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ORIGINAL RESEARCH

Impact of Electroacupuncture on the Expression Profile of the Anterior Cingulate Cortex in a Rat Model of Irritable Bowel Syndrome Based on Transcriptome Sequencing and Bioinformatics Analysis

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Purpose: Given that electroacupuncture (EA) is a promising yet mechanistically unclear intervention for IBS, and considering the anterior cingulate cortex (ACC)'s crucial role in visceral pain and emotion within the gut-brain axis, this study aims to investigate the transcriptomic alterations in the ACC of a rat IBS model and to evaluate the potential therapeutic effects of EA administered at the acupoints Tianshu (ST25) and Zusanli (ST36).

Methods: Rats subjected to water avoidance stress (WAS) were evaluated for IBS symptoms, including increased anxiety-like behavior, abnormal abdominal muscle activity, and elevated abdominal withdrawal reflex (AWR) scores—a validated measure of visceral hypersensitivity. The effect of EA at ST25 and ST36 was assessed on these symptoms, and the transcriptomic profile of the ACC was analyzed after WAS and EA treatment using RNA sequencing and subsequent validation by real-time PCR.

Results: The stress model induced significant IBS-like symptoms, including visceral hypersensitivity and anxiety-like behavior. EA at ST25 and ST36 significantly ameliorated these behavioral and physiological deficits. Notably, transcriptomic profiling of the ACC linked this behavioral improvement to central modulation, revealing dysregulation of 3 key genes involved in many pathways. RT-PCR validated the expression changes in 3 selected candidate genes, further supporting their role in EA's therapeutic effects.

Conclusion: EA alleviates IBS symptoms in rats, potentially through transcriptomic reprogramming in the ACC, which provides novel mechanistic insight into how peripheral acupuncture may exert central actions to treat IBS.

Keywords: anterior cingulate cortex, irritable bowel syndrome, transcriptome sequencing analysis, electroacupuncture

Introduction

Irritable bowel syndrome (IBS) is a common intestinal functional disorder characterized by symptoms such as abdominal pain, diarrhea, and constipation and represents a worldwide public health concern.[1,2] Epidemiological studies indicate that IBS affects an estimated 9–23% of the world's population and represents a major global burden.[3] The etiology and pathogenesis of IBS are complex, but as research continues to expand, the abnormal bidirectional interaction between the intestine and brain and structural neuronal changes in specific brain regions are increasingly recognized.[4–6] The six identified genetic loci, which regulate the expression of multiple genes associated with IBS, are expressed primarily in the brains of IBS patients. This intriguing finding suggests that central nervous system malfunction may play a part in the pathogenesis of this disease.[7] Substantial evidence suggests that genetic alterations in the anterior cingulate cortex, hippocampus, and other related brain regions may contribute to visceral hypersensitivity, pain, and intestinal motility

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issues associated with IBS, particularly when triggered by chronic exposure to environmental stress and fatigue.[8–10] However, the accurate genetic and epigenetic underpinnings of IBS remain unclear. Given the absence of specific biomarkers, the clinical diagnosis and treatment of IBS continue to pose significant challenges.[11] Currently, clinical treatment is limited to improving intestinal symptoms, with each drug targeting only one symptom. There is an urgent need to find more effective treatment approaches.

Acupuncture, which originated in China, has been shown to have unique advantages in the prevention and treatment of dysfunctions of the digestive system.[12–14] According to some preliminary studies, acupuncture has complicated effects on neurotransmitters, inflammation, visceral sensitivity, gastrointestinal dynamics, and negative emotions in animal models of IBS.[15–18] However, most existing studies have focused on exploring the peripheral mechanisms of acupuncture in treating IBS, whereas only a limited number of studies have examined the effects of acupuncture on the central nervous system (CNS). Zusanli (ST36) and Tianshu (ST25) are considered effective acupuncture points for the treatment of IBS,[19,20] although their pathways of action exhibit distinct differences. Stimulation at ST36 may primarily activate the vagus-adrenal axis, modulating systemic autonomic balance and the stress response system, thereby remotely regulating gene expression and neural function in the ACC. In contrast, the effects of stimulation at ST25 do not rely on the vagus nerve pathway.[21] Instead, its mechanisms may be mediated through spinal-brainstem ascending projections or local gut-brain axis circuits, allowing for more direct modulation of brain regions involved in visceral sensation and emotional integration. This specificity between acupoints highlights the need for further systematic elucidation.

The anterior cingulate cortex (ACC) is recognized as a key brain area involved in the generation and regulation of visceral pain and emotion.[22] The hyperactivity of neurons in the ACC plays a significant role in the pathophysiology of IBS, with associated neurotransmitters, receptors, signaling pathways, and neural circuits involved in the control of visceral pain and emotions.[23–26] In the pathogenesis of IBS, abnormal expression of multiple genes in the ACC collectively contributes to disease progression.[27] Some genes are upregulated, affecting glutamate receptor function;[28] others are involved in glycolipid metabolism disorders and insulin resistance; while still others act as immunomodulatory factors, exacerbating central sensitization and pain perception, thereby inducing stress and anxiety. Acupuncture is effective in improving pain aversion and reducing pain-related anxiety, fear, and other negative emotions through a mechanism related to the regulation of ACC function.[29] There is evidence that acupuncture modulates the ACC to have a therapeutic effect on IBS at the neuronal and neurotransmitter levels.[30,31] Existing research indicates that electroacupuncture treatment can modulate relevant metabolic pathways, regulate nervous system function, and reverse abnormal gene expression in the brain through mechanisms involving non-coding RNA or epigenetic modifications.[32,33] However, the specific extent to which electroacupuncture treatment modulates gene expression in the ACC has not been fully elucidated, and the mechanistic differences in how different acupoints regulate ACC gene expression remain to be further clarified. Therefore, to comprehensively investigate the potential effects of electroacupuncture (EA) on the expression of genes in the ACC of rats with IBS, we conducted advanced transcriptomic sequencing and rigorous bioinformatics analyses. This study was conducted to elucidate the scope of the primary molecular mechanisms of EA in the treatment of IBS, thereby providing guidance for the scientific understanding of its effects and providing support for evidence derived from animal studies that may inform the clinical application of EA in the prevention and treatment of IBS.

Materials and Methods

Experimental Animals and Groups

All animal procedures in this study strictly complied with the World Health Organization's International Guiding Principles for Biomedical Research Involving Animals and were approved by the Ethics Committee of Shandong University of Traditional Chinese Medicine (Certificate No. SDUTCM20220827001); the 3R principles (replacement, reduction, and refinement) were implemented throughout, with predefined humane endpoints and a health-monitoring checklist to safeguard animal welfare and data quality. Healthy, male, SPF-grade Wistar rats (180 ± 20 g) were purchased from Jinan Peng Yue Animal Breeding Co., Ltd.; upon arrival, animals underwent overt health inspection and short-term observation, were assigned unique identifiers and individual records documenting source, body weight, health status, and group code. Rats were housed in the Experimental Animal Center of Shandong University of Traditional Chinese Medicine with standard pelleted chow and sterilized water available ad libitum, at four per cage with appropriate environmental enrichment and shelter; environmental conditions were maintained at 18–24°C, 50–70% relative humidity, and a 12 h/12 h light-dark cycle, with routine documentation of parameters and scheduled bedding replacement and cage sanitation. Before formal experiments, a 7-day acclimatization was implemented to mitigate transport and environment related stress; prior to modeling, baseline assessments were conducted at a fixed time under consistent environmental conditions, including general clinical observations and visceral sensitivity determined by abdominal withdrawal reflex (AWR) scoring, performed according to recognized standards by trained personnel. Subsequently, group allocation was performed using the random-number table method, assigning the 50 Wistar rats to a control group (n = 10) and a model group (n = 40); the randomization sequence was generated and retained by personnel blinded to procedures, with cage assignment and labeling designed to prevent unmasking, and blinding maintained where feasible during outcome assessment and data analysis. To ensure rigor and reproducibility, original data, environmental records, and operational logs were established and archived contemporaneously to support audit trails and verification.

IBS Animal Model Establishment

The chronic water avoidance stress (WAS) paradigm was employed to establish the experimental IBS model.[34] The procedure of WAS can be described briefly. After all the tanks (50 cm × 50 cm × 30 cm) were filled with water, the rats in the model group spent one hour every day for ten days on the central platform (8 cm × 8 cm × 10 cm) in tanks that were 1 cm above the water surface. The Control group rats remained in tanks without water.

Forty rats assigned to the model group were randomized equally into four groups using the random-number table method: IBS (n = 10), ST25 (n = 10), ST36 (n = 10), and Sham EA (n = 10).

EA Treatment

Following validation of the IBS animal model, we first conducted a visceral pain threshold assessment.

Animals in the Control and IBS groups were maintained without intervention for 14 consecutive days under identical housing and routine husbandry conditions to minimize extraneous variables, whereas the remaining groups proceeded to EA procedures implemented on a unified platform and according to standardized workflows to ensure comparability. All needling-related procedures were performed under anesthesia induced with 2% isoflurane on a dedicated rat surgical table.[19] To control for anesthetic effects on outcomes, rats in the Control and IBS groups were likewise exposed to 2% isoflurane for the same duration. The anesthesia regimen, exposure window, and monitoring items were kept consistent, and procedure timing was fixed insofar as possible to mitigate circadian influences. The EA intervention followed the prespecified treatment protocol: two 15 mm acupuncture needles were bound together with insulating tape, placed 1 mm apart, and inserted 5 mm into the muscle; the needle tails were connected to the positive and negative terminals of a Han's Acupoint Nerve Stimulator, with stimulation parameters set to a sparse-dense wave of 2/15 Hz and a current intensity of 1 mA;[35] bilateral EA was administered at ST25 and ST36 in the ST25 and ST36 groups, respectively. A sham EA control was established by inserting needles at bilateral remote sham acupoints (located 10 mm lateral the iliac crest), with the needling method and session duration matched to the real EA. All interventions were delivered for 20 minutes per day for 14 days; acupoint localization was performed in accordance with the latest standard,[36] with the positioning procedure and adjudication criteria standardized and documented prior to study initiation.

Throughout intervention and measurement, animal welfare and humane endpoints were reinforced, with continuous monitoring of activity, respiration, reflexes, feeding, and adverse events; blinding and quality control were implemented wherever feasible, and the duration of anesthetic exposure, needling and stimulation parameters, acupoint verification, daily session start-stop times, and any deviations or adverse events were recorded in a standardized manner. Rats began the experiment at 40 days old. The timeline for key procedures was as follows: acclimation (Experimental Days 0–7; Postnatal Days [PND] 40–47), chronic stress modeling (Days 7–17; PND 47–57), the first AWR test (Days 17–18; PND 57–58), electroacupuncture intervention (Days 18–32; PND 58–72), the final AWR test (Days 32–33; PND 72–73), followed by behavioral assessments, electromyographic (EMG) measurements,and sample collection. The entire experimental design was shown in Figure 1.

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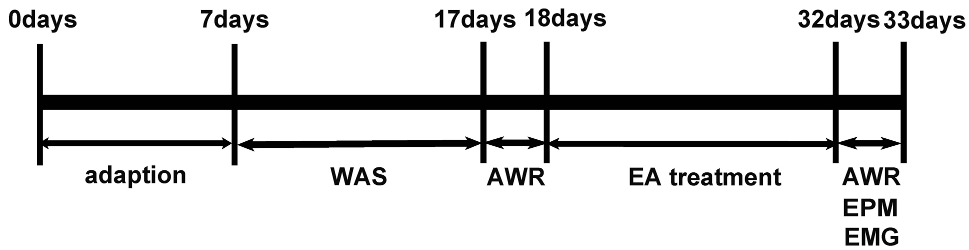

Figure 1 Experimental design.

Main Outcome Measurement

Visceral Sensation Measurement

Electromyographic (EMG) measurements, validated as effective measurements of visceral pain, were carried out to assess visceral hypersensitivity.[19] We assembled and calibrated a custom CRD apparatus by connecting a balloon and syringe to a benchtop sphygmomanometer via a three-way tubing manifold; pressure was read in mmHg, and the circuit was pre-primed and vented to eliminate dead space and ensure stable pressure transmission. Recognizing that electrode implantation is invasive, and to balance animal welfare with statistical power, five rats per group were selected for CRD evaluation using the random-number table method, with blinding of operators and assessors implemented whenever feasible. All selected animals were fasted for 24 hours but not deprived of water, anesthetized with 2% isoflurane for induction and maintenance, and positioned supine on a rat surgical platform; the abdominal skin was shaved and disinfected with povidone-iodine, thermal support was provided to maintain body temperature, and respiration and reflexes were monitored throughout. Bipolar EMG electrodes (inter-electrode distance 5 mm) were implanted by stabilizing the abdominal wall and rectus abdominis with the left hand and vertically inserting the electrodes into the rectus abdominis muscle layer to a depth of 5–7 mm with the right hand; electrodes were secured with subcutaneous sutures and film, externalized leads were connected to the acquisition system to confirm signal patency and baseline stability, and meticulous hemostasis was maintained to minimize tissue trauma. Following electrode placement, a medical-grade latex balloon lubricated with glycerol or petrolatum was gently advanced 6 cm through the anus and maneuvered with care to avoid mucosal injury. In order to avoid signal interference with electrophysiological measurements caused by struggling rats, we used the low concentration of isoflurane anesthesia to keep the rats quiet without blocking the production of visceral pain.[37] Rats received 1% isoflurane light anesthesia, and CRD was initiated via syringe-driven balloon inflation.[19,35] Distension was applied at 20, 40, 60, and 80 mmHg, each maintained for 20 seconds. The electrical signals from the rectus abdominis muscle were recorded and analyzed via a Power Lab system (AD Instrument Co., Ltd., New South Wales, Australia). The area under the curve (AUC) during CRD (20 seconds) was compared with the area under the curve (AUC) of baseline (20 seconds) to quantify electromyographic activity.[38]

The abdominal withdrawal reflex (AWR) was assessed in accordance with standardized procedures: 2% isoflurane was used only for brief inhalational anesthesia to facilitate catheter placement; a paraffin-oil-lubricated balloon catheter was gently advanced anus to approximately 6 cm from the anal verge and secured at the tail base with adhesive tape to prevent displacement. Anesthesia was then turned down to 1% isoflurane for about 15 minutes in a quiet setting. CRD was delivered by stepwise inflation via a syringe through a three-way valve at target pressures of 20, 40, 60, and 80 mmHg, each stimulus maintained for 20 seconds. Each pressure level was repeated 3 times and averaged. Pain pressure thresholds and behavioral responses were determined according to the standard AWR scoring criteria:[39] Grade 0, no visible response; Grade 1, brief head/neck movement or mild tension; Grade 2, slight contraction of the abdominal wall without lifting off the platform; Grade 3, tonic contraction of the abdominal wall with evident abdominal elevation lifting off the surface; Grade 4, pelvic elevation with body arching. AWR scores were recorded at each target pressure and the visceral pain threshold was calculated,[40,41] with continuous monitoring of respiration, activity, and adverse events throughout; any abnormalities prompted immediate termination and documentation.

Elevated Plus-Maze Test

Anxiety-related behavior in IBS rats was assessed using the elevated plus-maze (EPM).[42] The apparatus comprised two open arms and two closed arms (50 cm × 10 cm), with 75cm high walls on the closed arms, interconnected by a central square platform (10 cm × 10 cm). Rats were placed in the central area facing an open arm and allowed to explore for 5 minutes; we recorded the time spent in the open arms, central area, and closed arms, as well as the number of entries into the open and closed arms and the total arm entries during the 5-minute session. Based on these primary measures,[42] we calculated the percentage of open arm entries and the percentage of time spent in the open arms. Behavioral data were analyzed using behavior analysis software, which also generated trajectory plots.

Transcription Sequencing and Real-Time PCR

We selected three rats from each group by the random number table method, and the rats were anesthetized with 4% isoflurane for 15 minutes until death. The ACC from each group were freshly separated from brain tissues, stored in liquid nitrogen, and then dispatched to Majorbio (Shanghai, China) for transcriptional sequencing.[43]

TRIzol was used to extract total RNA, and we subsequently examined RNA integrity, concentration, and purity. cDNA was generated in the samples that satisfied the library formation requirements. The subsequent steps involved purifying the double-stranded cDNA, repairing the ends, and ligating sequencing adapters. After the library construction was completed, qPCR was used to detect the library concentration and ensure its quality. After filtering out low-quality data by FastQC program, the high-quality data obtained was aligned to the rat reference genome using HISAT2 software (NCBI assembly Rnor_6.0). Subsequently, HTSeq was used to count per-gene Read Counts, and FPKM normalization was applied for cross-gene expression comparison, while retaining raw counts for differential analysis. Differential expression was modeled with DESeq2/edgeR, computing fold changes and P values, and applying Benjamini–Hochberg FDR correction; DEGs were screened according to prespecified thresholds (fold change ≥2 or ≤0.05, FDR < 0.05, P < 0.05), with appropriate filtering of low abundance genes and, where necessary, batch effect correction and principal component analysis (PCA)/clustering to validate group separability. Results were visualized using volcano plots, MA plots, heatmaps, and PCA, and GO/KEGG functional enrichment analysis was performed to interpret major biological processes and pathways. Our attention was directed towards genes experiencing a decline in expression within the IBS group, followed by an elevation post-acupuncture therapy, alongside genes that demonstrated an increase in expression in the IBS group, subsequently undergoing a reduction after acupuncture.

GO annotation and enrichment analysis of differentially expressed genes (DEGs) in the ACC were performed using DAVID and GOseq, with GOseq applied to correct transcript-length dependent enrichment bias; GO terms were classified into biological process (BP), molecular function (MF), and cellular component (CC), and raw P values were subjected to Benjamini-Hochberg multiple-testing correction to obtain FDR. Using the KEGG annotation framework, DEGs in the ACC were mapped to the KEGG database for pathway annotation and enrichment to identify phenotype-relevant metabolic and signaling pathways; significant pathways were defined as those meeting adjusted P < 0.05 and FDR < 0.05. Candidate genes were selected based on expression abundance, magnitude of differential regulation (up or down regulation), and statistical significance; pyruvate dehydrogenase kinase 4 (PDK4), glutamate metabotropic receptor 2 (GRM2),and serum/glucocorticoid-regulated kinase 1 (SGK1) were prioritized and validated by quantitative real-time PCR (qRT-PCR), with ≥3 biological and technical replicates, and including melt-curve analysis and negative controls to ensure robustness and reproducibility.

Statistical Analysis

The statistical analysis of experimental data was performed using SPSS 26.0 software. For normally distributed data, one-way ANOVA was applied, with results expressed as mean ± standard error ($\bar{x} \pm$ SEM). If homogeneity of variance was satisfied, inter-group comparisons were conducted using the LSD method; otherwise, Dunnett's T3 method was employed. Non-normally distributed data were analyzed using the Kruskal–Wallis *H*-test, with results presented as median [M (Q25, Q75)] in quartile format. Statistical significance was determined using a threshold of P<0.05.

Results

EA Ameliorated the IBS Behavior

The findings revealed that the pain threshold for visceral sensations in the model group was considerably reduced compared to the control group (P<0.001), as illustrated in Figure 2A. When compared to the Control group, rats in the IBS group exhibited heightened AWR scores at pressure levels of 20 mmHg (P<0.01), 40 mmHg (P<0.001), and 60 mmHg (P<0.01). Subsequent to EA therapy at ST25, the AWR scores in the ST25 group were substantially lower than those in the IBS group at 20 mmHg (P<0.01), 40 mmHg (P<0.05), and 60 mmHg (P<0.05). Notably, the AWR scores in the ST36 group decreased significantly after EA therapy at the same pressure levels (20 mmHg, P<0.01; 40 mmHg, P<0.01; 60 mmHg, P<0.01) (Figure 2B).

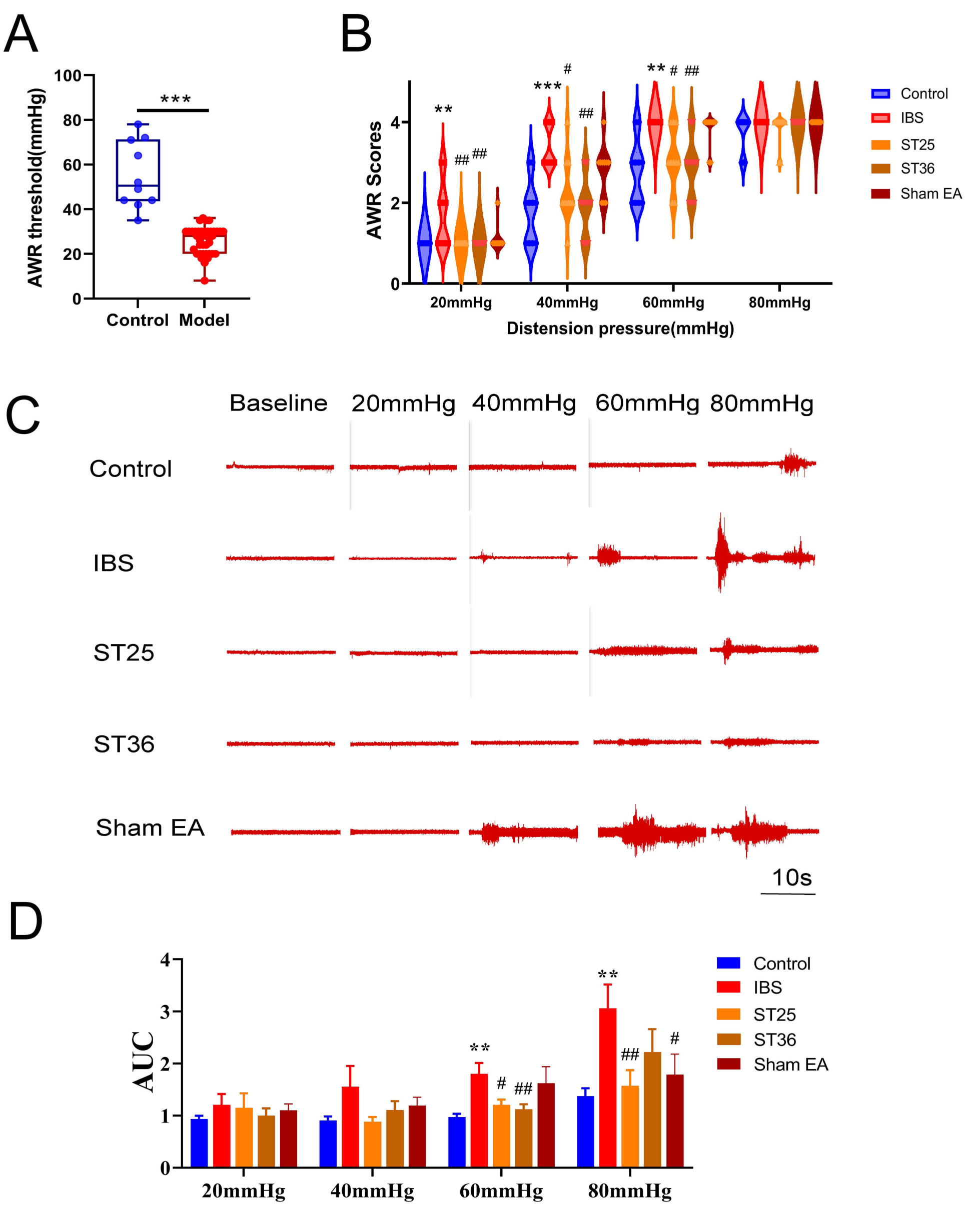

Figure 2 Assessing the effects of electroacupuncture at ST25 and ST36 in IBS rats. (**A**) Visceral pain threshold pressure measured via the AWR test, comparing the Model group (n=40) with the Control group (n=10). (**B**) AWR scores recorded at graded pressures of 20, 40, 60, and 80 mmHg (n=10). (**C**) Representative EMG traces under different pressures (20, 40, 60, and 80 mmHg). (**D**) AUC of EMG activity in response to graded distension pressures (n=5). Data are expressed as the mean ± standard error, $^{**}p < 0.01$ compared with the Control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the IBS group.

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EMG assessments were conducted in response to varying intensities of CRD, with concurrent scoring of AWR responses. Representative EMG traces are depicted in Figure 2C. The EMG AUC was larger in the IBS group compared to the Control group at 60 mmHg (P<0.01) and 80 mmHg (P<0.01). Following EA at ST25, the EMG AUC in the ST25 group was notably smaller than that in the IBS group at 60 mmHg (P<0.05) and 80 mmHg (P<0.01). Similarly, after EA therapy at ST36, the EMG AUC in the ST36 group was significantly smaller than that in the IBS group at 60 mmHg (P<0.01) (Figure 2D). The AWR scores and EMG AUC of the Sham EA group showed no statistically significant difference compared to the IBS group, indicating that Sham EA cannot treat IBS visceral pain.

Typical trials of EPM are shown in Figure 3A–E. Compared with those in the Control group, the percentage of the number of times the rats entered the open arms (OE%) and the percentage of the time the rats stayed in the open arms (OT%) and entered the arms (TE) in the IBS group were significantly lower (OE%, P<0.05; OT%, P<0.01), and the rats exhibited reduced activity, decreased curiosity about the new environment, and anxiety symptoms. Compared with those in the IBS group, the OE%, OT% and TE in the ST25 and ST36 groups were greater (ST25: OT%, P<0.05. ST36: OE%, P<0.05; OT%, P<0.05), and the anxiety behaviors of the rats were significantly improved. Sham EA cannot alleviate the anxiety of rats. The difference between the two EA groups was not statistically significant (Figure 3F–H).

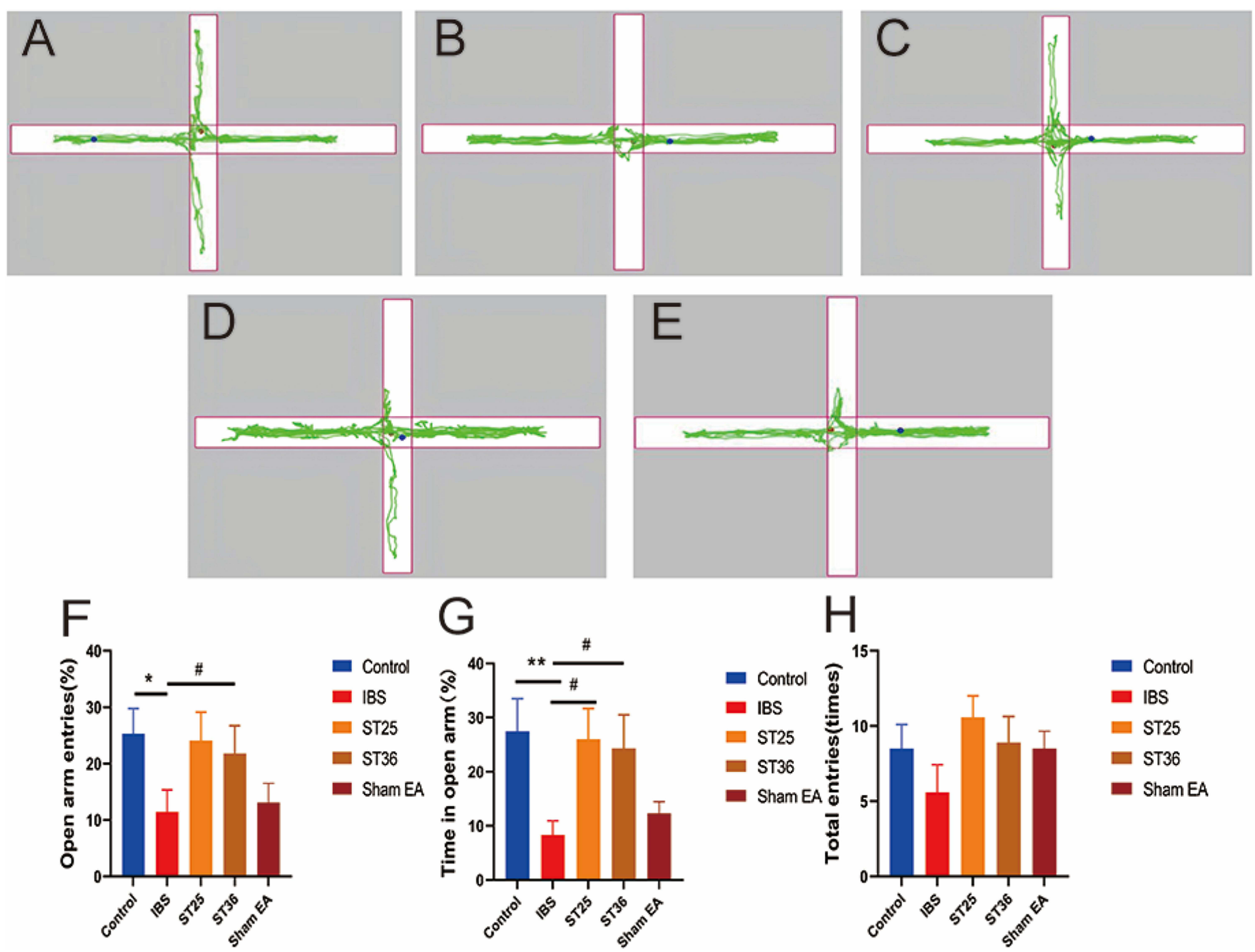

Figure 3 Compare anxiety-like behavior of rats across different groups in the elevated plus-maze. (**A**) Typical trajectory of the Control group. (**B**) Typical trajectory of the IBS group. (**C**) Typical trajectory of the ST25 group. (**D**) Typical trajectory of the ST36 group. (**E**) Typical trajectory of the Sham EA group. (**F**) Open arm entries (n=10). (**G**) Time spent in the open arms (n=10). (**H**) Total entries (n=10). Data are expressed as the mean ± standard error, *p < 0.05, **p < 0.01, compared with the Control group. #p < 0.05, compared with the IBS group.

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Identification of DEGs by EA Treatment in a Rat Model

The preceding experiments demonstrated that EA at ST25 and ST36 markedly improved visceral sensitivity and negative emotions in IBS rats. Accordingly, we conducted a transcriptome analysis of the ACC, which is intimately associated with visceral pain, exclusively in the Control, IBS, ST25 and ST36 groups.

To elucidate the potential correlation between the ACC transcriptome and IBS, we examined the mRNA expression of the rat ACC and performed unsupervised cluster analysis (Figure 4A–E). Figure 4B allows us to compare the variations in gene expression between the Control, IBS, ST25, and ST36 groups. The upregulated and downregulated genes from the ST25, ST36, IBS, and Control groups were used to produce heatmaps (Figure 4C). The relative expression level of genes in various groups is reflected in the log10 (TPM) value. As illustrated in Figures 4A and D, a total of 191 genes were identified as altered between the Control and IBS groups. Among these genes, 93 exhibited increased expression after IBS modeling, whereas 98 presented decreased expression. Following EA treatment at the ST25 point, 259 genes were differentially expressed, with 106 downregulated genes and 153 upregulated genes. A total of 374 genes were differentially expressed in IBS vs ST36 following EA treatment at ST36, with 295 downregulated genes and 79

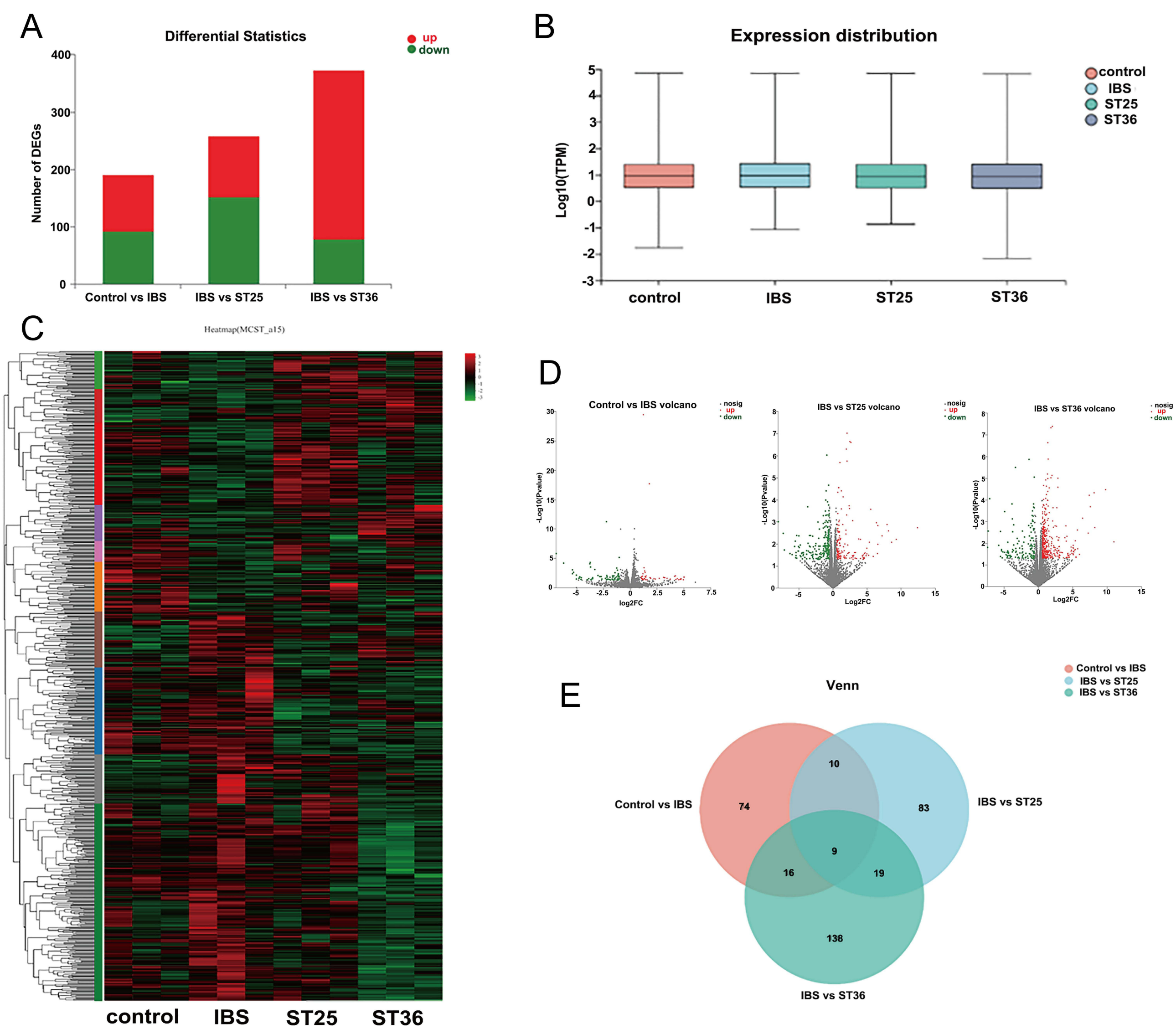

Figure 4 DEGs in the rat ACC across experimental groups (n=3). (**A**) Bar chart quantifying the counts of upregulated and downregulated DEGs in each group. (**B**) Distribution of gene expression levels in the Control, IBS, ST25, and ST36 groups. (**C**) Heatmap displaying DEG patterns across Control, IBS, ST25, and ST36. (**D**) Volcano plot visualizing significant DEGs across the groups. (**E**) Venn diagram highlighting shared and unique genes among the groups.

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upregulated genes. Sixteen genes, including Wnt6, PDK4, and GRM2, which are linked to stress and glucose metabolism, presented upregulated expression during IBS modeling and decreased expression following acupuncture treatment at ST25, as shown in Figure 4E. Eighteen genes, including Wnt6, PDK4, and SGK1, which are involved in the regulation of glucocorticoids and stress, exhibited upregulated expression after IBS modeling and downregulated expression after acupuncture treatment at ST36. All three groups had nine overlapping genes in total.

Based on differentially expressed genes (DEGs) in the rat ACC, we inferred biological roles via Gene Ontology (GO) enrichment covering biological processes (BP), cellular components (CC), and molecular functions (MF); the results showed that EA modulated DEGs were primarily enriched in cell parts, binding, cellular processes, biological regulation, and metabolic processes (Figure 5A), and across the four groups, the main functions centered on ion binding and response to stimulus (Figure 5B). To further delineate pathways associated with EA-target genes, we performed KEGG pathway enrichment, prioritizing metabolism-related categories; in line with the experimental design, comparisons were made relative to the IBS model group: in the ST25 group, DEGs were significantly enriched in five metabolic pathways metabolism of other amino acids, lipid metabolism, glycan biosynthesis and metabolism, carbohydrate metabolism, and amino acid metabolism (Figure 5C); in the ST36 group, DEGs were mainly enriched in three pathways nucleotide metabolism, metabolism of cofactors and vitamins, and carbohydrate metabolism (Figure 5D).

The comprehensive results of the protein–protein interaction (PPI) network analysis are detailed in Figure 6A and B. To further validate these findings, we selected three key genes, namely, GRM2, PDK4 and SGK1, for PCR analysis, as shown in Figure 6C–E, respectively. As clearly shown in Figure 6C, the mRNA expression level of GRM2 was significantly greater in the IBS group than in the Control group (Control group: 1.08 ± 0.27, IBS group: 2.84 ± 0.12, $P < 0.001$). In addition, we observed a marked reversal of GRM2 mRNA expression by EA at ST25 treatment (ST25 group: 1.3 ± 0.34, $P < 0.01$), suggesting a potential therapeutic effect of EA at ST25 in modulating GRM2 expression. Similarly, PDK4 mRNA expression was upregulated in response to IBS treatment, as shown by the comparison between the Control and IBS groups (Control group: 1.08 ± 0.21, IBS group: 2.57 ± 0.31, $P = 0.008$). The application of EA

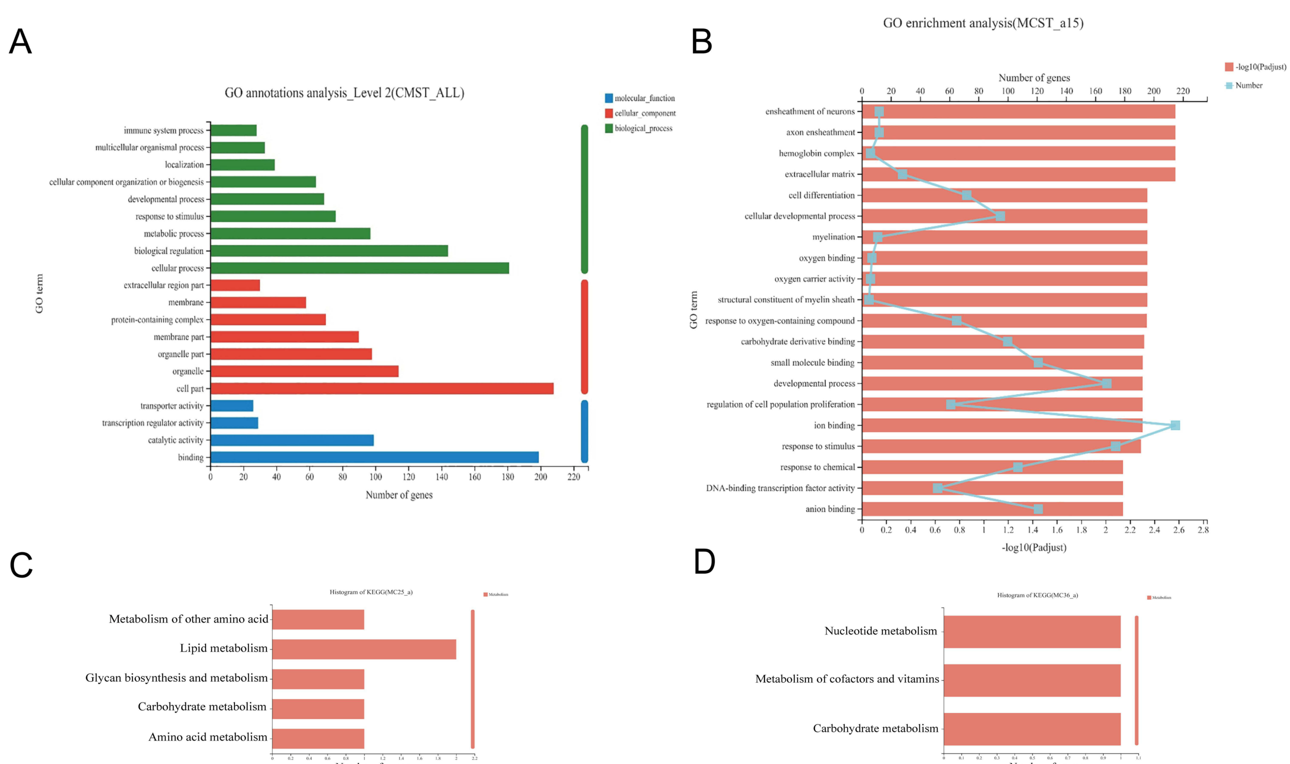

Figure 5 GO and KEGG functional annotations of DEGs in the ACC (n=3). (**A**) GO enrichment analysis (including BP, biological process; CC, cellular component; MF, molecular function). (**B**) GO annotation analysis of DEGs in the ACC. (**C**) KEGG analysis results for DEGs in the ACC after EA at ST25. (**D**) KEGG analysis results for DEGs in the ACC after EA at ST36.

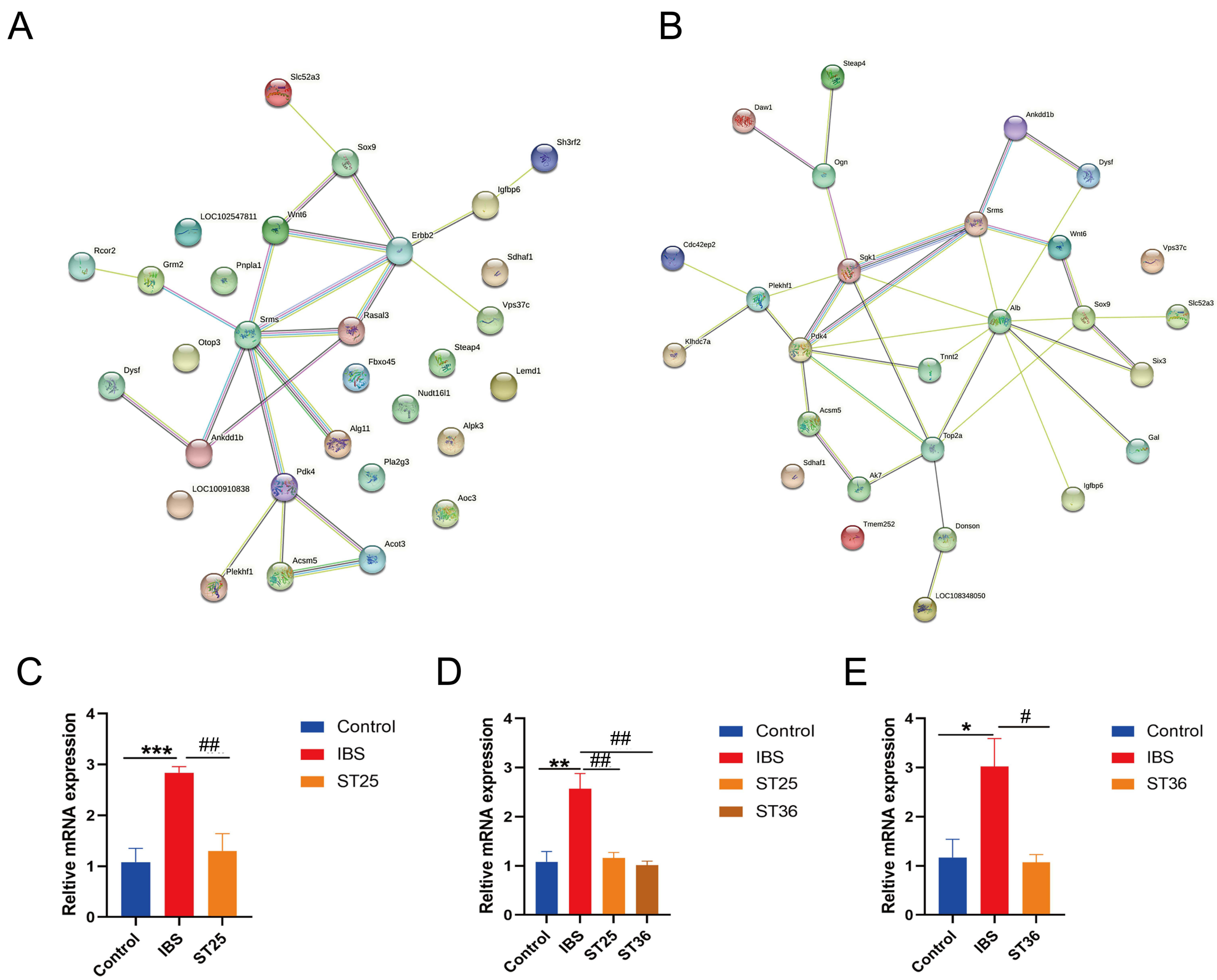

Figure 6 PPI analysis of ACC DEGs and hub genes validated by qPCR (n=3). (**A**) PPI network of ACC DEGs after EA at ST25. (**B**) PPI network of ACC DEGs after EA at ST36. (**C**) Changes of GRM2 expression in ACC. (**D**) Changes of PDK4 expression in ACC. (**E**) Changes of SGK1 expression in ACC. Data are expressed as the mean ± standard error, *p < 0.05, **p < 0.01, ***p < 0.001 compared with the Control group. #p < 0.05, ##p < 0.01, compared with the IBS group.

resulted in a significant downregulation of PDK4 mRNA expression, with both ST25 and ST36 showing efficacy (ST25 group: 1.16 ± 0.11, P = 0.005; ST36 group: 1.01 ± 0.09, P = 0.003). These findings further highlight the regulatory potential of EA in modulating gene expression associated with IBS. Furthermore, SGK1 mRNA expression was also greater in the IBS group than in the Control group (Control group: 1.17 ± 0.37, IBS group: 3.02 ± 0.57, P = 0.034). Treatment with EA at ST36 resulted in a significant reversal of SGK1 mRNA expression (ST36 group: 1.07 ± 0.16, P = 0.037), indicating a specific effect of EA at ST36 on SGK1 regulation. EA normalized dysregulated mRNA expression, supporting its potential as a transcriptome-level therapy for IBS.

Discussion

Our research indicated that EA applied at ST36 and ST25 led to an improvement in IBS symptoms, as evidenced by a reduction in AWR scores, an increase in the duration spent in the open arms of the elevated plus-maze test. In rats with IBS, EA at ST25 and ST36 dramatically increased the colonic pain threshold and lowered anxiety. Our transcriptomic analysis addresses how EA at ST25 and ST36 modulates gene expression to relieve symptoms and clarifies the differential effects of these two acupoints. The PCR validation of GRM2, PDK4, and SGK1 confirms the accuracy of our transcriptome data and highlights these genes as key players in the therapeutic pathway.

The ACC is crucial for the development of negative emotions and visceral pain sensitivity in the etiology of IBS.[25,44,45] By detecting genetic changes in the ACC, we can speculate on the signaling molecules or neurotransmitters that may be produced in the ACC, and furthermore, we can explore the pathways that are activated in the next step.[46] EA can change the expression of mRNAs or miRNAs in rat models.[32,33,47] The core of the neurogenetic mechanism of EA appears to be the targeted modulation of stress- and metabolism-related genes whose expression is altered by chronic visceral hypersensitivity.

EA at the ST25 acupoint demonstrated a specific and potent effect on downregulating GRM2 expression. The GRM2 is a possible candidate gene for Parkinson's syndrome, depression, anxiety, schizophrenia and other psychiatric diseases.[48,49] Research indicates that repeated stress significantly elevates GRM2 expression in the brains of animals.[50] The association between the GRM2 gene and anxiety is further supported by studies showing that GRM2-knockout mice do not exhibit cognitive impairments or increased anxiety levels under pressure in behavioral trials.[51] Collectively, this evidence directs our attention to the significant link between genetic alterations in the ACC, stress generation, and anxiety during the pathogenesis of IBS. Our study demonstrates that EA treatment can restore the elevated GRM2 expression in the ACC of IBS rats back to baseline levels. Changes in GRM2 gene levels may affect changes in group II metabotropic glutamate receptors,[52] which can regulate the release of neurotransmitters, neuronal growth and the regulation of emotions and play pivotal roles in stress-induced analgesia. EA may affect glutamate receptors and central sensitization through multiple indirect molecular pathways.[18,53] It may also directly alleviate stress-elevated pain thresholds and associated pain via neural circuits.[54]

In contrast, EA at the ST36 acupoint uniquely reversed the upregulation of SGK1. SGK1 is a well-established downstream target of glucocorticoids, involved in the central stress response system and the regulation of ion channel, and plays a pivotal role in the pathogenesis of various nervous system diseases and inflammatory disorders, including inflammatory bowel disease, Parkinson's disease, and major depressive disorders.[55,56] The upregulation of SGK1 in the IBS group is a clear molecular signature of chronic stress.[57,58] In addition to regulating energy metabolism in the brain,[59] recent in-depth research has revealed that SGK1 in the central nervous system may contribute primarily to the onset and progression of neurological diseases by promoting the accumulation of excitatory neurotransmitters.[60] The specific downregulation of SGK1 in the ACC induced by electroacupuncture at ST36 suggests its modulation of neuroendocrine. In IBS, chronic stress causes Hypothalamic-Pituitary-Adrenal (HPA) axis hyperactivity, elevating glucocorticoids and consequently SGK1. Electroacupuncture at ST36 likely normalizes the HPA axis.[61,62] This leads to decreased SGK1 expression, which suppresses ion channel activity and restores synaptic plasticity, thereby reducing ACC neuronal hyperexcitability. This cascade effectively dampens central sensitization, alleviating both the visceral pain and the stress components of IBS. SGK1 also negatively regulates the expression of both brain-derived neurotrophic factor (BDNF)[63] and vascular endothelial growth factor (VEGF).[64] Acupuncture may influence BDNF and VEGF by downregulating SGK1 in the ACC, which attenuates central sensitization and relieves symptoms associated with irritable bowel syndrome. Previous research has supported a portion of the theory.[65,66] By mitigating the central effects of stress hormones, EA at ST36 likely alleviates the stress-induced exacerbation of visceral hypersensitivity, representing a more "top-down" regulatory mechanism. This aligns with the classical understanding of ST36 as a key acupoint for systemic regulation and stress reduction.[67]

Despite their differences, both ST25 and ST36 treatments led to the significant downregulation of PDK. PDK4 is related to glucose and lipid homeostasis and is involved in insulin resistance.[68] In IBS rats, the sympathetic nervous system is hyperexcited to release catecholamines, and PDK4 and SGK1 gene expression levels are increased in the ACC. The two genes may synergistically switch the energy source from glucose to fatty acids to maintain blood glucose levels to increase metabolism in the ACC. Reduced aerobic glycolysis can reverse the course of bowel diseases when PDK4 gene expression is decreased.[69,70] EA at two acupoints seem to restore cellular energy metabolism[71] within the ACC, which is likely disrupted by the chronic stress of IBS. By normalizing glucose utilization and metabolic homeostasis,[72] both treatments may enhance neuronal resilience and function,[71] thereby contributing to the overall reduction in visceral pain and negative emotional states. The fact that the genes, including Wnt6 and PDK4, were commonly regulated by both treatments underscores this shared foundational mechanism of metabolic and stress-response correction.

In conclusion, our findings suggest that EA at ST25 and ST36 modulates gene expression in the ACC of IBS rats through distinct but overlapping neurogenetic mechanisms. ST25 appears to primarily target local synaptic transmission and pain processing circuit, while ST36 primarily regulates the central stress response. Both converge on the crucial pathway of improving cellular energy metabolism. Both ST25 and ST36 are effective, they likely initiate their therapeutic effects through distinct neurobiological pathways. This is supported by prior work from Ma's team,[21] which found that low-intensity EA at ST36 activates the vagus-adrenal axis, whereas EA at ST25 does not, indicating different initial modes of action. These distinct molecular fingerprints provide a scientific rationale for the principle of acupoint specificity in traditional Chinese medicine and offer a deeper understanding of how acupuncture can be used to treat the complex interplay of visceral sensitivity and stress in IBS.

This study has several limitations that should be considered when interpreting the results. First, the sample size for RNA-sequencing analysis was relatively small. While this is not uncommon in exploratory transcriptomic studies due to cost and analytical complexity, it inherently limits the statistical power to detect subtle gene expression changes and may affect the generalizability of the molecular findings. Future studies with larger cohorts are warranted to validate the identified signaling pathways and candidate biomarkers. Second, visceral pain sensitivity was assessed using the CRD test under light anesthesia. Although this is a standard methodological approach to ensure stability and minimize stress-induced confounders during measurement, anesthesia itself may modulate central and peripheral neural activity, potentially influencing nociceptive responses. Despite these limitations, the consistency observed across behavioral, molecular, and histological endpoints strengthens the overall validity of our conclusions.

Conclusion

To our knowledge, this is the first study to investigate the relationship between ACC RNA expression levels and the efficacy of acupuncture in treating IBS. In conclusion, our study revealed that EA treatment at ST25 and ST36 reduced the symptoms of IBS. The genes GRM2, PDK4 and SGK1 were found to be upregulated in IBS patients according to real-time PCR. While the SGK1 gene was downregulated by EA at ST36, the expression of the GRM2 gene in IBS patients tended to return to normal after EA therapy at ST25. The PDK4 gene tended to return to normal following EA at ST25 and EA at ST36, supporting both a separate mechanism of action following EA at the two acupoints and a therapeutic mechanism for IBS.

Abbreviations

IBS, irritable bowel syndrome; ACC, anterior cingulate cortex; AWR, abdominal withdrawal reflex; TCM, traditional Chinese medicine; WAS, water avoidance stress; CRD, colorectal distension; EA, electroacupuncture; OE, open arm entries; OT, open arm time; CT, close arm time; CE, close arm entries; CNS, central nervous system; EMG, electromyographic; AUC, the area under the curve; ECG, electrocardiogram; FPKM, fragments per kilobase of transcript per million mapped reads; DEGs, differentially expressed genes; FDR, error finding rate; BNDF, brain-derived neurotrophic factor; VEGF, vascular endothelial growth factor; PDK4, the pyruvate dehydrogenase kinase 4; SGK1, the serum/glucocorticoid-regulated kinase 1; GRM2, the subtype 2 metabotropic glutamate receptor gene.

Data Sharing Statement

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

Ethics Approval and Informed Consent

All animal procedures in this study strictly complied with the World Health Organization's International Guiding Principles for Biomedical Research Involving Animals. The animal experimental protocol was approved by Ethics Committee of the Center for Scientific Research with Animal Models at Shandong University of Traditional Chinese Medicine (Certificate No. SDUTCM20220827001).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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