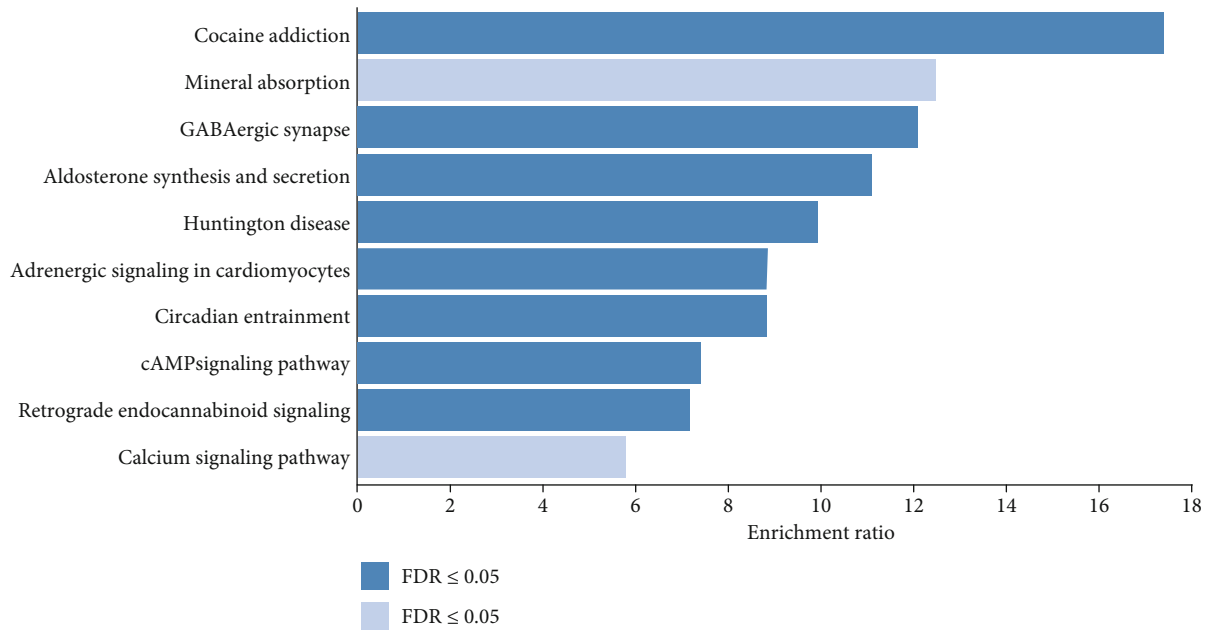
3.2. *Enrichment of Gene Sets Mediated by Sevoflurane.* To investigate the functional properties of the sevoflurane-mediated gene sets, we performed the KEGG pathway enrichment analysis using WebGestalt 2019. The resulting gene list (Figure 1) contained 77 user IDs; of these user IDs, 50 were unambiguously mapped to the unique Entrez Gene IDs, and 27 could not be mapped to any Entrez Gene ID. As shown in Figure 2(a), the Gene Ontology (GO) Slim summary was based on the 50 unique Entrez Gene IDs. In total, 50 genes were included in the biological process, cellular component, and molecular function categories. Among the 50 unique Entrez Gene IDs, 35 IDs were annotated to the selected functional categories and also in the reference list, which were used for the enrichment analysis. The reference list was mapped to 61,506 Entrez Gene IDs, and 7469 IDs were annotated to the selected functional categories that are used as the reference for the enrichment analysis. Benjamini and Hochberg (BH) [19] was used to control the false discovery rate (FDR) of enrichment analysis. As described in Figure 2(b) and Table 3, the top 10 KEGG pathways were

as follows: Huntington’s disease (17 genes); aldosterone synthesis and secretion (5 genes); retrograde endocannabinoid signaling (5 genes); calcium signaling (5 genes); GABAergic synapse (5 genes); cAMP signaling pathway (7 genes); cocaine addiction (4 genes); adrenergic signaling in cardiomyocytes (6 genes); circadian entrainment (4 genes); and mineral absorption (3 genes). After 35 annotated genes were mapped to a gene set, 7 genes were enriched in the cAMP signaling pathway, 5 genes in GABAergic synapse, and 5 genes in the calcium signaling pathway (Table 3), which are shown in a Venn diagram in Figure 2(c). Three candidate pathways including the GABAergic synapse, cAMP signaling pathways, and calcium signaling pathway were further selected to identify the candidate DPT-associated genes.

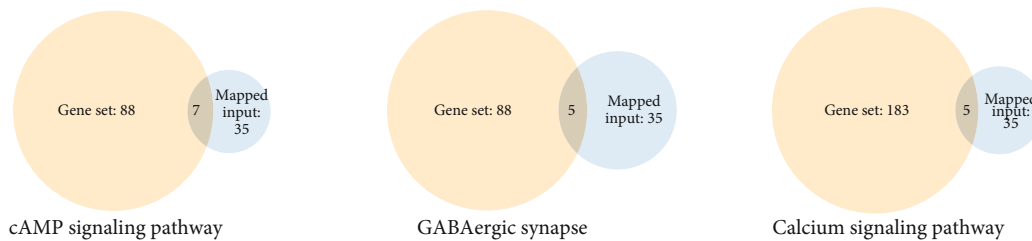
3.3. *Visualization of the Sevoflurane-Linkage Networks by Cytoscape.* A relevant biological network containing the DPTs and their associated genes (Figure 3) was constructed using Cytoscape version 3.8.0. The major associated-genes, namely, GABARAP, NSF, DNM1, GABARAPL2, UBQLN1,



(a)



(b)



(c)

FIGURE 2: GO enrichment analysis of 77 proteins associated with DPTs by WebGestalt 2019. Only 50 user IDs of 77 proteins associated with DPTs were mapped to the unique Entrez Gene IDs. WebGestalt 2019 was used to evaluate the biological process (red column), cellular component (blue column), and molecular function (green column) categories (a). Among the 50 unique Entrez Gene IDs, 35 were annotated to the selected functional categories and reference list, which were used for the enrichment analysis. The bar chart shows the top 10 pathways in which 35 IDs were enriched (b). The Venn diagram shows that 7 genes in the cAMP signaling pathway, 5 genes in the GABAergic synapse, and 5 genes in the calcium signaling pathway were mapped in the gene set (c).

TABLE 3: TOP 10 pathway enrichment from 77 interactive proteins analyzed by WebGestalt.

ID	Pathway	Gene	Expect	FDR
hsa05030	Cocaine addiction	CREB1/DLG4/GRIA2/BDNF	0.22960	4.1e-03*
hsa04978	Mineral absorption	CLCN2/FTH1/ATP2B1	0.239	5.49e-02
hsa04727	GABAergic synapse	GPHN/GABARAPL2/GABARAPL1/NSF/TRAK2	0.41240	4.1e-03*
hsa04925	Aldosterone synthesis and secretion	ATP2B1/ATP2B2/ATP2B4/CAMK2G/CREB1	0.44990	4.1e-03*
hsa05016	Huntington disease	DLG4/NDUFS7/NDUFA2/NDUFS2/NDUFS3/BDNF/SOD1/SOD2/CREB1	0.90440	1.0e-4*
hsa04261	Adrenergic signaling in cardiomyocytes	CACNG2/CREB1/ATP2B1/ATP2B2/ATP2B4/CAMK2G	0.67480	4.1e-03*
hsa04713	Circadian entrainment	CREB1/GRIA2/PER1/CAMK2G	0.4499	4.03e-02*
hsa04024	cAMP signaling pathway	CREB1/GRIA2/ATP2B1/ATP2B2/ATP2B4/BDNF/CAMK2G	0.93250	4.1e-03*
hsa04723	Retrograde endocannabinoid signaling	GRIA2/NDUFS7/NDUFA2/NDUFS2/NDUFS3	0.69350	2.67e-02*
hsa04020	Calcium signaling pathway	ATP2B1/ATP2B2/ATP2B4/CAMK2G/STIM1	0.85750	5.4e-02

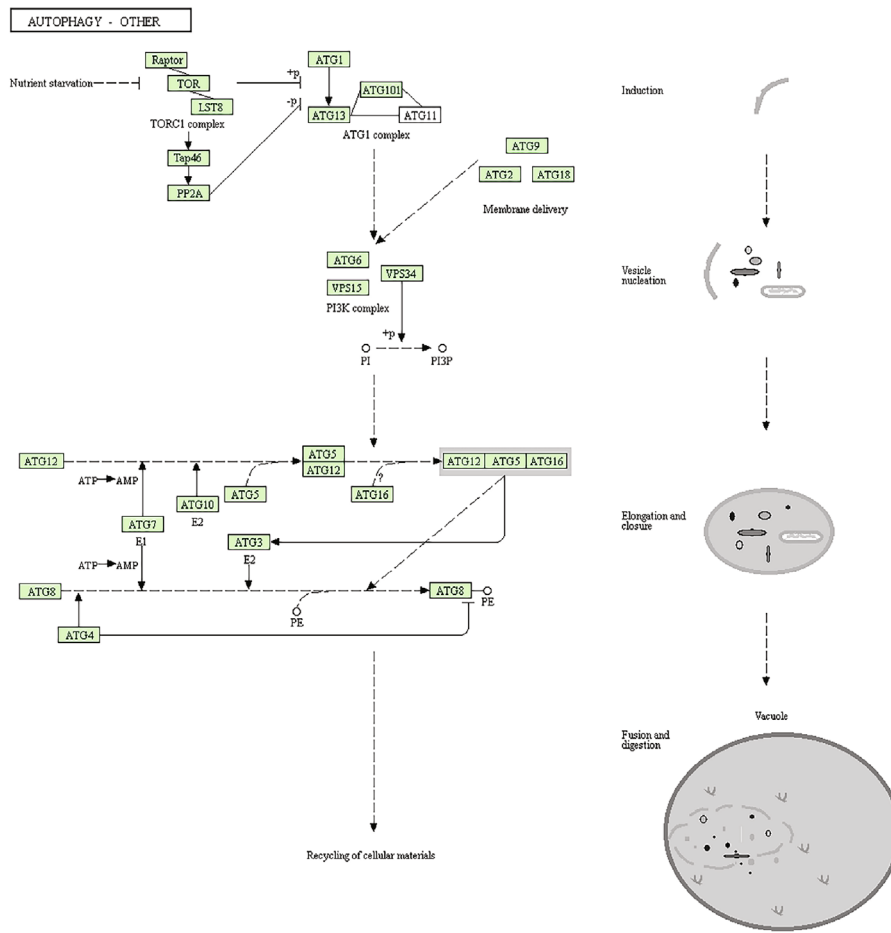
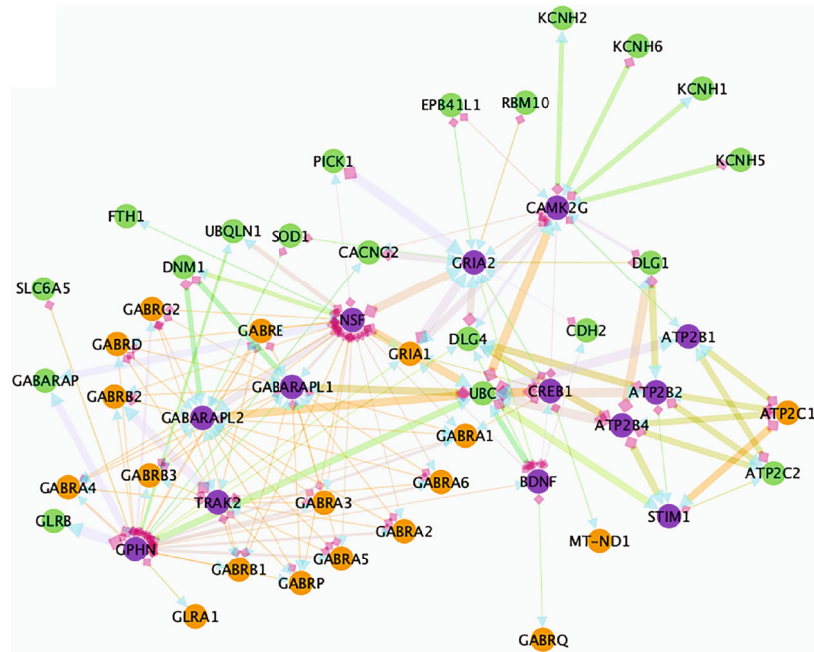


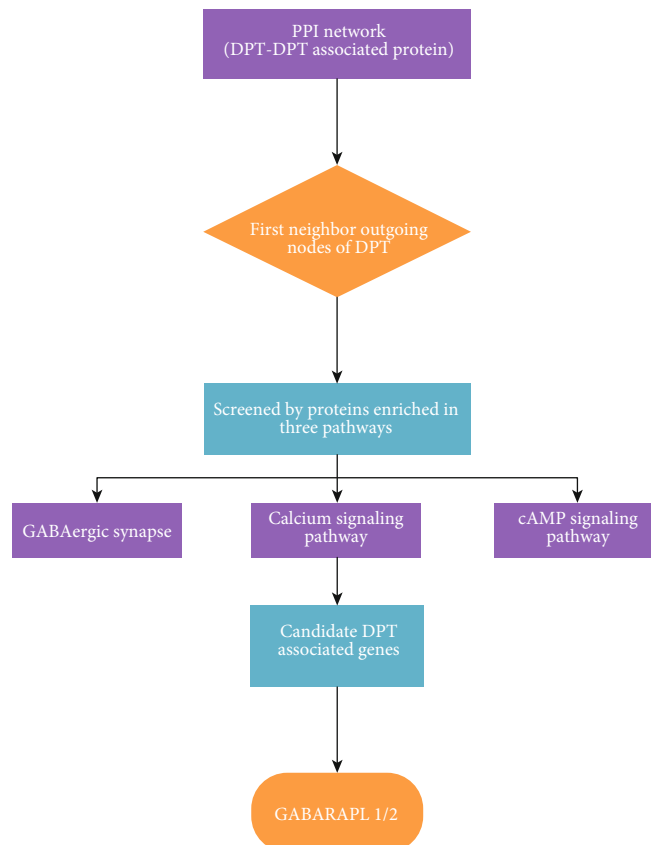
FIGURE 3

and 7(b)). The network (Figure 8) derived from the STRING database showed that GABARAPL 1 and 2 strongly interact with ATGs including ATG3, ATG4A, ATG4B, ATG4C, ATG4D, ATG5, ATG7, ATG12, BECN1, and PIK3C3 (Figure 6).

3.5. *GABARAPL1 and 2 Characteristics Identified by Bioinformatics Analysis.* The GABARAPL1 and 2 genes were identified at chr12 (P13.2): 10212805-10223130 and chr16 (q23.1): 75566351-75577881, respectively. Among 100 vertebrates, the 4 exons of GABARAPL1 and 3 exons of the



(a)



(b)

FIGURE 5: Subnetwork analysis of proteins enriched in candidate signaling. PPI subnetwork was constructed using proteins included in the GABAergic synapse, calcium signaling, and the cAMP signaling pathway with the first neighbor interacted proteins. Node color was mapped to different kinds of proteins. Green presented DPTs of sevoflurane. Orange meant DPT-associated proteins. Purple showed the proteins enriched in GABAergic synapse, calcium signaling, and cAMP signaling. Edge size was mapped to experimentally determined interactions with low values to small size, and edge color was mapped to the combined score with low values to bright colors. Blue arrow indicated target. Pink diamond showed source (a). Flow diagram of screening candidate DPT-associated genes (b).

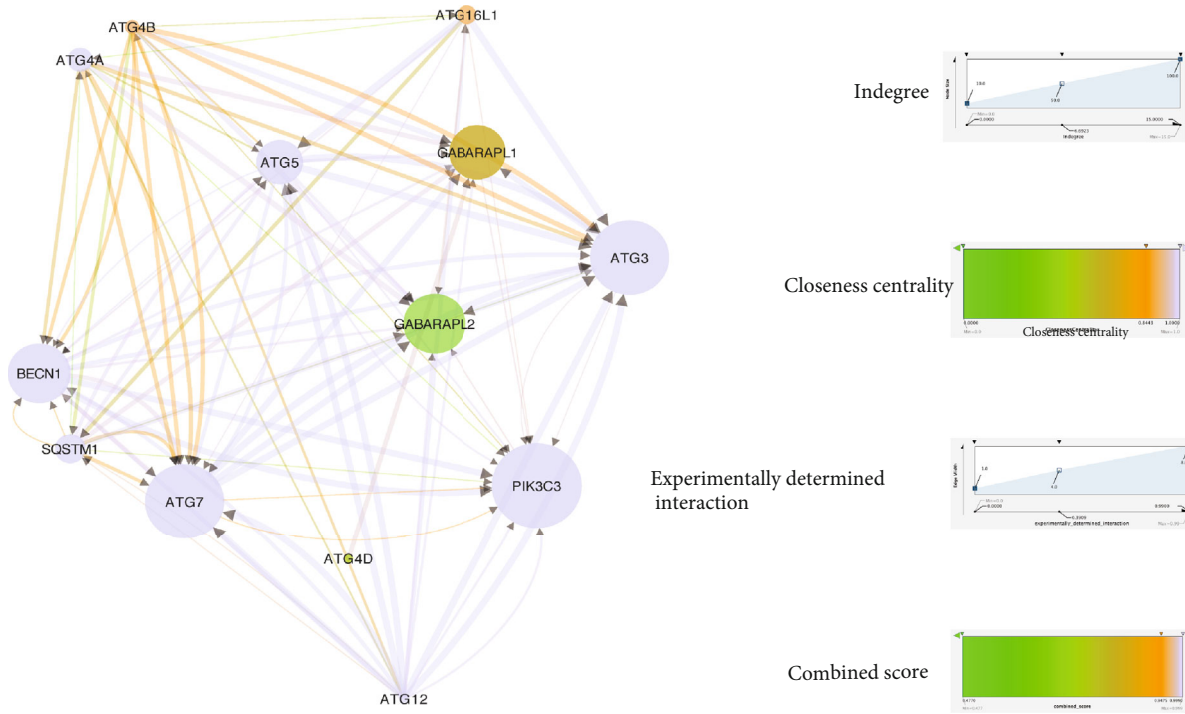


FIGURE 6: PPI network of GABARAPL1 and 2 with ATG. The PPI network of GABARAPL1 and 2 was built using Cytoscape according to the data from the STRING database. Node size was mapped to indegree with low values to small sizes, and node color was mapped to closeness centrality with low values to bright colors. Edge size was mapped to experimentally determined interactions with low values to small size, and edge color was mapped to combined colors with low values to bright colors. Arrow indicates target.

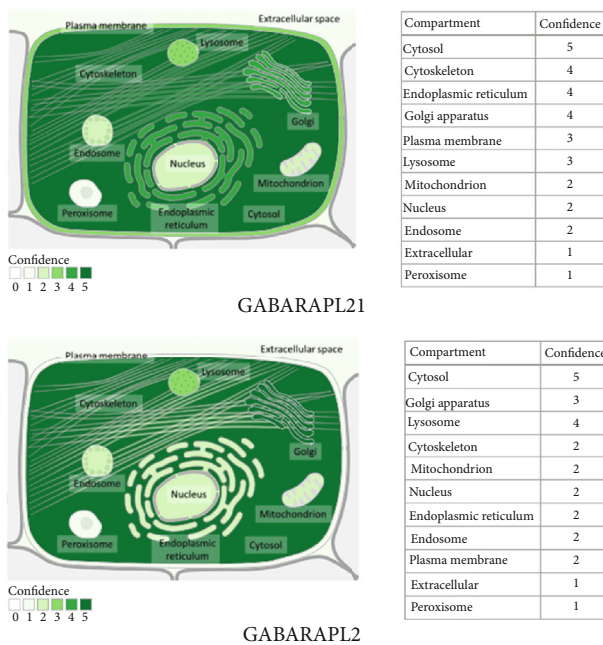


FIGURE 7

study, GABARAPL1 and 2 were identified to be core targets of direct protein associated with sevoflurane in the subnetwork. Further analysis demonstrated that GABARAPL1 and 2 strongly interacted with other core ATGs, such as

ATG7, which agreed with a previous report showing the role of the ATG8 family in the elongation and maturation of autophagosomes [27]. Furthermore, our results have revealed that GABARAPL1 and 2 are evolutionarily conserved among numerous vertebrates, highly expressed in the nervous system, and mainly located in the cytosol, lysosome, and the Golgi apparatus. Collectively, these findings indicated that GABARAPL1 and 2 function as regulators of autophagy in the nervous system.

DTome is an authorized database to identify the DPTs of drugs [17]. Proteins enriched in the top 10 pathways interacted with DPTs of sevoflurane from DTome. Because the GABAergic synapse [28], cAMP signaling pathways [29], and calcium signaling [30] are strongly related to neurotoxicity and neurodevelopment, the genes included in these signaling pathways were selected to investigate DPT-associated genes. Interestingly, bioinformatics analysis of the candidate genes revealed that GABARAPL1 and 2 were core proteins among the DPT-associated proteins. More importantly, GABARAPL1 and 2 not only are known to be homologs of LC3 but also act as crucial regulators of the autophagosomes [31]. A recent study illustrated that proteasome inhibitor treatment caused a dramatic induction of GABARAPL1 rather than GABARAPL2. Knockdown of GABARAPL1 reduced cell survival [32]. In addition, GABARAPL2 was also identified to be involved in Parkinson’s disease [33]. Consistent with our analysis, GABARAPL1 and 2 activity was processed by ATG4B, whereas the ATG8 family except GABARAPL1 was inhibited by the inactivity of ATG4B,

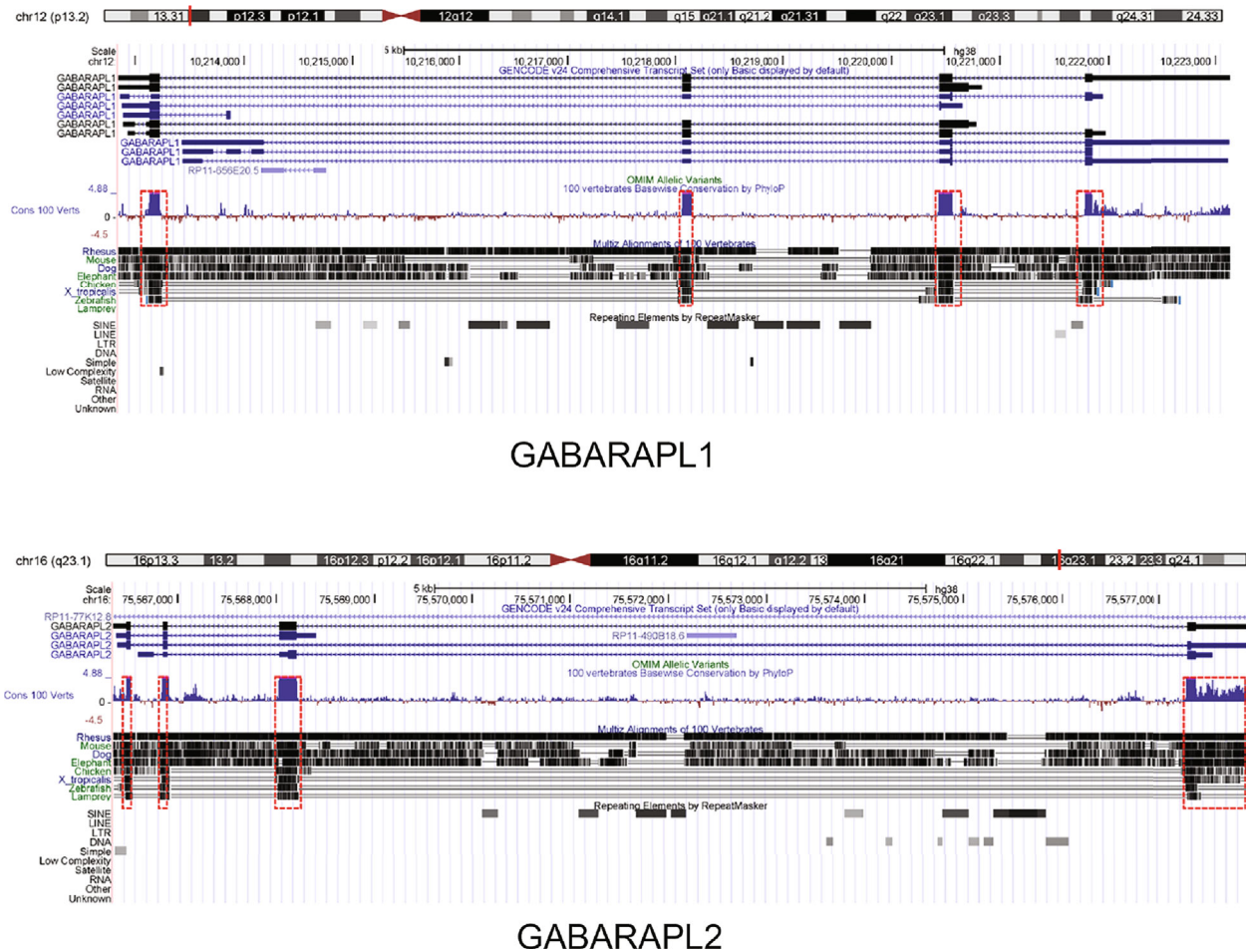


FIGURE 8

resulting in neurotoxicity induced by hyperglycemia [34]. However, another new study has shown that GABARAPL1 and 2 played an important role in neurotoxicity with axonal transport deficiency disrupting autophagic flux [35]. In line with our finding, current data supported that GABARAPL1 and 2 may regulate autophagy flux to decide the dual effect of sevoflurane on neurons.

Despite our data originating from authorized databases, including DTome and GeneCards, and STRING, this study has several limitations. Most importantly, the results of the bioinformatics analysis should be further demonstrated using experimental approaches. GABARAPL1 and 2 should be identified by preconditioning and postconditioning brains with sevoflurane in animals suffering from ischemia and by sevoflurane exposure during early or late life. A recent report has suggested that exposing aged rats to sevoflurane leads to the downregulation of LC3, a homolog of GABARAPL1 and 2 [20]. The dual effect of sevoflurane on neurons may be controlled by GABARAPL1 and 2. Thus, it is necessary to perform further experiments to determine if there are DPTs of sevoflurane upstream of GABARAPL1 and 2. This study was designed to investigate only one of the many available molecular mechanisms involved in the dual effect of sevoflurane. Finally, other potential roles of DPT-associated genes

enriched in the top 10 pathways other than the GABAergic synapse and cAMP signaling pathways may play a role in regulating the dual effect of sevoflurane on neurons.

Sevoflurane plays a dual role in neuroprotection during brain ischemia and neurotoxicity induced by sevoflurane exposure during early or late life. Our work suggested that sevoflurane controls autophagy flux by targeting DPTs. Emerging evidence has shown that brain ischemia referred to as excitotoxicity is reduced and limited by the activation of autophagy [10]. Using H4 human neuroglia cells, sevoflurane exposure has been shown to induce neurotoxicity and significantly enhanced the level of LC3-II, and these effects are reversed by inhibiting autophagy [21]. In contrast to these reports, the present study constructed a network of DPT-autophagy proteins and expanded our understanding of autophagy regarding the dual effect of sevoflurane on neurons.

5. Conclusion

In summary, this study used bioinformatics analysis to demonstrate that the network of sevoflurane-DPT-GABARAPL1 and 2 associated with autophagy is responsible for the dual effect of sevoflurane on neurons.

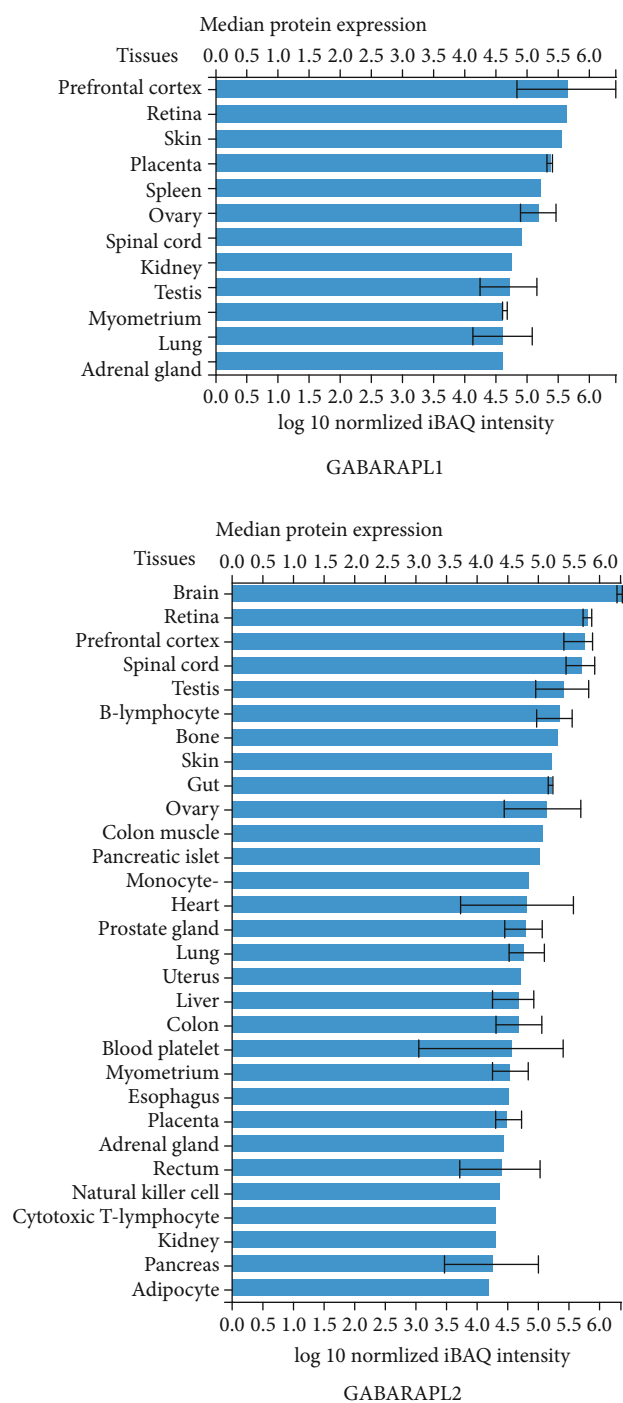


FIGURE 9

Abbreviations

- DPT: Direct protein target
- PPI: Protein-protein interaction
- GABARAPL: Gamma-aminobutyric acid receptor-associated protein-like
- GO: Gene Ontology
- FDR: False discovery rate
- ATG: Autophagy-related protein
- LC3: Light chain 3.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Importantly, the funding institution did not contribute to the design of the study, data collection, data analysis, data interpretation, or manuscript.

Conflicts of Interest

All authors declare that they have no competing interests.

Authors' Contributions

GL and DR designed the study, collected and analyzed the data, and wrote the manuscript. MZ and LX designed the study and collected and analyzed the data. LZ, SZ, and YW controlled the quality and collected and analyzed the data. All authors read and approved the final manuscript. Guolin Lu and Dongdong Rao contributed equally to this study.

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