Study on the mechanism of SND in the treatment of anxiety disorders based on network pharmacology, molecular docking and experiment validation

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Author contributions
Shi XP, Ma SB and Shi YJ conceived and designed the work. Wang FY, Yuan P, Zheng ML, Guo XD performed experiments. Wang FY, Wang J and Li L carried out network pharmacology and molecular docking. Wang FY and Yuan P wrote the manuscript, Shi XP and Ma SB revised the manuscript. Yuan P and Miao S collated and analyzed data. All authors read and approved the final manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abstract
Background: Sini decoction (SND) is a classic traditional Chinese medicine (TCM) formulation that can be used to treat anxiety-related disorders, but the active substance and underlying molecular mechanism of its anxiolytic effects are unknown. In this study, network pharmacology, molecular docking research and experimental verification methods were used to preliminarily explore the bioactive compounds and potential target mechanisms of SND anxiolytic. Methods: The active components and corresponding targets of SND were collected by TCMSP. GeneCards, OMIM, PharmGkb, TTD and Drugbank were used to search for the targets of anxiety disorders. The core target of SND in the treatment of anxiety was screened by PPI R language was used to analyze the intersection targets of SND in the treatment of anxiety disorders by GO and KEGG enrichment analysis. AutoDock Vina was used for molecular docking, and Discovery Studio was used for visual conformation analysis after docking. The anti-anxiety effect and molecular mechanism of SND were studied by in vivo experiment. Results: Based on network pharmacological analysis, we obtained 112 active ingredients and 350 effective targets related to anxiety from SND. In PPI analysis, 26 targets such as STAT3, MAPK3, MAPK1, MAPK14, SRC, HSP90AA1, TP53 and PIK3CA were identified as core targets. GO and KEGG analysis showed that the anxiolytic mechanism of SND may be related to the neuroactive ligand-receptor interaction pathway and inflammatory pathway. Molecular docking showed that quercetin, naringenin, licochalcone A had high affinity with JAK2, MAPK14 and MAPK3. Animal experiments have shown that SND reverses the upregulation of GluN2B (NMDAR) and GluA1 (AMPAR) proteins, and SND improves anxiety disorders by regulating glutamate transmitter levels, which may be related to neuroactive ligand-receptor interaction pathways, particularly glutamate receptors. Conclusion: This study shows that SND can improve FS-induced behavioral changes in mice and can modulate hippocampal synapse-associated protein defects, partially reversing glutamate receptor expression through the neuroactive ligand-receptor interaction pathway, and further improved anxiety disorders. At the same time, combined with network pharmacology and molecular docking, the key components, core targets and related pathways of SND are discussed, which shows that the active components of SND play an effective role in anxiety through multi-targets and multi-pathways, which provides a reference for the material basis and mechanism of SND.

Keywords: SND; anxiety disorders; network pharmacology; molecular docking; pharmacological mechanisms
**Introduction**

Anxiety disorders is the most common mental disease, which is characterized by persistent anxiety, tension, panic and other anxiety, accompanied by autonomic nervous disorder, muscle tension and motor restlessness [1]. Anxiety disorders often lead to disorders of memory, intelligence and social activities of patients, and are one of the top 10 leading causes of non-fatal disability in the world [2]. At present, the drugs commonly used clinically to treat anxiety include benzodiazepines (eg, diazepam). Selective 5-HT and norepinephrine reuptake inhibitors (SNRIs) (eg, venlafaxine and duloxetine). Selective 5-HT reuptake inhibitors (SSRIs) (eg, fluoxetine and sertraline) [3]. Although these drugs have shown some efficacy in the treatment of anxiety disorders, but unfortunately, the long-term use of these drugs is prone to drug resistance, addiction and drug withdrawal recur phenomenon [4]. In addition, patients are often accompanied by adverse reactions such as dizziness, headache, gastrointestinal dysfunction and sexual dysfunction [5]. Therefore, it is very necessary to develop anti-anxiety drugs with good clinical efficacy and little side effects.

A growing number of studies have shown that TCM plays an increasingly prominent role in the field of anti-anxiety, showing the advantages of safety and effectiveness, less side effects, overall treatment and so on. Suzaoren decoction, Xiaoao Powder and Banxia-Houpu decoction are considered as important alternative therapies because of their remarkable efficacy in the treatment of anxiety diseases [6]. SND is the main prescription in Treatise on Typhoid Diseases by Zhang Zhongjing in Han Dynasty, which is composed of Aconitum carmichaelii Debx (Fuzi), Zingiber officinale Rosc (Ganjiang), Glycyrrhiza uralensis Fisch (Gancao) [7]. Modern clinical studies have shown that SND is mainly used for diseases such as cardiovascular system and nervous system. Studies have shown that SND provides direct evidence for SND in the treatment of mental illness by inhibiting the increase of corticosterone and CRH mRNA levels induced by chronic unpredictable stress (CUS) in mice [8]. In addition, recent studies have also shown that SND and some of the active ingredients in SND can treat and improve the symptoms of depression and anxiety [9]. However, unfortunately, the underlying anti-anxiety mechanism of SND is still unknown.

Network pharmacology can analyze the action mechanism of TCM compound prescription and TCM components as a whole, and independently present the disease-related “component-target-pathway” to effectively reveal the material basis of the efficacy of TCM and its molecular mechanism, which can systematically reveal the complex overall biological network relationship among drugs, components, targets and diseases. It provides a new perspective for analyzing and predicting the pharmacological mechanism of drugs [10]. Molecular docking technique predicts the binding conformation and binding free energy of ligands and receptor active sites by simulating the geometric structure of ligands and targets and the interaction between molecules. Explore the interaction between small molecules and receptors [11]. Therefore, this study used network pharmacology, molecular docking studies and experimental validation to preliminarily explore the material basis and molecular mechanism of SND in anti-anxiety disorders and to provide a basis for further elucidation of the molecular mechanisms of SND in the treatment of anxiety disorders.

**Materials and methods**

Reagents and materials

Fuzi, Gancao and Ganjiang were purchased from their main producing areas (20220507 in turn were purchased from the Chinese Herbal Pharmacy of Xijing Hospital of Air Force military Medical University). C57BL/6 male mice (6–8 weeks, body weight 20–25 g) were selected, mice were housed individually in standard plastic cages and the room was kept at a controlled temperature (22 ± 2 °C) and humidity (50–60%) in the absence of specific pathogens. Purchased from the Experimental Animal Center of Air Force military Medical University (certificate No. SCXK (SHAN) 2019-001). All procedures were approved by the Fourth Military Medical University Animal Care and Use Committee (certificate No.202300085, Grade II). The Open Field device (DigBehv-LR4, Shanghai Jiliang, China) and the Elevated Plus Maze device (DigBehv-LR4, Shanghai Jiliang, Shanghai, China) were utilized in the experiment. The anti-p-actin antibody (AS5316) were procured from Sigma-Aldrich (Saint Louis, Missouri, USA), while the antibodies against GluN2B (ab65783), p-GluN2B-S1303 (ab81271) and GluA1 (ab31232) were obtained from Abcam (Cambridge, UK). The antibodies against p-GluA1-S485 (ab5849) and p-GluA1-S831 (ab5847) were sourced from Millipore (Billerica, MA, USA), and the antibodies against p-GluN2B-T1472 (p4208 s) were purchased from Cell Signaling Technology (Danvers, MA, USA). The BCA and ECL were obtained from Pierce (Rockford, IL, USA). The PVDF membrane was purchased from Roche (Mannheim, Germany). The workflow of this study is shown (Figure 1).

**Screening pharmacological activity of active components of SND.** The compounds of each herb in SND were obtained through the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://old.tcmpe.com/tcms-p.php) [12]. It is a unique systematic pharmacological platform for capturing the relationship between herbs, targets and diseases. The amount of medicine that enters the circulatory system after it enters the body is referred to as OB and DL is the similarity between a chemical and a known drug. Therefore, based on the absorption, distribution, metabolism and excretion (ADME) characteristics of drugs in the body, the compounds with OB > 30% and DL > 0.18 in SND were identified as compounds with pharmacological activity [13, 14]; in addition, by consulting the literature, it was found that some components with high content in the three medicinal herbs were not included in the TCMSP database, which was added to the final chemical constituents of SND [15, 16].

**Screening potential targets of compounds.** TCMSP database, the PharmMapper database (http://lilab.eust.cn/pharmmapper/) and the SwissTargetPrediction database (http://www.swistargetprediction.ch/) were used to convert the protein targets corresponding to the screened active chemical components into corresponding gene targets [17, 18]. The molecular structure (sdf format) of the active compounds required for PharmMapper and SwissTargetPrediction was queried through the PubChem database [19]. Then, we submitted the collected targets into Uniprot database (https://www.uniprot.org/) and limited the species to “Homo sapiens”. The official symbols of all gene targets were obtained.

**Screening disease-related targets.** The targets related to anxiety disorders were searched from five databases of TTD (http://bidd.nus.edu.sg/group/ctjd/), GeneCard (https://www.genecards.org/), Drugbank (https://go. drugbank.com/), DisGeNet (https://disgenet.org/) and PharmGkb (https://www.pharmgkb.org) [20]. The species was set to “Homo sapiens ”. Note that some targets from the database need to be submitted to the Uniprot database for conversion to official gene symbols. Then, the genes found in the five databases associated with anxiety disorders were merged to eliminate duplicate target genes.

**Construction of component-disease network.** The online tool Venny2.1.0 was used to plot the intersection of the active ingredient genes of SND and the anxiety-related genes, and obtain the potential targets of SND against anxiety disorders. The intersection targets of the SND components screened in the previous step corresponding targets and anxiety disorders targets were selected, and the component-disease target diagram was constructed through Cytoscape3.8.2 software [21]. Nodes were calculated using the CytoHubba application in Cytoscape software.

**PPI network construction.** In order to explore the interaction between SND and anxiety disorders target proteins, we used Cytoscape software and String database (https://string-db.org/) to
build a visual protein-protein interaction (PPI) network. The intersection target of SND and anxiety disorder was imported into the string database to obtain information about the PPI network. The filter condition of the organism is set to “Homo sapiens”, and the minimum interaction score is required to be “the highest confidence level (0.9)”. The downloaded protein interaction data were imported into Cytoscape software to adjust the values of Betweenness, Closeness, Degree and other parameters among target proteins, and the most closely related core targets were obtained.

**GO and KEGG enrichment analysis.** In order to explore the regulatory effect of SND on potential intracellular signal transduction pathway, biological process (BP), molecular function (MF) or cellular component (CC), the functional enrichment of the intersection target protein obtained from 2.2.4 was analyzed by R4.0.2 language, and the functional enrichment of (GO) BPS, MFS, CCS and KEGG pathway analysis were obtained. Install and use the Colorspace, Dose, ClusterProfiler, Enrichplot, and ggplot2 packages in R4.0.2 software to generate histograms and bubble charts [22]. Significant rich BPS, MFS, CCS and KEGG pathway of $P < 0.05$ was selected for further study, in which the top 10 BPS, MFS, CCS and top 30 KEGG pathways were visualized in R language.

**Construction of drug-component-disease-target-pathway network.** The top 20 pathways in KEGG enrichment analysis were selected as the core pathways, and the active compounds corresponding to the 20 core targets screened by PPI were found. Based on the above information, Cytoscape software was used to construct a multidimensional network diagram and the relationship among SND-chemical components-core target-anxiety disorder-key pathway was established to explore the possible mechanism of SND in the treatment of anxiety disorders.
Molecular docking
Molecular docking techniques between small molecules and related targets can predict the binding mechanism and activity between active components and target proteins to some extent [23]. Firstly, the candidate components in Cytoscape network are searched for 3D structure through Pubchem database and saved into SDF format. Secondly, the 20 targets with the closest interaction in PPI were selected, and the targets were found in the RCSB (https://www.rcsb.org/) database, and the proteins were dehydrated, liganded, and hydrogenated. Then molecular docking was carried out by AutoDock Vina software. The results take the binding energy as the evaluation standard of the degree of binding with the compound. In general, when the conformational binding of the compound molecule to the receptor is stable, the energy is relatively low, the possibility of action is greater, and the docking result is more reliable [24]. We chose the best four for final conformational analysis. The PDB format structure of JAK2, MAPK14, MAPK3 proteins were downloaded from the RCSB, and then, they were uploaded to Maestro 11.1 to get docking scores [25]. The complex compound with protein was visualized using Pymol software, and Discovery Studio 2020 was used for visualization of 2D structures [26].

Pharmacodynamic study of SND on anxiety disorders
Preparation of SND. SND was prepared according to the 2020 edition of Chinese Pharmacopoeia. Fuzi:Ganjiang:Gancao = 3:2:3. Fuzi and Gancao were boiled twice in water, and filtered with decoction, Ganjiang is extracted from volatile oil by steam distillation with water, and the ginger residue is decocted with water for 1 h, and then all decoctions were combined with reduced pressure concentration and freeze-dried.

Model preparation and drug therapy. The animals need to adapt to laboratory conditions for one week before the experiment. 40 male C57BL/6 mice were randomly divided into control group, Forced swimming (FS) group, SND low, middle and high dose groups. The SND dose group was given intragastric administration of 2.6 g/(Kg.d), 5.2 g/(Kg.d) and 10.4 g/(Kg.d) respectively, while the other groups were given the same volume of normal saline and 0.1 mL/10 g respectively, once a day for 9 days. In the last two days of administration, the mice in the other groups except the control group were made by FS [27]. The mice were made to swim continuously in an open container with diameter 10 cm and high 30 cm for 2 days, each time 30 min, the container was filled with water (22 ± 2 °C) to 20 cm. The body weight and status of the mice were observed after FS, and the behavior was tested on the second day after modeling.

Open field test. The behavior experiment was carried out on the second day after FS treatment, and the OFT device was composed of a square box of 30 × 30 × 30 (cm). All animals were admitted to the test laboratory 2 h in advance, and mice were allowed to move freely for 15 min, and locomotor activity was filmed with a video camera fixed on top of the box. The central 15 × 15 (cm) area of the OF is considered to be the central area, accounting for 1/4 of the total area. Finally, the motion tracking system was used to analyze the time and distance of the mice moving in the central area [28].

Elevated plus maze. The elevated device consists of two open arms (25 cm × 8 cm × 0.5 cm) and two closed arms (25 cm × 8 cm × 12 cm), with a central platform (8 cm × 8 cm), and the instrument is raised to 50 cm above the ground. All the mice were accustomed to 2 h before the behavior test to eliminate the influencing factors. Individual mice were placed in the central square, facing their open arms and allowed to move freely for five minutes. The movement was recorded by the camera, and the times of entering the arm and the time of opening the arm of the mouse were analyzed and calculated by the video tracking system [29].

Western-blot analysis. Western-blot Analysis was used to detect the effect of SND on the protein expression of GluA1 and GluN2B in anxiety mice. The hippocampal tissue of each group was separated by ultrasonic lysis with 300 μL RIPA lystate, and then the supernatant was obtained by centrifugal 30 min. The protein concentration was measured by BCA and adjusted to make the concentration of each group consistent. The protein was denatured in 95 °C water bath for 10 minutes. Then configure separation gel (8%) and concentrated gel (3%), sample, electrophoresis, transfer film. Seal at room temperature for 2 hours with 5% milk. After incubating with the first antibody at 4 °C for one night, it was incubated with the second antibody at room temperature for 1 h. ECL detection system (Tanon5200, China) was used to visualize PVDF membrane. The data obtained are processed by ImageJ software [30].

Data analysis
All data were expressed as mean ± SD and statistically analyzed by GraphPad Prism9.0.0. Single factor analysis of variance (ANOVA) was used. P < 0.05, which is statistically significant.

Results
Network pharmacology
Construction of SND active ingredient target. Computer was used to search the TCMSp database for the active ingredients of the SND. A total of 493 active ingredients were identified. Subsequently, 104 key active ingredients were further screened based on ADME parameters of OB ≥ 30% and DL ≥ 0.18. Interestingly, stigmastanol is found in all three Chinese herbal medicines under the number MOL000359. It is worth noting that some of the active ingredients of the three Chinese herbal medicines were not included in the TCMSp. The results of the review are added as follows: Mesoaconitine, Aconitine, benzoylconacine, Benzoylemasacone, Benzylohpacacine in aconite, 10-Gingerol, Zingiberene in Ginger Rhizome and Liquiritigenin, Glycyrrhizic acid ammonium salt, Glycyrrhizic acid in Liquorice were added to the SND active ingredient. 112 active components were obtained by deleting repetitive components by two methods. Based on TCMSp, PharmMapper database and Swiss Target Forecast database, 505 targets of active components of SND were obtained. In order to better understand the complex relationship between SND compound and its target, the compound-target network diagram of SND was constructed (Figure 2).

Acquisition of potential targets for anti-anxiety disorders of SND. With "anxiety disorder" as the search term, anxiety-related disease target information was obtained by using DisGeNET, Genecards, DrugBank, TTD and OMIM databases (Figure 3A). Intersect it with the main active ingredient target of SND, draw the venny diagram in R (Figure 3B), and the intersecting part of the two circles represented 350 intersection targets of SND for the treatment of anxiety. In order to further clarify the interaction between SND potential targets and anxiety disease targets, it is necessary to build an interactive network between active components and disease targets, which is completed by Cytoscape3×9.1 (Figure 3C). The results showed that 5529 genes associated with anxiety disorders were predicted. The left node represented the active ingredient of the SND (the yellow node represents Liquorice, the green node represents Aconite, the purple node represents Ginger Rhizome and the node containing three colors represents the common ingredients) and the right node was the anti-anxiety target of the active ingredient of the SND, with a total of 462 nodes and 1538 edges. It reflects the multi-component and multi-target characteristics of SND.

Construction of PPI interaction networks and core targets. The 350 intersection targets of SND and anxiety disorder were imported into STRING15.5 database to construct PPI network. Then imported into Cytoscape and used the CytoNCA plug-in to analyze the key indicators of the core target, and the screening standard is that the target with DC, BC, CC, EC, LAC and NC greater than the median is the core target. The PPI result is shown in Figure 4A. The PPI results include 170 nodes and 665 edges, each node represents a target protein, and the node size is proportional to its corresponding degree of binding. All of these parameters can be used to analyze the attributes of nodes in the interactive network. We select the target node with six key indicators higher than the corresponding median in the PPI network, and set the filter conditions 1 and filter 2, the results...
Figure 2 SND-component-target network diagram. The left rhombus pink node is Liquorice, the right purple rhombus node and green rhombus node are Ginger Rhizome and Aconite respectively, and the red rhombus node is the common component of the three. The middle blue rectangular node is the target corresponding to the three components.

Figure 3 Composite target network for SND treatment of anxiety disorders. (A) DisGeNET, Genecards, DrugBank, TTD and OMIM 5 databases search for anxiety related targets. (B) Venny diagram of anxiety targets and SND targets, overlapping targets for 350 diseases and drugs. (C) The composite target network consists of three TCM, 112 compounds and 350 targets, including 462 nodes and 1538 edges.
Figure 4 Diagram of interaction network between SND and anxiety disorder core protein (PPI). (A) PPI analysis with STRING database. According to conditions 1 DC > 5, BC > 60.01, CC > 0.07, EC > 0.02, LAC > 1.33, and NC > 2.25, the pink diamond node is the protein of the complex condition, and the protein of the green round node is removed. (B) According to condition 2 DC > 11, BC > 15.44, CC > 0.54, EC > 0.11, LAC > 5.8, and NC > 7.11, the core target is further screened, and the pink diamond node is the protein of compound condition, which removes the protein of green round node. (C) 26 core targets screened.

are shown in Figure 4A–4B. Among them, the top 26 proteins are shown in Figure 4C, which visually show the interaction between targets and screen out the key targets of SND in the treatment of anxiety disorders. Table 1 lists the 20 key nodes with above-average values in the composite target network and their topology parameters. Through the enrichment analysis of the GO and KEGG pathways, we used R language to analyze the BP, CC and MF terms of 350 intersection targets of SND anti-anxiety disorder. The results showed that a total of 3393 GO terms were significantly rich (P < 0.05), including 2957 BP terms, 157 CC terms and 278 MF terms. The top 10 terms that are significantly rich in the BP, CC, and MF categories are displayed visually (Figure 5A). The results showed that the main terms of BP are response to drug, response to molecule of bacterial origin, response to lipopolysaccharide, cellular response to chemical stress, peptidyl-tyrosine phosphorylation and reactive oxygen species metabolic process. The main terms of CC are membrane rafts, plasma
Table 1: The core targets of anxiety disorders are DC, BC, CC, EC, LAC, and NC values.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>BC</th>
<th>CC</th>
<th>DC</th>
<th>EC</th>
<th>LAC</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>71.113</td>
<td>0.590</td>
<td>22.000</td>
<td>0.176</td>
<td>9.545</td>
<td>13.135</td>
</tr>
<tr>
<td>AKT1</td>
<td>309.127</td>
<td>0.632</td>
<td>30.000</td>
<td>0.200</td>
<td>8.467</td>
<td>16.269</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>62.579</td>
<td>0.545</td>
<td>16.000</td>
<td>0.117</td>
<td>5.750</td>
<td>7.351</td>
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<tr>
<td>IL2</td>
<td>61.763</td>
<td>0.554</td>
<td>19.000</td>
<td>0.147</td>
<td>8.105</td>
<td>10.108</td>
</tr>
<tr>
<td>MAPK14</td>
<td>220.104</td>
<td>0.615</td>
<td>27.000</td>
<td>0.190</td>
<td>8.222</td>
<td>13.928</td>
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<tr>
<td>MAPK1</td>
<td>262.833</td>
<td>0.655</td>
<td>34.000</td>
<td>0.236</td>
<td>10.294</td>
<td>21.677</td>
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<tr>
<td>MAPK3</td>
<td>270.606</td>
<td>0.661</td>
<td>35.000</td>
<td>0.246</td>
<td>11.029</td>
<td>23.575</td>
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<td>MAP2K1</td>
<td>44.171</td>
<td>0.537</td>
<td>14.000</td>
<td>0.114</td>
<td>6.286</td>
<td>7.365</td>
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<tr>
<td>PIK3CA</td>
<td>165.203</td>
<td>0.615</td>
<td>27.000</td>
<td>0.185</td>
<td>7.704</td>
<td>13.653</td>
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<td>STAT3</td>
<td>556.161</td>
<td>0.673</td>
<td>38.000</td>
<td>0.248</td>
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<td>27.197</td>
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<td>VEGFA</td>
<td>66.775</td>
<td>0.526</td>
<td>15.000</td>
<td>0.100</td>
<td>6.000</td>
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<td>CCND1</td>
<td>66.460</td>
<td>0.545</td>
<td>17.000</td>
<td>0.116</td>
<td>6.588</td>
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<td>JAK2</td>
<td>73.282</td>
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<td>19.000</td>
<td>0.149</td>
<td>7.579</td>
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<td>RB1</td>
<td>88.075</td>
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<td>19.000</td>
<td>0.125</td>
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<td>HSP90AA1</td>
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<td>33.000</td>
<td>0.229</td>
<td>10.061</td>
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<td>LYN</td>
<td>64.235</td>
<td>0.558</td>
<td>18.000</td>
<td>0.146</td>
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<tr>
<td>TNF</td>
<td>93.685</td>
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<td>17.000</td>
<td>0.097</td>
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<tr>
<td>JAK1</td>
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<td>0.554</td>
<td>19.000</td>
<td>0.146</td>
<td>8.211</td>
<td>10.249</td>
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<tr>
<td>SRC</td>
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<td>0.673</td>
<td>37.000</td>
<td>0.244</td>
<td>10.270</td>
<td>24.230</td>
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</table>

Figure 5: Network pharmacology prediction of SND treatment for anxiety disorders. (A) Results of the GO enrichment analysis. The histogram shows the top 10 terms in the BP, CC, and MF categories in the GO analysis, with the abscissa being the number of annotated genes, the ordinate being the GO item, the length of the histogram corresponding to the number of annotated genes in the entry, and the color corresponding to the corrected q-value. (B) Results of the KEGG enrichment analysis. The size of the bubble represents the number of enriched genes, the larger the bubble and the redder the color, the more significant the degree of enrichment. (C) SND-Chemical Constituents-Core Target-Anxiety Disorder-Pathway Map.
Construction of drug-component-disease-target-pathway network. In order to fully clarify the possible mechanism of SND in the treatment of anxiety disorders, the top 20 pathways in the results of KEGG analysis were selected as the key pathway, and the active components corresponding to 26 core targets screened by PPI were identified. A total of 80 chemically active components corresponding to 26 core targets were found. The data were introduced into cytoscape to construct the SND-chemical composition-core target-anxiety disorder-key pathway network, as shown in Figure 5C. Using the tool Analyze Network, the results include 156 nodes and 360 edges, resulting in the parameters of the top 6 active ingredients of degree and 26 core targets. As shown in Table 2. The results show Quercetin, 6-gingerol, Zingerone, (8)-Gingerol, licochalcone A and Naringenin may be the more important active ingredients, predicting MAPK14, MAPK3, MAPK1 and PIK3CA as the main targets of the SND for the treatment of anxiety. SND may improve anxiety disorders through multiple pathways and targets. Neuroactive ligand – receptor interaction and PI3K-Akt pathway may be an important approach to the treatment of anxiety disorders mediated by the SND.

### Table 2 SND anti-anxiety top 6 active ingredient list

<table>
<thead>
<tr>
<th>Drug</th>
<th>Composition</th>
<th>Mol ID</th>
<th>2D Structure</th>
<th>Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Glycyrrhiza uralensis</em> Fisch</td>
<td>Quercetin</td>
<td>MOL000098</td>
<td><img src="image" alt="Quercetin" /></td>
<td>14</td>
</tr>
<tr>
<td><em>Zingiber officinale</em> Rosc</td>
<td>6-Gingerol</td>
<td>MOL002467</td>
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Molecular docking. Based on the previous analysis results, in order to clarify the binding ability between the core target protein and the potential anti-anxiety components of SND. Therefore, we performed molecular docking on the 20 key targets and the first 6 bioactive components screened in the PPI network analysis. In general, the lower the binding energy, the stronger the interaction between drugs and protein molecules, which is lower than –5.0 kcal/mol, that is, the drug component has strong binding activity to the target [31]. It can be seen from Figure 6A that the binding energy of most of the active components of SND to the core target is lower than –7.0 kcal/mol, indicating that there is a strong correlation between the potential active components of SND and the core target of disease anxiety. The docking results of representative active components Quercetin, licochalcone A, narigenin and core target MAPK14, MAPK3, JAK2 were visualized in 3D and 2D by using PyMol and DS. The results of molecular docking show Narigenin and JAK2 bind to LEU-932 mainly through hydrogen bonds (Figure 6B). Quercetin and MAPK14 are mainly bound to ILE-166, ASP-168 GLU-71, ARG-67 and ARG-70 through hydrogen bonds (Figure 6C), licochalcone A and MAPK14 are mainly combined with ARG-70 through hydrogen bonds (Figure 6D), and Quercetin and MAPK3 are mainly combined with ASP-187, LYS-73, LEU-52, MET-121 and THR-186 through hydrogen bonds (Figure 6E). Based on this, we speculate that the three compounds quercetin, narigenin and licochalcone A bind well to the three core targets JAK2, MAPK14 and MAPK3, and predict that all these compounds may play a key role in the treatment of anxiety through these targets.

Pharmacodynamic study of SND on anti-anxiety mice

In order to induce anxiety, we chose the stress model of FS. Recent studies in rodents have shown that acute stress can increase glutamate release, reduce spinal density, induce drastic molecular changes in the hippocampus, amygdala and prefrontal cortex, and impair cognitive and emotional behavior within hours or even days after initial stress exposure [32]. AmaliaFloriou-Servou exposed mice to novel stress, restraint stress and FS stress [33]. The strongest changes in gene expression induced by swimming stress were observed at the proteome level, and behavioral assessment showed an increase in anxiety [34]. There are also experiments that use FS stress models to study the neural circuits of stress responses and to understand stress-related learning and memory processes [35]. Our experiment further confirms previous studies that mice are more likely to avoid rather than approach behavior after FS. Therefore, the acute swimming stress model seems to be more suitable for studying the potential molecular changes in the brain after acute stress. We further used OFF and EPM experiments to verify the effects of SND in anti-FS-induced anxiety mice. The experimental flowchart is shown in Figure 7A. OFF behavioral results showed that compared with the FS stress group, the total exercise distance (Figure 7B–7C), central region exercise distance (Figure 7B–7D) and central movement time (Figure 7B–7E) of the control group increased, indicating that FS acute stress induced anxiety-like behavior in mice. Compared with the FS stress group, the total exercise distance (Figure 7B–7C) and central region exercise distance of mice (Figure 7B–7D) in SND + M and SND + H dose groups increased, and the central exercise time of mice in SND + H dose group also increased (Figure 7B–7E). The results showed that SND could improve anxiety-like behavior in mice, and the SND + H dose group had the best therapeutic effect. Using EPM to determine the behavioral differences among the three groups, we found that compared with the FS stress group, the percentage of the open arm entry time (Figure 8A–8D) in the control group increased, while the percentage of closed arm entry time (Figure 8A–8D) in SND + H dose group increased, while the percentage of closed arm (Figure 8A–8C) entry time decreased. It can be seen from the picture that there is no difference in the total arm number of mice in each group, indicating that the mice are in a state of anxiety and did not reach a state of depression. The results also showed that SND could improve the anxiety-like behavior of mice.

![Figure 6](https://example.com/fig6.png)

**Figure 6.** The molecular docking between main chemical components of SND and target proteins. (A) Heatmap of binding energies. (B) Molecular docking of JAK2 with narigenin. (C) Molecular docking of MAPK14 with quercetin. (D) Molecular docking of MAPK14 with licochalcone A. (E) Molecular docking of MAPK3 with quercetin.
Figure 7 Effects of SND treatment on OFT behavioral performance of anxious mice. SND was given intragastrically for 9 days, then forced swimming (swimming for 30 minutes for 2 days) was used to stress. Finally, EPM and OFT experiments were used to test the behavior of mice. OFT and EPM tests were used to determine anxiety-like behavior. (A) Experimental flow chart. (B) Movement trajectories of mice in the OFT experiment. Total distance moved by mice. (C) Distance. (D) Time. (E) Moved in the center region. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (n = 6).

Figure 8 Effects of SND treatment on EPM behavioral performance of anxious mice. (A) Movement trajectories of mice in EPM experiments. Number of times. (B) That mice entered the arm in each group and the time of entry in the closed arm. (C) Open arm. (D) *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (n = 6).
Western-blot analysis

GO and KEGG results indicated that SND for anxiety disorders mainly focused on neurotransmitter receptors and anti-inflammation, so we used western blot analysis to detect the expression of GluA1 and GluN2B proteins in the hippocampal tissues of mouse brain (Figure 9). Compared with FS stress group, the expression levels of GluN2B (P < 0.001), p-GluN2B-T1472 (P < 0.001), p-GluN2B-S1303 (P < 0.001), GluA1 (P < 0.001), p-GluA1-S831 (P < 0.001) and p-GluA1-S845 (P < 0.001) in control group decreased significantly. Compared with FS stress group, the expression levels of p-GluN2B-T1472 (P < 0.05) and p-GluA1-S831 (P < 0.05) decreased in SND+L dose group. Compared with FS stress group, the expression levels of GluN2B (P < 0.05), p-GluN2B-T1472 (P < 0.01), p-GluN2B-S1303 (P < 0.01), GluA1 (P < 0.05), p-GluA1-S831 (P < 0.01) and p-GluA1-S845 (P < 0.05) were significantly decreased in SND+M dose group. Compared with FS stress group, the expression levels of GluN2B (P < 0.001), p-GluN2B-T1472 (P < 0.001), p-GluN2B-S1303 (P < 0.001), GluA1 (P < 0.001), p-GluA1-S831 (P < 0.001) and p-GluA1-S845 (P < 0.01) were significantly decreased in SND+H dose group. In summary, compared with the FS stress group, all dosing groups except the SND+L dose group significantly reduced glutamate transmitter protein expression associated with glutamate. These results suggest that the anti-anxiety effect of SND is associated with negative regulation of glutamate receptors.

Figure 9 Effect of SND on GluN2B, p-GluN2B-T1472, p-GluN2B-S1303, GluA1, p-GluA1-S831 and p-GluA1-S845 expression in the hippocampus (n = 6). (A) The protein levels of GluN2B, p-GluN2B-T1472 and p-GluN2B-S1303 determined by Western blots in mice hippocampus. (B) Protein expression levels of GluN2B in the hippocampus. (C) Protein expression levels of p-GluN2B-T1472 in the hippocampus. (D) Protein expression levels of p-GluN2B-S1303 in the hippocampus. (E) The protein levels of GluA1, p-GluA1-S831 and p-GluA1-S845 determined by Western blots in mice hippocampus. (F) Protein expression levels of GluA1 in the hippocampus. (G) Protein expression levels of p-GluA1-S831 in the hippocampus. (H) Protein expression levels of p-GluA1-S845 in the hippocampus. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Discovery

SND is an important prescription for the treatment of mental related diseases. Previous studies have shown that SND can improve the depressive state of mice [8]. However, due to the complex chemical composition of SND, its potential active components and the exact pharmacological mechanism for the treatment of anxiety have not been fully understood. In this study, we used animal experiments combined with network pharmacology to study the mechanism of SND in the treatment of anxiety disorders. Our results showed that the anxiety model of mice was successfully replicated by FS. Behavioral tests showed that mice in SND group had the desire to explore open and new environment, and reversed the expression of glutamate-related proteins, suggesting that SND could improve the anxiety state of mice.

According to the properties of absorption, distribution and clearance, a total of 112 chemical constituents were selected based on database and literature search SND. They are mainly polysaccharides, flavonoids, alkaloids, terpenoids and gingerols, which have been found to improve the abnormalities of the nervous system [36]. Among the active ingredients predicted by molecular docking, quercetin and licochalcone A have been shown to have anti-anxiety. Quercetin decreased the level of ROS and MMP, increased the rate of OCR and ATP production in anxious mice, and also significantly decreased the activation of astrocytes and the level of IL-1β, improved mitochondrial function and regulated neuroinflammation to alleviate anxiety-like behavior in mice [37]. In addition, it has been reported that licochalcone A has anti-inflammatory and neuroprotective effects, which can significantly reduce neuropathic pain, but also can effectively block the activation of BV2 cells and inhibit the release of p38 phosphorylation for axiolytic effects [38]. In addition, other active components of SND also have anti-anxiety effects. After injection of Fuzipolysaccharide-1 (FPS), the expression of BDNF and the number of DG neurons increased significantly in the brain of mice. TrKB inhibitor blocked DG-enhanced cell proliferation and FPS-induced antidepressant effect in mice, suggesting that the antidepressant and neurogenic effects of FPS may be related to BDNF-TrKB signaling pathway [39]. In addition, some studies have confirmed that 6-gingerol and 8-gingerol in Ginger Rhizome have 5-HT3 receptor antagonist activity [40]. It can be seen that SND has a positive effect on anxiety disorders, which is worthy of further discussion [41].

The results of PPI network analysis showed that the core targets of SND on anxiety included 26 such as STAT3, MAPK3, MAPK1, MAPK14 and SRC. Among them, MAPK3, MAPK1 and MAPK14 were the targets with the most gene entries in the KEGG pathway, and the results of molecular docking show that the binding energy is higher. Mitogen-activated protein kinase (MAPKs) can transmit extracellular signals into cells and participate in many biological processes such as proliferation, differentiation and apoptosis of eukaryotic cells [42]. MAPKs is expressed in postmitotic neurons of adult mammalian brain and regulates neuronal activity and synaptic plasticity [43]. It has been reported that the expression of MAPK is often increased in the serum of patients with neurodegenerative diseases such as Alzheimer's disease with insomnia and anxiety, but when the anxiety symptoms are relieved, the expression of MAPK will also decrease [44]. Available data show that ERK1 (MAPK3)/2 (MAPK1) plays a key role in various neuropsychiatric disorders, including anxiety disorders [45]. At the receptor level, ERK is coupled to a variety of neurotransmitter receptors, including serotonin, adrenergic, dopamine and glutamate receptors. Activation of different subtypes of these receptors leads to phosphorylation of ERK. Interestingly, in addition to ERK, the direct upstream regulator of ERK brain-derived neurotrophic factor (BDNF) and the downstream transcription factor of ERK cAMP response element-binding protein (CREB) are down-regulated in parallel and play similar roles to ERK in anxiety disorders [46]. In addition, the study found that P38a (MAPK14) is widely distributed in different neurons, including dendrites, cytoplasm, and nuclei. It plays a key role in cellular responses to infection-associated stressors and is a drug development target that blocks cytokine production [47]. We also know that when chronic inflammation occurs, persistent inflammatory stimulation may produce damage rather than protective effects. p38MAPK is involved in glial cell-mediated neuroinflammation, while p38a appears to be the main subtype involved in inflammation [48]. Based on the above analysis, we speculate that MAPK is the main target of SND in the treatment of anxiety disorders.

The pathogenesis of anxiety disorders is related to abnormal concentrations of neurotransmitters in the synaptic spaces, among which 5-HT, DA, GLU, GABA are closely related to the pathogenesis of AD. The results of GO enrichment showed that the BP of SND in the treatment of anxiety was mainly enriched in chemical synaptic transmission regulation and cross-synaptic signal regulation. The expression process of GC mainly involves synaptic membrane and intrinsic components of presynaptic membrane. MF-related processes mainly include neurotransmitter receptor activity and postsynaptic neurotransmitter receptor activity. Acute stress can cause the involvement of a variety of neurotransmitters and neuromodulators. Among them, FS stressors are widely used to induce anxious behaviors. Previous studies have shown that stress has a significant effect on key brain regions and brain diseases, and that a key target of acute stress response is the hippocampus [49]. Glutamatergic synapse, long-term enhancement, and activity-related neuroprotection, and anti-inflammatory enrichment in the dorsal hippocampus (dHFC), neuroactive ligand receptor activation, serotoninergic synapses, and γ-aminobutyric acid synaptic synapses in the ventral hippocampus (vHC) [33].

Clinical studies and experimental animals have confirmed that neurons in related brain regions have unique signal pathways to adapt during the development of anxiety disorder. Long-term adaptive changes in these signaling pathways are involved in the remodeling of different forms of neuronal and synaptic plasticity, which are essential for persistent anxiety-like behavior. Among them, the neuroactive ligand-receptor interaction pathway is of great significance, which focuses on all the receptors and ligands related to extracellular and extracellular signal pathways on the plasma membrane. The pathway is divided into class A (rhodopsin), class B (secretagogue), class C (metabolic glutamate/phenomones), and channels/other receptors based on the structure of the ligand [50]. As important neurochemical messengers, neurotransmitters play an integral role in maintaining normal physiological processes in mammals. Abnormal neurotransmitter activity has been associated with a range of neurological disorders, including Parkinson's disease, Alzheimer's disease, Huntington's disease, and anxiety disorders [51]. Glutamate is an excitatory neurotransmitter of the central nervous system that plays a central role in the complex communication network established between neurons, astrocytes, oligodendrocytes, and microglia, and multiple abnormal triggers such as energy deficiency, oxidative stress, mitochondrial dysfunction, and calcium overload can lead to abnormal glutamate signaling. Different from normal people, the number of neurons in some areas of the brain in patients with anxiety decreased significantly. These changes are mainly caused by apoptosis induced by oxidative stress and glutamate excitotoxicity, which eventually lead to nervous system diseases [52]. N-methyl-D-aspartate receptors (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) receptors (AMPAR) are two important subtypes of glutamate receptors, both of which are related to anxiety. The subunits of AMPAR are GluA1, GluA2, GluA3 and GluA4. The subunits of NMDAR include GluN1, GluN2A and GluN2B. A study has shown that acute stress increases extracellular glutamate in the medial prefrontal cortex (mPFC) and hippocampus, while glutamate-mediated excitotoxicity induces neuronal atrophy in these brain regions through the action of extra-synaptic NMDAR [53]. In this study, SND reversed the upregulation of NMDAR and AMPAR proteins induced by regulatory alleviated anxiety-like behavior in mice, possibly due to inhibition of excessive glutamate release and reduced excitotoxicity. Therefore, there is ample evidence that overstimulation can lead to nerve cell...
damage or death, excitotoxicity, and inhibition of excitatory glutamate system is essential for an appropriate response to stress. In summary, based on the above results, our studies show that SND can improve FS-induced behavioral changes in mice and regulate hippocampal synaptic-related protein defects, especially glutamate receptors to play an anti-anxiety effect. These findings enrich the understanding of the biological activity of SND and contribute to the development and application of SND in encephalopathy including anxiety disorders and neurological diseases. However, There are still some limitations in the current research. First of all, there are obvious defects in predicting the effective anti-anxiety components of TCM based on network pharmacology, that is, the content of effective components in TCM is not taken into account. When the predicted active ingredient content is too low, it cannot be used as an effective anti-disease ingredient in TCM. In addition, molecular docking screening of anti-anxiety substances in SND lacks this part of the experimental verification, because the advantages of non-chemical drug structure of TCM monomers can provide a new strategy for new drug research and development. Therefore, we need to further clarify the anti-anxiety effect and key mechanism of SND in future research.

References
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