Abstract

Genome-Wide Association Studies (GWASs) have reported numerous associations between risk variants and Major Psychiatric Disorders (MPDs) including Schizophrenia (SCZ), Bipolar Disorder (BD), Major Depressive Disorder (MDD), and others. We reviewed all of the published GWASs, and extracted the genome-wide significant (p<10^{-6}) and replicated associations between risk SNPs and MPDs. We found the associations of 6 variants located in 6 genes, including L Type Voltage-Gated Calcium Channel (LTCCs) subunit alpha1 C gene (CACNA1C), that were genome-wide significant (2.0 × 10^{-8} ≤ p ≤ 1.0 × 10^{-6}) and replicated at single-point level across at least two GWASs. Among them, the associations between MPDs and rs1006737 within CACNA1C are most robust. Thus, as a next step, the expression of the replicated risk genes in human hippocampus was analyzed. We found CACNA1C had significant mRNA expression in human hippocampus in two independent cohorts. Finally, we tried to elucidate the roles of venlafaxine and ω-3 PUFAs in the mRNA expression regulation of the replicated risk genes in hippocampus. We used cDNA chip-based microarray profiling to explore the transcriptome-wide mRNA expression regulation by ω-3 PUFAs (0.72/kg/d) and venlafaxine (0.25/kg/d) treatment in Chronic Mild Stress (CMS) rats. ω-3 PUFAs and venlafaxine treatment elicited significant CACNA1C up-regulation. We concluded that CACNA1C might confer the genetic vulnerability to the shared depressive symptoms across MPDs and CACNA1C might be the therapeutic target for depressive endophenotype as well.

Keywords: CACNA1C; Major Psychiatric Disorders (MPD); Schizophrenia (SCZ); Bipolar Disorder (BD); Major Depressive Disorder (MDD); Genome-Wide Association Study (GWAS)
activates as the result of membrane depolarization. It could conduct Calcium (Ca\(^{2+}\)) into the cell. Activation of Cav1.2 could initiate physiological responses such as secretion, and gene transcription, etc. Calcium (Ca\(^{2+}\)) is important for the normal function of immunity. Ca\(^{2+}\) could control the complex and crucial immune functions in lymphocytes [3]. Altered Ca\(^{2+}\) concentration in lymphocytes leads to various pathological conditions such as immunodeficiency, inflammatory and autoimmune syndromes [3], Ca\(^{2+}\) is also required for microglial M1-like pro-inflammatory activation [4,5].

DSM-5 and ICD-10 have guided psychiatry since the early 1980s. They have discrete categories that are based largely on the symptoms. DSM-5 and ICD-10 have discrete categories such as schizophrenia, major depressive disorder and bipolar disorder. So far biologists still have been unable to find any biological such as genetic or neuroscientific evidence to divide complex mental disorders into separate categories [6]. Some psychiatrists suggested to divide the disease into several endophenotypes, among which depressive symptom is commonly observed in BD, MDD, and SCZ disorders [7]. Interestingly, pharmacological activation of VDCCs induces a depressive-like phenotype [8], while knockdown of or down regulation of CACNA1C (Cav1.2) in prefrontal cortex could induce the antidepressant-like effect in mice [9].

Omega-3 Polyunsaturated Fatty Acids (omega-3 PUFA) have potential effect on the resolution of inflammation [10]. Omega-3 PUFA include Eicosapentaenoic Acid (EPA; 20:5 omega-3) and Docosahexaenoic Acid (DHA; 22:6 omega-3) [11]. EPA and DHA serves as a substrate for the formation of the specialized pro-resolving mediator Resolvin E1 (RvE1) and Resolvin D1 (RvD1), which stimulates the resolution of inflammation. RvE1 and RvD1 produce antidepressant effects induced by the lipopolysaccharide induced depression model [12] and chronic mild stress model [13] rodents. Clinical studies have reported low levels of omega-3 PUFA in patients with depression [14,15]. In Randomized Controlled Trials (RCTs), supplementation with omega-3 PUFA has been reported to have beneficial effects on severe psychiatric disorders [16], esp. depressive symptoms [17].

In this context, we reviewed all of the published VDCCs (CACNA1C, CACNA2D1, CACNB2, CACNB3, and CACNG5-G4), GWASs, and extracted the genome-wide significant (p<10\(^{-8}\)) and replicated associations between risk SNPs and severe psychiatric disease including SCZ, BD, and MDD. These associations per se are not sufficient to suggest a cause-effect relationship. However, if the biological functions of the risk SNPs can be demonstrated, or if their regulatory targets are abundant in specific human brain regions which are related with depressive symptoms (e.g., hippocampus), we could have more clues for the mechanisms underlying depressive symptoms. Thus, in the next step, the expression of the replicated risk genes in human hippocampus was analyzed. Finally, we tried to elucidate the roles of antidepressant venlafaxine and omega-3 PUFA in the mRNA expression regulation of the replicated risk genes in hippocampus.

**Materials and Methods**

**Identification of replicated associations between risk genes and severe psychiatric disease**

We searched for the literatures using the keywords "(GWAS OR Genome-wide association study) AND (schizophrenia OR depression OR bipolar disorder OR mood disorder) AND the following VDCC (CACNA1C, CACNA2D1, CACNB2, CACNB3 and CACNG5-G4)". From these literatures, the most reliable associations between VDCC and schizophrenia, depression or bipolar disorder were extracted. We notice that although most of the distinct VDCCs have been associated with schizophrenia, depression or bipolar disorder, the associations at individual marker level replicated across studies are not very common. Such replicated associations were reported in six genes located at five genomic regions by a total of seven studies (Table 1).

**Detection of mRNA expression of risk genes in human hippocampus**

mRNA expression of risk genes was examined in the human hippocampus by two independent cohorts free of neurodegenerative disorders. The first cohort included human hippocampus tissues of 134 Europeans from UK Brain Expression Consortium (UKBEC) [18]. mRNA expression of the risk genes in this first cohort was examined with Affymetrix Human ST 1.0 exon arrays, which were validated by qPCR. When a normalized intensity was >36, that is, a log2 (normalized intensity) was >5.17, it was taken as “expressed”. The second cohort included 13 human hippocampus tissues extracted from 173 Americans (The Genotype-Tissue Expression (GTEx) project [19]). mRNA expression of the risk gene in this second cohort was examined using RNA-Seq (validated by qPCR). The expression levels with TPM values >1 were taken as “expressed”.

**Hippocampal expression of risk genes regulated by omega-3 PUFA and venlafaxine in rats**

**Animals**: 18 male Sprague-Dawley rats, weighing 100 g to 120 g, were purchased from the Laboratory Animal center (Shanghai, China). All rats were given at least 3 days to acclimate to the environment before the experimentation. All rats were housed single in cages (40 cm × 25 cm × 20 cm) with bedding. Food and tap water were available freely during the experiments unless otherwise noted. The housing environment maintained on a 12 h light/dark cycle (lights on at 7:00) under controlled temperature of (22°C ± 1°C) until otherwise noted. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and P.R. China legislation on the use and care of laboratory animals.

**Materials**: Fish oil and Venlafaxine were purchased from Sigma (St Louis, MO, USA) and Wyeth Pharmaceuticals, Inc (Collegeville, PA, USA), respectively. 10,891 cDNA clones were used in the cDNA chip (provided by Shanghai Biochip Ltd, Shanghai, China). RNA isolation and purification reagents were purchased from Qiagen, Inc. (Gaithersburg, MD, USA). Cy3, Cy5 fluorescent dye was purchased from Amersham Biosciences (Waltham, MA, USA). G2655AA chip scanners and G2938b analysis software were purchased from the U.S. Agilent Technologies (Santa Clara, CA, USA).

**Chronic mild stress (CMS)**: The CMS procedure was performed as described previously with slight modification. Rats were subjected to two periods of food and water deprivation, 45°C cage tilt, soiled cage (300 ml water in sawdust bedding), paired housing, and low intensity stroboscopic illumination (300 flashes/min), and one period of white noise (90 db) and overnight illumination. The above procedure was applied randomly and continuously every week. Each period of stress was 12 h to 14 h duration.

**Sucrose intake tests**: After 14 h period of food and water deprivation, water and sucrose (1%) bottles were supplied. All rats consumed the sucrose for 1 h. Then the bottles were weighed, and
Rats in CMS+omega-3 PUFAs group were administered with fish oil of menhaden (omega-3 PUFAs) at a dosage of 3.16 ml/kg p.o., q.d. The menhaden oil contained 25% w/w omega-3 PUFAs, and the concentration was 0.91 g/ml. As such, rats in CMS+omega-3 PUFAs group received a dose of 0.72 g/kg/omega-3 PUFAs. Rats in CMS+VEN group received a dose of 2.5 mg/kg/d venlafaxine. Rats in CMS+NS group were subjected 0.9% saline to 2.5 ml/d, which is equal to CMS+omega-3 PUFAs and CMS+VEN group. All drugs and saline were administered for a total of 4 weeks from week 5 to week 8 (Figure 1).

**Procedure**: At week 0, all rats were divided into 2 groups randomly: CMS group (N=18) and CON (n=6). Rats in CMS group went through the 4-week chronic mild stress procedure. Rats in CON group were treated normally without any additional treatment. At the end of the week 4, rats in CMS group were subdivided into 3 groups: CMS+omega-3 PUFAs group, CMS+VEN and CMS+NS group. Rats in CMS+omega-3 PUFAs group were administered with fish oil of menhaden (omega-3 PUFAs) at a dosage of 3.16 ml/kg p.o., q.d. The menhaden oil contained 25% w/w omega-3 PUFAs, and the concentration was 0.91 g/ml. As such, rats in CMS+omega-3 PUFAs group received a dose of 0.72 g/kg/omega-3 PUFAs [21]. Rats in CMS+VEN group received a dose of 2.5 mg/kg/d venlafaxine. Rats in CMS+NS group were subjected 0.9% saline to 2.5 ml/d, which is equal volume with that in CMS+omega-3 PUFAs and CMS+VEN group. All drugs and saline were administered for a total of 4 weeks from week 5 to week 8 (Figure 1).

**Microarray hybridization experiment**: Total RNA was extracted and purified from the bilateral hippocampal tissues from CMS+omega-3 PUFAs and CMS+VEN group (3 rats in each group randomly) and saline group (3 rats randomly). The resulting RNA was used to measure the absorbance values (A260 and A280) at 260 nm and 280 nm with an ultraviolet spectrophotometer. RNA purity was determined by the A260/A280 ratio (standard range 1.8 to 2.1). The results showed that all RNA quality was high (RIN>7). A total of 3 control group RNAs were mixed as a negative control. The total volume of the reaction system was 20 μL. RNA was transfected with 1 μL of MMLV reverse transcriptase into cRNA. 4 μg cRNA was added to Cy3 and Cy5 fluorescent dyes. The omega-3 PUFAs and venlafaxine group cRNA was labeled with Cy3, and the saline mixed control group was labeled with Cy5. A pair of samples of one omega-3 PUFAs or venlafaxine group and mixed control group were used for each chip. RNA was labeled with the same amount of probe hybridization: 42°C 16 hour’s hybrid, 55°C wash tablets. The chip results were scanned with Agilent microarray scanner. Finally, we used the GeneSpring to carry out the homogenization processing analysis. The chip results were then analyzed using GeneSpring. The chip was scanned with Agilent microarray scanner. Finally, we used the GeneSpring to carry out the homogenization processing analysis. The final ratio of Cy3/Cy5 ≥ 1.5 or ≤ 0.6 indicates that the gene has a differential expression. According to the gene number provided by GeneSpring, we carried out Gene Informatics Analysis via U.S. National Medical Biology Information Center (NCBI).

**Statistical analysis**: Behavioral data were analyzed using Multivariate Analysis of Variance (MANOVA) and then Fisher Least Significant Difference (LSD) post hoc tests and Nyholt correction [20]. We used one-way ANOVA to test the antidepressant effect of omega-3 PUFAs and venlafaxine.

## Results

**Genome-wide significant associations between SNPs of 6 VDCC genes and major psychiatric diseases**

We only found the associations of 6 variants located in six genomic regions (CACNA1C [21-24], CACNA2D2 [25], CACNB2 [26], CACNB3 [24], and CACNG5-G4 [27]) that were genome-wide significant and replicated at single-point level across at least two GWASs (2.0 × 10^-8 ≤ p ≤ 1.0 × 10^-6). The protein encoded by CACNA1C, CACNB3, CACNB4, CACNB5, CACNA2D1, and CACNA2D2 that were genome-wide significant and replicated at single-point level across at least two GWASs (2.0 × 10^-8 ≤ p ≤ 1.0 × 10^-6). The protein encoded by the above genes belongs to the voltage-gated calcium channel alpha (CACNA1C, CACNA1D, CACNA1E, CACNA2D1 and CACNA2D2), beta (CACNB2 and CACNB3) and gamma (CACNG4 and CACNG5) subunit family. Among them, the associations between MPDs and variants rs1006737 within CACNA1C are most robust, because they were replicated across three GWASs (3 × 10^-8 ≤ p ≤ 7 × 10^-8) [21-23]. Part of these associations has been validated by functional studies (Table 1).
We determined mRNA expression levels for the risk genes reported in GWAS. Six protein-coding genes, including CACNA1C, CACNA2D1, CACNB2, CACNB3, CACNG4 and CACNG5 had significant mRNA expression in human hippocampus in two independent cohorts (Table 2). All log2 (normalized intensity) values for nine genes in the UK Europeans >1.6 (5.25 to 7.76); TPM values for six genes in the Americans >1 (1.0 to 10.4).

### mRNA expression of risk genes in human hippocampus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNA1C</td>
<td>4q42</td>
<td>2.4 ± 0.17*</td>
</tr>
<tr>
<td>CACNA2D1</td>
<td>17q12.3</td>
<td>0.98 ± 0.04</td>
</tr>
<tr>
<td>CACNB2</td>
<td>17q12.3</td>
<td>1.07 ± 0.87</td>
</tr>
<tr>
<td>CACNB3</td>
<td>17q12.3</td>
<td>13.1 ± 5.35</td>
</tr>
<tr>
<td>CACNG4</td>
<td>17q12.3</td>
<td>1.34 ± 0.48</td>
</tr>
<tr>
<td>CACNG5</td>
<td>17q12.3</td>
<td>13.1 ± 5.35</td>
</tr>
</tbody>
</table>

Data are presented as mean sucrose intake ± SEM (n=6 ml/h). We set the 1.6 and 0.6 as the threshold for up or down regulation.

#### Table 2: The mRNA expression of VDCC genes in hippocampus in normal human brain.

<table>
<thead>
<tr>
<th>Gene</th>
<th>UK European (n=134)</th>
<th>American (n=173)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNA1C</td>
<td>6.4</td>
<td>0.9</td>
</tr>
<tr>
<td>CACNA2D1</td>
<td>7.69</td>
<td>5.4</td>
</tr>
<tr>
<td>CACNB2</td>
<td>6.76</td>
<td>2.2</td>
</tr>
<tr>
<td>CACNB3</td>
<td>6.8</td>
<td>10.4</td>
</tr>
<tr>
<td>CACNG4</td>
<td>6.89</td>
<td>10.2</td>
</tr>
<tr>
<td>CACNG5</td>
<td>5.25</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The numbers in UK European cohort are log2-transformed normalized intensity values from Affymetrix Human ST 1.0 exon arrays; the numbers in American cohort are TPM values from RNA-Seq.

#### Table 3a: Sucrose intake (mg/l) in ω-3 PUFA and venlafaxine treated rats (n=6).

<table>
<thead>
<tr>
<th>Week</th>
<th>CMS+ω-3 PUFA</th>
<th>CMS+VEN</th>
<th>CMS+NS</th>
<th>CON+NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>8.26 ± 3.37</td>
<td>8.48 ± 3.46</td>
<td>7.32 ± 2.99</td>
<td>8.81 ± 3.6</td>
</tr>
<tr>
<td>Week 1</td>
<td>7.48 ± 3.06</td>
<td>8.3 ± 3.39</td>
<td>7.73 ± 3.16</td>
<td>5.66 ± 2.31</td>
</tr>
<tr>
<td>Week 2</td>
<td>6.7 ± 2.74</td>
<td>8.12 ± 3.31</td>
<td>8.15 ± 3.33</td>
<td>10.69 ± 4.36</td>
</tr>
<tr>
<td>Week 3</td>
<td>7.96 ± 3.25a</td>
<td>5.78 ± 2.36b</td>
<td>5.83 ± 2.38b</td>
<td>12.08 ± 4.93</td>
</tr>
<tr>
<td>Week 4</td>
<td>4.38 ± 1.79a</td>
<td>5 ± 2.04b</td>
<td>4.88 ± 1.96b</td>
<td>10.35 ± 4.22</td>
</tr>
<tr>
<td>Week 5</td>
<td>3.58 ± 1.46a</td>
<td>6.55 ± 3.49</td>
<td>7 ± 2.86</td>
<td>8.96 ± 3.68</td>
</tr>
<tr>
<td>Week 6</td>
<td>6.85 ± 2.79b</td>
<td>9.42 ± 3.84</td>
<td>5.33 ± 2.18b</td>
<td>13.42 ± 5.48</td>
</tr>
<tr>
<td>Week 7</td>
<td>13.68 ± 5.58b</td>
<td>17.85 ± 3.31</td>
<td>4.83 ± 1.98b</td>
<td>13.1 ± 5.35</td>
</tr>
<tr>
<td>Week 8</td>
<td>12.75 ± 5.21a</td>
<td>18.9 ± 7.72a</td>
<td>4.65 ± 1.9b</td>
<td>11.42 ± 4.66</td>
</tr>
</tbody>
</table>

Data are presented as mean sucrose intake ± SEM (n=6 ml/h). CMS: Chronic Mild Stress; VEN: Venlafaxine (2.5 mg/kg/d diluted in 2.5 ml saline); NS: Equal volume with that in rats 2.5 ml/d; ω-3 PUFAs: ω-3 PUFAs (0.72 g/kg/d) a: P<0.05, compared with CMS+NS group; b: P<0.05, compared with CON+NS group. 1% sucrose intake was significantly decreased after CMS from week 3. 1% sucrose intake was significantly improved by a single dose of n-3 PUFAs (0.72 g/kg/d), and venlafaxine (2.5 mg/kg) at week 7 and week 8 after Independent Samples t-test.

### Sucrose intake ratio in omega-3 PUFA and venlafaxine treated rats

Unpredictable Chronic Mild Stress (CMS) produced anhedonia in rodents, a core symptom of depression that shows loss of interest in normally pleasurable and rewarding activities. The sucrose intake in rodents, a core symptom of depression that shows loss of interest in normally pleasurable and rewarding activities. The sucrose intake ratio in CMS rats was significantly decreased after CMS from week 3. We also found CACNA1C might be the therapeutic target for the depressive endophenotype in CMS rats. To the best of our knowledge, the present study is the first research in detecting the pharmaceutical target of CACNA1C gene expression for omega-3 PUFA treatment.

### Omega-3 PUFA and venlafaxine treatment regulated gene expression

After the last sucrose test, all rats were sacrificed. Hippocampus was analyzed by Affymetrix Human Gene 1.0 ST arrays. We used the regulatory effects of omega-3 PUFAs and venlafaxine in CMS rats, by comparing to saline treatment. We focused on the six genes reported in GWAS. Omega-3 PUFA treatment elicited significant upregulating genes including CACNA1C, Venlafaxine treatment elicited significant upregulating genes including CACNA1C, as well.

#### Discussion

In the current study, we identified a significant gene, i.e. CACNA1C, for the shared etiology of severe psychiatric disease. We also found CACNA1C might be the therapeutic target for the depressive endophenotype in CMS rats. To the best of our knowledge, the present study is the first research in detecting the pharmaceutical target of CACNA1C gene expression for omega-3 PUFA treatment.

Severe psychiatric diseases are genetically complex diseases. Increasing evidence suggested a shared genetic etiology among them [1]. We first searched the GWASs and meta-analyses of GWASs literatures. We notice the most replicable six SNPs located in six VDCC genes (CACNA1C, CACNA2D1, CACNB2, CACNB3, CACNG4 and CACNG5) contributed to the risk of severe psychiatric diseases. The protein encoded by the above genes belongs to the voltage-gated calcium channel alpha (VDCC) subunit family. We focused on these 6 replicable genes in the following study. Among which GWASs and meta-analyses of GWASs have shown the most robust associations of Single-Nucleotide Polymorphisms (SNPs) of CACNA1C with severe psychiatric diseases [21-24,29-31].

Secondly, we detected the mRNA expression of the 6 genes (CACNA1C, CACNA2D1, CACNB2, CACNB3, CACNG4 and CACNG5) in hippocampus. We found all of the 6 genes are abundant in hippocampus in 134 Europeans [18] and 173 Americans individually [19] cohorts. VDCC is important in providing calcium influx to cortical pyramidal neuron in hippocampus [32]. VDCC channels are rich in the soma and proximal dendrites of hippocampal pyramidal neurons and in axons, gial processes and axon terminals in all hippocampal subfields as well [33]. Multiple VDCC also activates transcription regulators that regulate adult hippocampal neurogenesis [34]. Embryonic deletion of VDCC in glutamatergic neurons in forebrain could induce the emotional behavior endophenotypes [35].
Furthermore, based on the evidence that VDCC contribute to shared etiology among severe psychiatric disorders, including SCZ, BD and MDD, and the contribution of VDCC on the inflammatory etiology on the depressive like phenotype. In our following-up step, we tried to illustrate whether VDCC contributed to the therapeutic target of antidepressants. CMS paradigm produced anhedonia, a core feature of depression. 1% sucrose solution intake is a standard criterial to evaluate the anhedonia induced by CMS. We found 1% sucrose intake was significantly improved by venlafaxine (2.5 mg/kg) after treatment of 3 weeks and 4 weeks. Next, we used cDNA chip-based microarray profiling to explore the transcriptome-wide regulatory effects of venlafaxine in CMS rats, by comparing to saline treatment. We found CACNA1C was significantly upregulated by 2.4 times by venlafaxine. In accordance with our findings, Du's research also found the Cav1.2 mRNA expression was doubled after fluoxetine hydrochloride treatment (10 mg/kg) for two weeks [36]. All of the above findings suggested that Cav1.2 might be the pharmacutic target of the antidepressants.

Lastly, since VDCC contribute to the normal function of immunity and etiology of depressive endophenotype, we hypothesis that VDCC might be the pharmacutic target of omega-3 PUFAs, which has potential effect on the resolution of inflammation [10]. Our data showed 1% sucrose intake was significantly improved by omega-3 PUFAs after treatment of 3 weeks and 4 weeks. This result confirmed the previous hypothesis that omega-3 PUFAs have potential antidepressant effects [14,15]. In consistent with our findings, previous research also showed omega-3 PUFAs have potential effect on the resolution of inflammation [10] and potential antidepressant effect in both animal model of depression [13] and human patients [14,15]. cDNA chip-based microarray data showed that CACNA1C were most significantly regulated by omega-3 PUFAs among all 6 protein-coding genes we previously found. Among which CACNA1C was most highly upregulated by 2.4 times by omega-3 PUFAs. VDCC contribute to the normal glial function, which could produce active cytokine cells in the brain [37]. Drugs targeted at VDCC might have promising management for neuroinflammatory disease [38].

The modest sample size is one limitation of our study. We recruit 3 rats for the cDNA chip-based microarray analysis. However, our data consistently showed omega-3 PUFAs and venlafaxine treatment elicited significant upregulating genes CACNA1C. We concluded that there was a significant increase of CACNA1C mRNA expression in omega-3 PUFAs and venlafaxine treated CMS rats in hippocampus, suggesting CACNA1C might be a therapeutic target for antidepressants of anti-inflammatory biomarker in depressive endophenotype.

Acknowledgement

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Author Contributions

Conceived and designed the experiments: X.G. X.L. K.J. Performed the experiments: X.L. X.G. Y.F. Y.Z. Analyzed the data: X.G. X.L. I.L. K.W. Wrote the manuscript: X.G. X.L. K.J.

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