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Article

TSPO ligand etifoxine enhances memory reconsolidation in novel object recognition memory

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Abstract: Etifoxine is a mixed drug ligand of TSPO and $GABA_A$. It plays an important role in the treatment of anxiety disorders by promoting the synthesis of neurosteroids. Anxiety disorders are closely related to learning and memory. Etifoxine was evaluated in novel object recognition (NOR) task mouse model by injecting etifoxine into the abdominal cavity. However, few studies have investigated the effect of etifoxine on memory reconsolidation. The behavioral results showed that etifoxine-enhanced NOR memory performance when injected into mice 0 h after reactivation rather than following a 6 h delay. Conversely, administering etifoxine 24 h after sampling without reactivation did not affect NOR memory performance. Notably, etifoxine enhances memory performance not because of its effect on nonspecific responses, such as motor activity and exploratory behavior. The transcriptomic results suggested that etifoxine could regulate learning and memory-related expression genes such as CCL5, CSF3R, CXCL1, Fos, ITIH2, LRG1 and RGS1, which enhanced learning and memory in mice. In conclusion, etifoxine can improve memory reconsolidation in mice by regulating memory-related genes in the hippocampus.

Keywords: etifoxine; memory reconsolidation; object recognition; mice

1. Introduction

Learning and memory can be divided into several functionally distinct phases. Memory consolidation is the phase in which a new, labile memory is stabilized and strengthened following acquisition. When reactivated, previously consolidated memory becomes temporarily labile and is then restabilized undergoing a phase, which is named reconsolidation [1]. During the labile periods of reconsolidation, memory is able to be modified, strengthened or deleted by various factors, such as stress, sleep, novel learning situations [2] and pharmacological manipulations especially [3]. Indeed, administration of inhibitors or agonists of functional proteins or receptors can impair or promote memory reconsolidation by modulating endogenous processes [4,5].

Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride) is a mixed TSPO and $GABA_A$ drug ligand, it has been originally approved for the treatment of anxiety disorders and shows less side effects and addiction than benzodiazepines [6]. Recently, studies of the pharmacological effects of etifoxine on the central nervous system have shown that etifoxine promote acceleration of peripheral nerve regeneration, functional recovery and axonal regeneration [7]. Etifoxine could alleviate LPS-induced cognitive dysfunction and neuroinflammation by enhancing the synthesis of allopregnanolone [8]. In addition, as a positive allosteric modulator of $GABA_A$ receptor function, etifoxine can directly bind to β2-containing $GABA_A$ receptor and stimulate the production of neurosteroids,

which indirectly act on $GABA_A$ receptor [9]. Growing evidence demonstrated that the GABAergic system may play a key role in cognitive processes, including memory formation and consolidation [10,11]. For example, rotenone decreases GABAergic synaptic transmission and impairs long-term synaptic depression and conditioned taste aversion memory [12]. Up to data, however, whether etifoxine administration affects memory reconsolidation remains unclear.

Here, we hypothesized that etifoxine promotes memory reconsolidation and further confirmed the improving effect of etifoxine on memory reconsolidation using a novel object recognition memory (NOR) task, a visual recall task that relies on rodents' natural tendency for novelty and commonly used to detect memory reconsolidation [13]. Multiple brain regions, including the hippocampus, prefrontal cortex and entorhinal cortex are involved in this task. In the current study, NOR task was used to evaluate the effects of etifoxine administration on the reconsolidation of memory in mice. To explore its potential molecular mechanism, RNA-sequencing technique was used to find the target genes in the hippocampus of mice after etifoxine treatment.

2. Materials and methods

2.1. Animal

Seven-week-old male ICR mice (35 ± 2 g) were purchased from Hunan SJA Experimental Animal Co., Ltd. These mice were maintained in a standard feeding environment (temperature: 25 ± 2℃, Humidity: 50 ± 5%) with a 12-hour light/dark cycle (lights on at 7 am). The mice had free access to standard rodent chow and water. All experiments were conducted during the cycle of illumination and in accordance with Shaoyang University guidelines for the care and use of experimental animals.

2.2. Drugs

Etifoxine was purchased from Adooq Bioscience, Irvine, California, USA and was dissolved in saline containing 1.0% Tween 80 solution. Previous studies indicate that 12.5, 25 and 50 mg/kg etifoxine can improve memory in mice [8,14]. In the present study, these dosages of etifoxine were intraperitoneally (i.p.) inject into mice.

2.3. Novel object recognition (NOR) task

The NOR task procedure was conducted as previous studies [15,16]. Briefly, on the sample trial, each mice was individually placed in the training cage (30 cm × 30 cm × 60 cm) and allowed to explore two identical objects for 10 min. 24 h after the sample trial, reactivation trial was conducted with the mice were individually lifted into the training cage and re-exposed to two identical objects for 5 min. The two objects are exactly the same as the sample trial and not be moved. After 24 h, on the test trial, mice were placed in the training cage again for the 5 min test wherein one object was replaced by a novel. All the behaviors of mice were recorded by the camera, and the time of each mice spent on exploring objects was analyzed. DI is calculated based on each set of data: Discrimination indexes (DI) = $[(T_{novel} - T_{familiar})/(T_{novel} + T_{familiar}) \times 100\%]$. Higher DI indicates a stronger memory.

2.4. RNA isolation and integrity test

Total RNA was extracted from hippocampus tissues using Transzol Up (Transgen, Beijing, China) under the cooling of liquid nitrogen and then was stored immediately in the refrigerator. The RNA concentration was measured using NanoDrop ND-1000 and their integrity of RNA samples were anaylzed by denaturing agarose gel electrophoresis (Bio-Rad, USA). Only the high-quality RNA samples were used for subsequent procedures, including RNA sequencing and reverse transcription. 1.5 μg RNA was further reverse transcribed to cDNA strand for RT-PCR using cDNA Reverse Transcription Kits (Applied Biosystems, USA).

2.5. RNA-sequencing analysis

Mice were subjected to NOR reconsolidation procedure, with etifoxine injection immediately after reactivation. 2 h after etifoxine injection, mice were sacrificed to obtained hippocampus. Samples were extracted from the hippocampus by using Transzol-Up reagent (Transgen, Beijing, China). And RNA-sequencing analysis was performed at Shanghai OE Biotech Co., Ltd.

2.6. Real time-quantitative PCR

To evaluate the data of transcription profile and to explore the mechanism of etifoxine, SYBR® Select Master Mix kit (Applied Biosystems, USA) was used to do real time-quantitative PCR on CFX96 PCR system (Applied Biosystems, USA). Six upregulated expression genes and six downregulated expression genes were select to analyze relative mRNA expression levels and β-actin according to the manufacturer's protocol (Applied Biosystems, USA). PCR Primers are designed using the software Primer 6.0 and PCR primer sequences: *Allc*: sense sequence, 5' CAA ATG GGT GGA TGG ATG GGA GAC 3'; antisense sequence, 5' CAG GTT CGC TGC TTG GAT TGA CA 3'. *Cd80:* sense sequence, 5' CAA TAC GAC TCG CAA CCA CAC CAT3'; antisense sequence, 5'ATG ATG ACA ACG ATG ACG ACG ACT G3'. *Rgs1*: sense sequence, 5' TCC AGG TAG AGA CTT GAG GAA GCA T3'; antisense sequence, 5' TAC ACA ACA GCA CGC AGC ATA CAT3'. *Cxcl1*: sense sequence, 5'AAC CGA AGT CAT AGC CAC ACT CAA G3'; antisense sequence, 5' GAA GCC AGC GTT CAC CAG ACA G3'. *Fos*: sense sequence, 5' AAG ACC GTG TCA GGA GGC AGA G 3'; antisense sequence, 5' GCA ACG CAG ACT TCT CAT CTT CAA G3'. *Melk*: sense sequence, 5' GCT GCT TCA CCT TCT GTC CTG TTC3'; antisense sequence, 5' GCC ACC TGT CCC AAT CGT TTC ATA T3'. *Ccl5*: sense sequence, 5'GAC ACC ACT CCC TGC TGC TTT G3'; antisense sequence, 5'TCT TCT CTG GGT TGG CAC ACA CT3'. *Lrg1*: sense sequence, 5' TGA CAA GAA CCT GGC GGA CCT T3'; antisense sequence, 5' GGA ATG AGG AAT CTG CAC ACT GAC A3'. *Csf3r*: sense sequence, 5' ACC TGT GTA GTG ACC TGG CTC TG3'; antisense sequence, 5' GAT TGA TGG CAC GCT GGA GTC C3'. *Itih2*: sense sequence, 5' CGG CTA ACA CAG AAT TGG TCT TGG A3'; antisense sequence, 5'TGG CAG TAA GCG GAG TCA CGA T 3'. *Npas4*: sense sequence, 5' CGA CCA GAT CAA CGC CGA GAT TC3'; antisense sequence, 5' GGT AGT GCT GCC ACA ATG TCT TCA3'. *Apoa2*: sense sequence, 5' CAA TGG TCG CAC TGC TGG TCA C3'; antisense sequence, 5' CCT GGC TCT GAA TCT CTG AGG TCT T3'. All RT-qPCR

reactions were performed starting with denaturation at 95 ℃ for 4 min, then 42 cycles of 95℃ for 18 s followed by 56℃ for 1 min. The specificity of the PCR reactions was checked by 1.8% agarose gel electrophoresis (Bio-Rad, USA). After PCR amplification, the relative expression levels of mRNA were determined by the software of the PCR system.

2.7. Statistical analyses

Statistical analysis was performed using Sigma Stat 12.5. All data are presented as mean ± SEM. The performance of NOR was analyzed using one-way analysis of variance (ANOVA). Tukey HSD method was used for Post-hoc comparisons. *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Immediate etifoxine administration after reactivation enhanced the memory performance of NOR tasks

To evaluate the effect of etifoxine on memory reconsolidation, we first investigated whether etifoxine administration immediately after reactivation facilities memory performance in the NOR task. For this, mice were randomized into four groups, each group containing 10 animals and treated with vehicle or various doses of etifoxine (12.5, 25 or 50 mg/kg body weight) 1 h pre-sampling respectively. The timeline of the NOR task and etifoxine treatment was illustrated in **Figure 1A**.

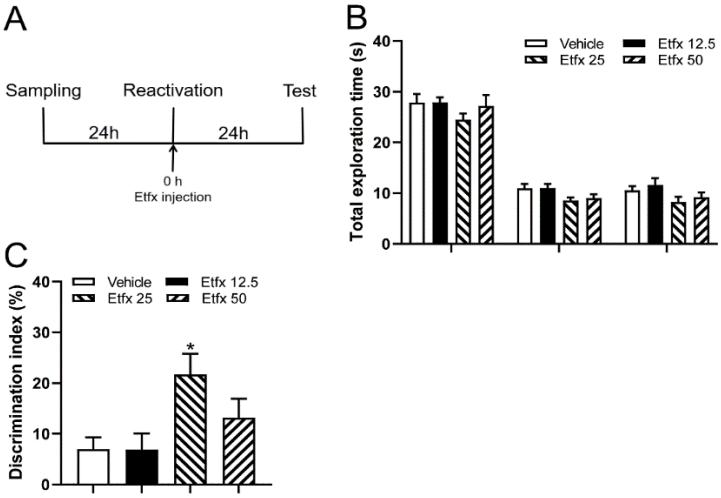

Figure 1. Effect of Etifoxine injection 0 h after reactivation on memory performance in ORM task. UCA (12.5, 25, 50 mg/kg) injection 0 h after reactivation. **(A)** The timeline of NOR task and Etfx injection; **(B)** total exploration time during sampling, reactivation and test phases; **(C)** discrimination index during test period.

*: $p < 0.05$ versus vehicle mice. ORM, novel object recognition memory; DI, discrimination index. Data represent group mean ± SEM. ORM, novel object recognition memory; Etfx, etifoxine; n =10 per group.

In NOR task, the total time spending on exploring both objects of mice in the four groups have no significant differences during sampling, reactivation or testing phase ($p > 0.05$; **Figure 2A**). However, the discrimination indexes of mice in the four groups

have a significant difference during testing phase ($p < 0.05$). In detail, mice in 25 but not 12.5 or 50 mg/kg etifoxine group showed a better discrimination index than vehicle group ($p < 0.05$). Additionally, *t*-tests revealed that the discrimination indexes were not differ significantly form zero for vehicle-treated group.

3.2. Etifoxine administration 6 h after reactivation had no effects on memory performance in NOR task

Then, we asked whether etifoxine administration 6 h after reactivation facilities memory performance in the NOR task. For this, mice were randomized into two groups, each group containing 10 animals and treated with vehicle or 25 mg/kg etifoxine. This dose of etifoxine were verified that enhanced memory performance significantly, which was used in the following experiments. The timeline of the NOR task and etifoxine treatment was illustrated in **Figure 2A**.

The NOR task showed that the total time spending on exploring both objects of mice between groups have no significant differences during sampling, reactivation or testing phase ($p > 0.05$; **Figure 2A**). Similarly, the discrimination indexes of mice between groups have no significant difference during testing phase ($p < 0.05$). Additionally, one-sample *t*-tests revealed that the discrimination indexes were not differ significantly form zero for vehicle-treated group.

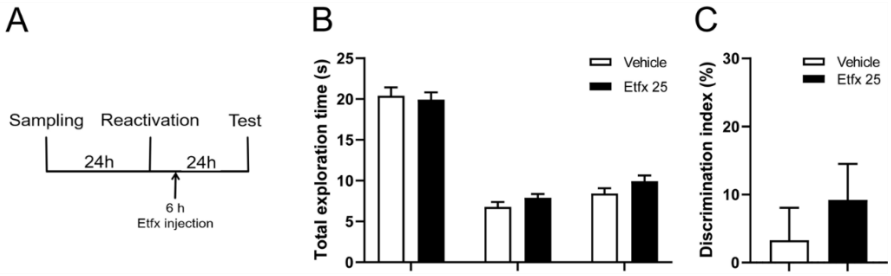

Figure 2. Effect of Etfx injection 6 h after reactivation on memory performance in ORM task. (AeC) Etfx (25 mg/kg) injection 6 h after reactivation. **(A)** The timeline of NOR task and Etfx injection; **(B)** total exploration time during sampling, reactivation and test phases; **(C)** discrimination index during test period. ORM, novel object recognition memory; DI, discrimination index.

Data represent group mean ± SEM. ORM, novel object recognition memory; Etfx, etifoxine; $n = 10$ per group.

3.3. Etifoxine administration 24 h after sampling with absence of reactivation had no effects on memory performance in NOR task

We next asked whether etifoxine administration without reactivation facilities memory performance in the NOR task. For this, mice were randomized into two groups, each group containing 10 animals and treated with vehicle or 25 mg/kg etifoxine 24 h after sampling with absence of reactivation. The timeline of the NOR task and etifoxine treatment was illustrated in **Figure 3A**.

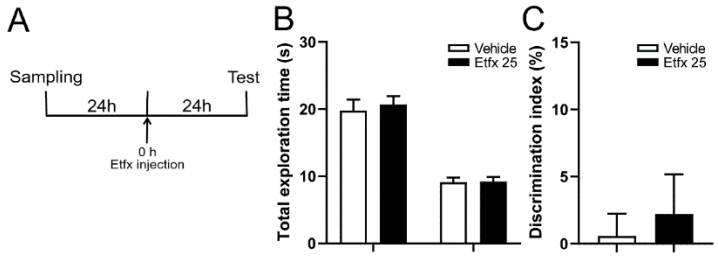

Figure 3. Effect of etifoxine injection 24 h after sampling on memory performance without the reactivation in ORM task. (AeC) Etfx (25 mg/kg) injection 24 h after sampling phase. **(A)** The timeline of NOR task and Etfx injection; **(B)** total exploration time during sampling, reactivation and test phases; **(C)** discrimination index during test period. ORM, novel object recognition memory; DI, discrimination index.

Data represent group mean ± SEM. ORM, novel object recognition memory; Etfx, etifoxine; *n* =10 per group.

The NOR task showed that the total time spending on exploring both objects of mice between groups have no significant differences during sampling or testing phase ($p > 0.05$; **Figure 2A**). Similarly, the discrimination indexes of mice between groups have no significant difference during testing phase ($p < 0.05$). Additionally, one-sample *t*-tests revealed that the discrimination indexes were not differ significantly form zero for vehicle-treated group.

3.4. Etifoxine administration does not affect nonspecific responses

Lastly, we asked whether etifoxine administration altered nonspecific responses such as locomotor activities and anxieties levels. For this, mice were randomized into two groups each group containing 10 animals and treated with vehicle or 25 mg/kg etifoxine 24 h after habitation with the test performed 24 h after the 10 min habitation period. The timeline of the OFT and etifoxine treatment was illustrated in **Figure 4A**.

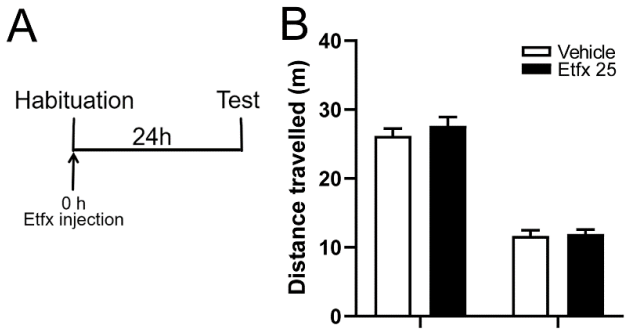

Figure 4. Effect of etifoxine (25 mg/kg) injection 0 h after habituation on nonspecific responses in ORM task. **(A)** The timeline of NOR task and Etifoxine injection; **(B)** The total distance travelled of mice during habituation and test phases were shown. ORM, novel object recognition memory.

Data represent group mean ± SEM. ORM, novel object recognition memory; Etfx, etifoxine; *n* = 10 per group.

The OFT showed that the distance traveled of mice between groups have no significant differences during habitation or testing phase ($p > 0.05$; **Figure 2A**). Similarly, the central times of mice between groups have no significant difference

during habitation or testing phase ($p > 0.05$).

3.5. Etifoxine modulated expression profile of hippocampus

RNA-sequencing was used to obtain an etifoxine-modified expression profile of hippocampus. After mice were treated with 25 mg/kg etifoxine, expression profile data indicated that etifoxine increased expressions of 30 genes (fold change > 2, $p > 0.05$; **Table 1**). Etifoxine decreased expression of 59 genes (fold change < 0.5, $p > 0.05$; **Table 2**). All comparisons were based on the vehicle group. Hierarchical clustering analysis was performed and showed that the samples from different groups could be divided into 4 regions in **Figure 5**.

Table 1. List of upregulated expression genes after etifoxine treatment.

Gene Name	Etfx/Vehicle Fold Change	Etfx/Vehicle *p* Value
Rgs1	6.42	0.0206
Allc	5.09	0.0249
Cd80	4.73	0.0167
Tesmin	4.72	0.0368
Lefty2	4.35	0.0396
Dmc1	3.80	0.0210
Aire	3.75	0.0034
Entpd4	3.37	0.0241
Cryaa	3.22	0.0213
Melk	3.03	0.0500
Neil3	2.94	0.0335
Dusp27	2.89	0.0364
Abhd11os	2.81	0.0309
Klk6	2.81	0.0354
Akr1c14	2.72	0.0117
Upk1a	2.70	0.0367
Uchl1os	2.66	0.0116
Cort	2.66	0.0153
Lctl	2.63	0.0466
Cftr	2.54	0.0470
Lin28b	2.52	0.0314
Egr2	2.51	0.0105
Lgals2	2.49	0.0207
Cxcl1	2.41	0.0244
Reep4	2.27	0.0045
Tmem273	2.12	0.0359
Fos	2.10	0.0147
Hspa1l	2.07	0.0304
Cnn1	2.03	0.0335
Tmem150b	2.03	0.0187

Table 2. List of downregulated expression genes after etifoxine treatment.

Gene Name	Etfx/Vehicle Fold Change	Etfx/Vehicle *p* Value
Srarp	0.50	0.0178
Csf3r	0.50	0.0003
Ncf4	0.49	0.0300
Npas4	0.49	0.0484
Cnga3	0.48	0.0195
Ptch2	0.48	0.0368
Grpr	0.48	0.0118
Naip5	0.47	0.0062
Lilrb4a	0.47	0.0020
Gpr179	0.46	0.0374
H2-T23	0.46	0.0260
Nkx3-1	0.46	0.0001
Hectd2os	0.45	0.0157
Itih2	0.44	0.0094
Fgfbp1	0.44	0.0257
Cdhr2	0.43	0.0157
Endou	0.43	0.0322
Insm2	0.43	0.0451
Cd40	0.43	0.0274
Mpeg1	0.42	0.0488
Sult1a1	0.42	0.0532
Klhl38	0.41	0.0277
Syt15	0.41	0.0124
Hsf5	0.40	0.0026
Mzb1	0.38	0.0106
Dhh	0.38	0.0011
Cybb	0.38	0.0105
C2cd4a	0.37	0.0233
Chrnb1	0.37	0.0142
F13a1	0.37	0.0008
P2rx2	0.37	0.0167
Adam32	0.36	0.0487
Cdc42ep5	0.35	0.0234
Clcnka	0.35	0.0023
Gpr160	0.33	0.0022
Sh2d6	0.33	0.0484
Apoa2	0.31	0.0007
Plekhg4	0.30	0.0184
Tnnt3	0.30	0.0134
Mir17hg	0.29	0.0059
Ccl5	0.27	0.0149

Table 2. (*Continued*).

Gene Name	Etfx/Vehicle Fold Change	Etfx/Vehicle *p* Value
Nlrp1b	0.26	0.0058
Jchain	0.24	0.0241
Serpina3m	0.24	0.0079
Stab2	0.24	0.0012
Hmx3	0.23	0.0007
Lrg1	0.23	0.0071
Cd101	0.22	0.0241
Kcne3	0.22	0.0176
Wfdc17	0.21	0.0253
DXBay18	0.21	0.0248
Dpep1	0.19	0.0353
Serpina9	0.18	0.0326
Clec4a1	0.18	0.0008
Vil1	0.17	0.0282
Oasl1	0.13	0.0170
Nmur1	0.12	0.0209
Vwce	0.11	0.0009
Ly9	0.10	0.0108

Online software for bioinformatics (http://www.bioinformatics.com.cn) was used to perform gene ontology (GO) analyses. The results indicated the 89 differentially expressed genes were involved in various functions and the top 10 functions included "Biological Process (BP)", "Cellular Component (CC)" and "Molecular Function (MF)". These genes are associated with positive regulation of angiogenesis, regulation of peptidase activity, positive regulation of acute inflammatory response, NADPH oxidase complex, transmembrane transporter complex, transforming growth factor beta receptor binding, etc. (**Figure 6**). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed etifoxine might interfere with the lipid and atherosclerosis, Toll−like receptor or NOD−like receptor signaling pathway, neuroactive ligand−receptor interaction, etc. (**Figure 7**).

To further explore the interrelationships of differentially expressed genes, online software STRING (https://string-db.org/cgi/) was used to determine the gene interaction network. We found that 77 genes formed a predictive network in etifoxine-treated mice hippocampus (**Figure 8**). Ccl5, Csf3r, Cxcl1, Fos, Itih2, Lrg1 and Rgs1 could be the key genes in the network.

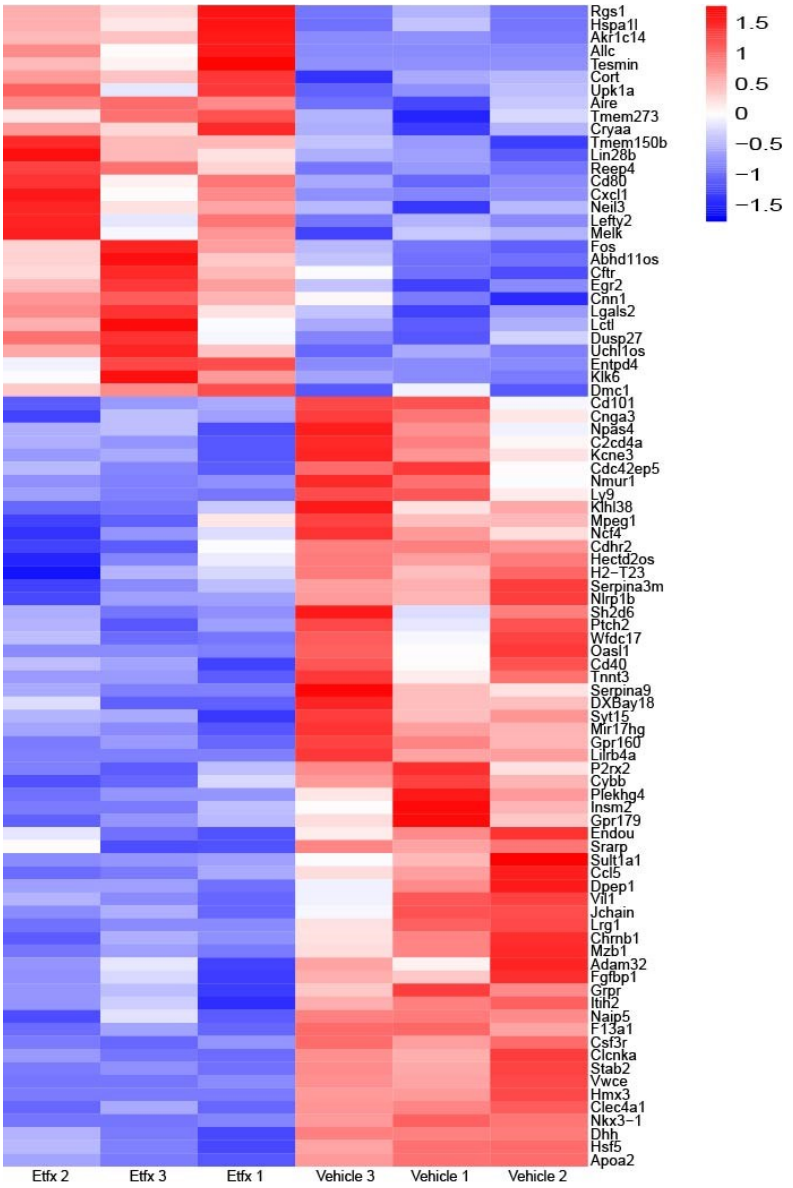

Figure 5. Cluster analysis showed the differentially expressed genes caused by etifoxine in the tissues of hippocampus. 89 differentially expressed genes (fold change > 2 or fold change < 0.5, $p < 0.05$) were identified by expression profile analysis, $n = 3$ per group.

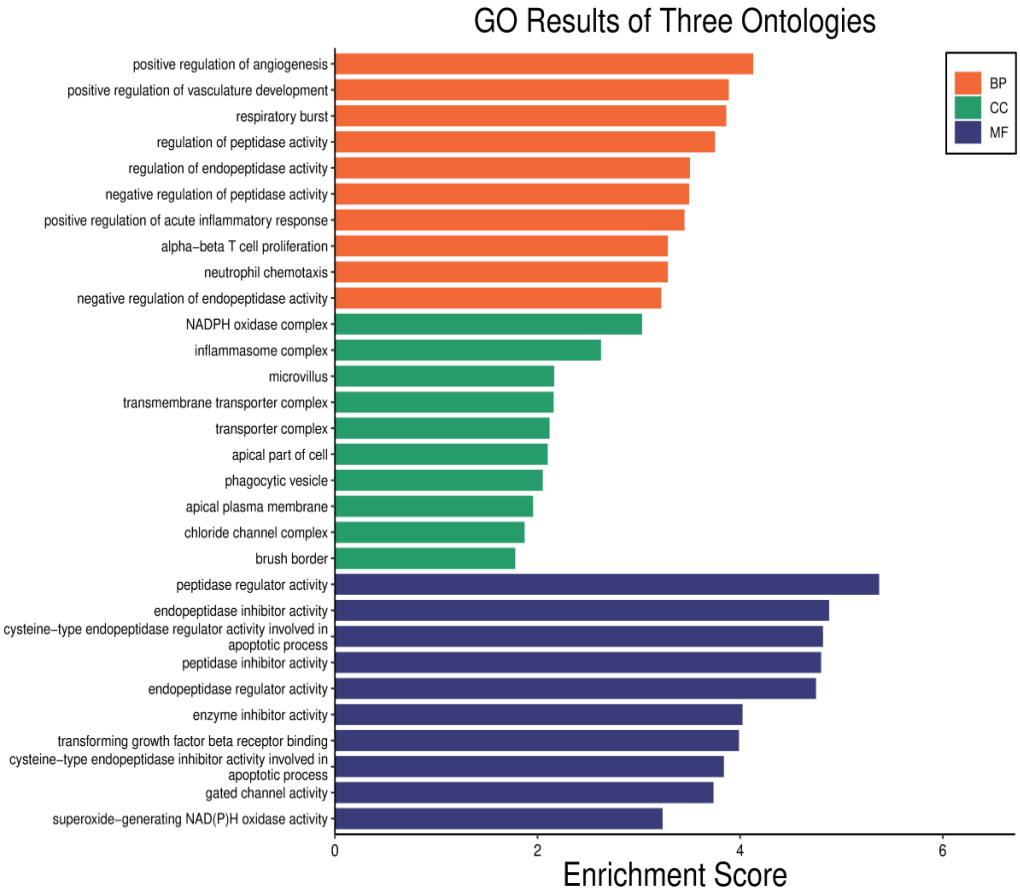

Figure 6. The top 10 Gene ontology (GO) terms with enrichment score selected for each category. Biological Process (BP), Cellular Component (CC), Molecular Function (MF) are listed in the image.

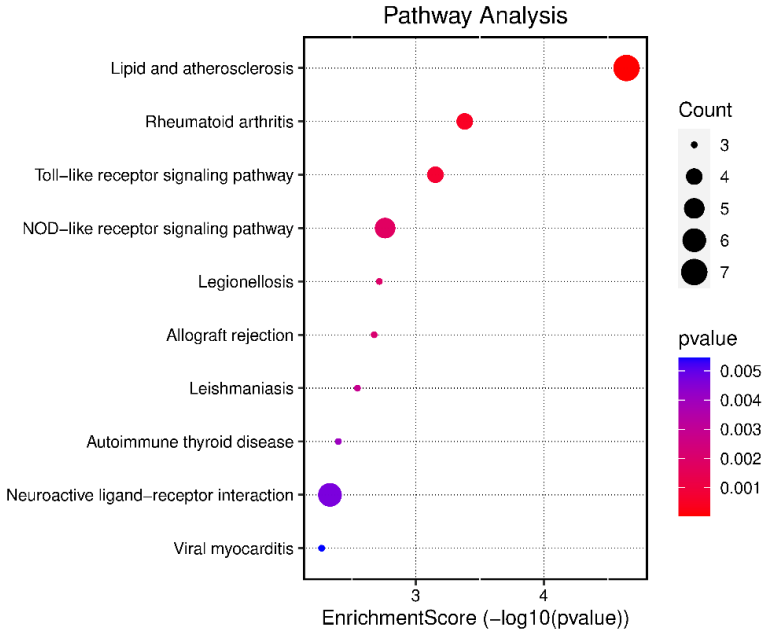

Figure 7. The Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze the differentially expressed genes in hippocampus.

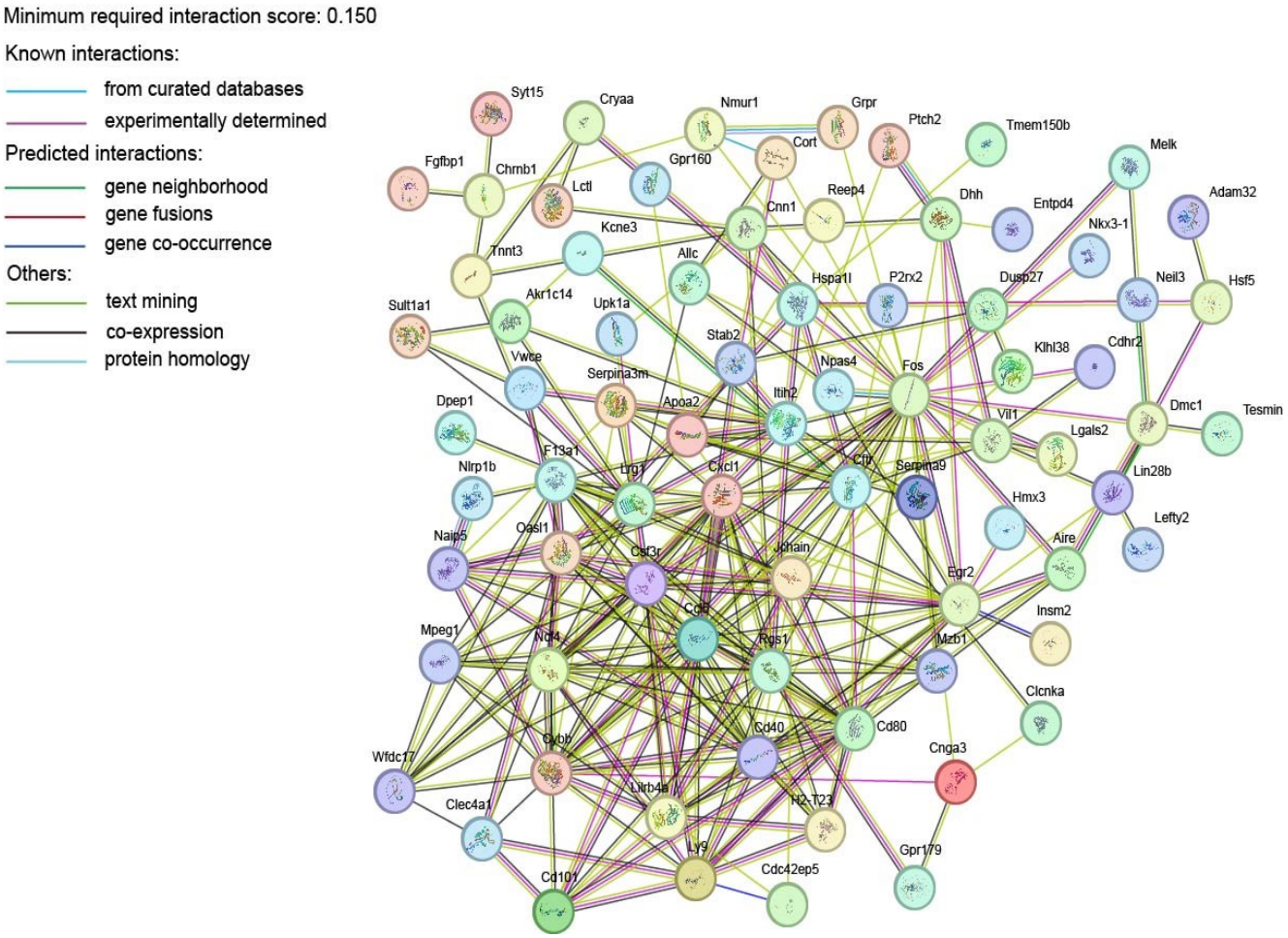

Figure 8. Effect of etifoxine on the protein-protein interaction network of differentially expressed genes in hippocampus. Upload the differential expression gene data to the STRING database and a network of 77 genes was generated.

3.6. RT-PCR confirmed depression-related gene expressions in the hippocampus

Among these different genes, 6 up-regulated and 6 down-regulated genes were further confirmed by RT-PCR and these genes are closely associated with recognition memory. From the RNA sequence data, expression levels of Allc, Cd80, Rgs1, Cxcl1, Fos, Melk were up-regulated by etifoxine treatment for 5.09, 4.73, 6.42, 2.41, 2.1 and 3.03 fold in the tissues of hippocampus; Ccl5, Lrg1, Csf3r, Itih2, Npas4 and Apoa2 were down-regulated by etifoxine treatment for 0.27, 0.23, 0.50, 0.44, 0.49 and 0.31 fold, respectively. RT-qPCR analysis showed that expression levels of up-regulated genes were 6.14, 4.52, 5.78, 2.89, 2.31 and 4.03 fold, respectively. For those up-regulated genes, the Pearson correlation of RNA sequencing analysis and RT-qPCR was 0.821. The expression levels of down-regulated genes were All the above 0.22, 0.19, 0.45, 0.34, 0.41 and 0.39 fold, respectively. For those down-regulated genes, the Pearson correlation of RNA sequencing analysis and RT-qPCR was 0.717. It suggests that the results of RNA sequencing analysis were reliable and etifoxine ameliorates recognition memory via modulating these gene expressions.

4. Discussions

The present study evaluated the effects of post-reactivation etifoxine administration on memory reconsolidation of NOR task in mice. We observed that administration of 25 rather than 12.5 or 50 mg/kg dose of etifoxine immediately following the reactivation enhanced memory performance in NOR task. However, administration of etifoxine at dose of 25 mg/kg 6 h after reactivation have no effect on NOR memory performance. Additionally, administration of his dose of etifoxine at 24 h after sampling without reactivation also have no effect on NOR memory performance. Furthermore, administration of etifoxine has no effect on locomotor activities and total exploration etc. nonspecific responses.

Present study showed that administration of etifoxine 0 h after reactivation enhanced memory performance in NOR task indicating that etifoxine tend to enhanced memory reconsolidation process. To futher confirm this role of etifoxine, we explored the effect of administration of etifoxine 24 h after sampling without reactivation on memory performance in NOR task. We observed that, in this procedure, etifoxine have no effect on NOR memory performance. These data suggested that enhancement effect of etifoxine on memory reconsideration is dependent on memory reactivation and futher indicated memory enhancing role of etifoxine did due to facilitated memory reconsolidation process. Moreover, it is important to rule out the nonspecific responses (exploration level and locomotor activity) that may influenced the role of etifoxine in the NOR task performance. For this, the exploration levels were evaluated in NOR task, while locomotor activates were evaluated in OFT task. There are an equal exploration level between groups in sample, reactivation and test of NOR task. We also observed that there have an equal locomotor activity between groups in habitation and test of OFT task. These data indicating that the role of etifoxine in memory performance of NOR task is not due to influence these nonspecific responses. Although present study excludes the effect of etifoxine on locomotor activities and exploration time in NOR task in mice, considering that anxiety is an important factor affecting the memory process and that etifoxine has a significant anxiolytic effect, is it possible that etifoxine promotes memory reconsolidation by affecting anxiety. Thus, this possibility needs to be further explored in future studies.

During memory reconsolidation process, growing evidence suggests that there exits a critical time window after reactivation, usually 1–6 h. In this time window, memories are unstable and is able to strengthened or deleted easily, while past this period memories become stable and is difficult to modified [17]. For example, administration of nicotine, urocanic acid and ketamine 0 h after reactivation enhanced memory reconsolidation, while their memory improvement were not occur when these drugs were administrated 6 h after reactivation. Similarly, the present results showed that administration of etifoxine 0 h rather than 6 h post-reactivation facilitated NOR memory reconsolidation, which indicates that etifoxine facilitated NOR memory reconsolidation dependent on a 6 h time window and futher supported the hypothesis that existence of a specific time window after memory reactivation.

Mounting evidence suggests that unstable memories undergo stabilization following acquisition or reactivation, this stabilization probably limited to a specific time window, 1–6 h [18]. And memory can be modified, strengthened or deleted by

various agents. However, administration beyond this time window will be ineffective [19]. Previous studies have suggested that multiple brain regions play a crucial role for NOR task, including hippocampus and prefrontal cortex. Etifoxine acts as a weak direct GABA receptor enhancer that potentiates GABA receptor function through a direct positive allosteric modulation effect. The GABA receptors are key mediators of primary inhibitory neurotransmission and occurred in brain regions such as the cerebral cortex, hippocampus and prefrontal cortex [20]. Growing evidence determined that GABA receptor plays role in synaptic transmission and memory processes, including long-term object recognition memory and working memory [21,22]. For example, taking GABA for one month significantly increased the recognition index in the NOR test and the accuracy rate of the T-maze test [21]. Notably, previous work suggests that $GABA_A$ receptor agonist, such as zolpidem, showed a positive role on overnight memory performance [23]; and $GABA_A$ receptor antagonist, such as bicuculline, impaired reference memory and working memory [24]. Therefore, it is reasonable to assume that increased $GABA_A$ is of crucial importance in etifoxine' s role to enhance memory reconsolidation.

Many evidences showed that etifoxine enhances GABAergic transmission through binding to the mitochondrial outer membrane translocator protein (TSPO), and then increasing synthesis of certain neurosteroids [25]. Moreover, TSPO protects the brains of Alzheimer's patients by decreasing neuroinflammation, restraining the opening of mPTP, and reducing formation of of reactive oxygen species [26]. Besides, a study in chronically stressed mice found that AC-5216, an agonist for TSPO, shows memory-enhancing effects in NOR task and increase the levels of proteins associated with the mTOR signaling pathway in PFC [27]. Thus, we speculate that the effect of etifoxine on memory reconsolidation may be related to the mTOR signaling pathway.

In this study, the results of RNA-sequencing suggested that etifoxine could regulate the transcriptional profile of mice hippocampus. Through expression profiling, we found significant changes in some genes. Bioinformatics analyses suggested that these alterations might be related to lipid synthesis and metabolism, immune recognition and neuroactive ligand-receptor interaction pathway.

Previous publications indicate that Ccl5, Csf3r, Cxcl1, Fos, Itih2, Lrg1, and Rgs1 might be the potential target genes. CCL5 is known for its role in neuroinflammation and cognitive function, Elevated CCL5 levels are associated with neuroinflammation and cognitive decline [28]. In our study, etifoxine treatment reduced CCL5 expression in the hippocampus, suggesting a potential anti-inflammatory effect that may enhance cognitive performance. The modulation of CCL5 may contribute to the improvement in learning and memory by reducing neuroinflammatory processes [29]. Traditionally associated with hematopoiesis, CSF3R has been implicated in neurogenesis and synaptic plasticity [30]. Our findings suggest that etifoxine upregulates CSF3R, which may indicate enhanced neurogenesis and cognitive resilience. The neurogenic effects of CSF3R could support the observed improvement in memory performance, highlighting its role in hippocampal plasticity and cognitive function. CXCL1 is involved in neuroinflammatory responses and has been linked to cognitive impairments [30]. Regulation of CXCL1 expression by etifoxine suggests a role in mitigating neuroinflammation, which could contribute to cognitive enhancement. The modulation of CXCL1 might help alleviate neuroinflammatory conditions that

negatively impact learning and memory. FOS, a marker of neuronal activation, is crucial for synaptic plasticity and memory formation [31,32]. Increased FOS expression following etifoxine treatment indicates enhanced neuronal activation and plasticity, supporting the molecular processes underlying learning and memory. The role of FOS in promoting long-term potentiation (LTP) in the hippocampus underscores its importance in memory processes. ITIH2 is involved in extracellular matrix stabilization and inflammatory processes. Although its direct role in cognition is less clear, modulation of ITIH2 by etifoxine may influence the structural environment of neurons, potentially impacting synaptic plasticity and cognitive function [33]. LRG1 is associated with angiogenesis and inflammatory responses. Its regulation by etifoxine may contribute to maintaining vascular health and mitigating inflammation in the CNS [34]. Improved vascular health and reduced inflammation could enhance cognitive function, highlighting the role of LRG1 in supporting cognitive performance [35]. RGS1 (Regulator of G-Protein Signaling 1): RGS1 modulates GPCR signaling, which is involved in various neurological processes, including learning and memory [36]. Regulation of RGS1 by etifoxine might affect synaptic transmission and plasticity, contributing to the observed cognitive enhancement. These findings highlight the complex and multifaceted mechanisms through which etifoxine may exert its effects on learning and memory, underscoring the need for further research to fully elucidate these pathways.

Etifoxine mechanism of action, which involves GABAergic modulation and the synthesis of neurosteroids, distinguishes it from other treatments that enhance memory reconsolidation through different pathways. For example, nicotine enhances memory reconsolidation by modulating cholinergic systems, specifically through its interaction with nicotinic acetylcholine receptors [17]. In contrast, urocanic acid achieves similar effects by modulating neuroinflammatory pathways. Ketamine, another comparator, enhances memory reconsolidation by antagonizing NMDA receptors, thereby increasing synaptic plasticity. Unlike these agents, etifoxine effects are mediated through the modulation of GABAergic transmission and neurosteroid levels, offering a complementary approach. This unique mechanism highlights etifoxine potential as an alternative or adjunctive therapy for cognitive impairments, particularly in conditions where other pathways may be less effective.

The present study acknowledges several limitations that need to be addressed in order to contextualize the findings and guide future research. Firstly, the sample size utilized in this study may restrict the generalizability of the results. Larger cohort studies are necessary to validate the effects of etifoxine on memory reconsolidation and gain a better understanding of its interactions with cognitive processes. Secondly, our study was confined to specific post-reactivation time points. To obtain a more comprehensive understanding of etifoxine's effects, future research should explore a wider range of time points and incorporate additional behavioral assays. Lastly, although key genes and pathways were identified, the direct causal relationships and detailed mechanisms underlying etifoxine's effects remain unclear. Further studies employing specific assays are required to elucidate these mechanistic pathways, which would provide deeper insights into the molecular underpinnings of etifoxine's role in enhancing memory reconsolidation.

In conclusion, this study demonstrates that etifoxine administration enhances

memory reconsolidation in a time-dependent manner in the NOR task in mice, with its effects contingent on memory reactivation. However, further research is required to confirm the role of etifoxine in other memory reconsolidation paradigms and to explore whether its memory-enhancing effects are influenced by its impact on anxiety. Additionally, the genes Ccl5, Csf3r, Cxcl1, Fos, Itih2, Lrg1, and Rgs1 have been identified as potential targets of etifoxine according to hippocampal transcriptomics. The relationship of these genes with GABAA and TSPO receptors remains unclear, and future studies should aim to elucidate the molecular mechanisms underlying etifoxine's memory-enhancing effects.

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Ethical approval: The study was conducted according to the Guidance for the Care and Use of Laboratory Animals, Shaoyang University (protocol code 2023A1124, approved date 23 may 2022).

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