Original Article
Endogenous neuroprotective mechanism of ATP2B1 in transcriptional regulation of ischemic preconditioning

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Abstract: Ischemic stroke is the main cause of disability and mortality in the world. Clinical studies have shown that patients who undergo mild transient ischemic attack (TIA) before more severe ischemic stroke have lower clinical severity of stroke and better functional prognosis. This phenomenon is called ischemic preconditioning (IPC). IPC is a powerful intrinsic protection of the brain against ischemic injury, but the underlying mechanism of IPC-mediated endogenous protection of the brain is not clear. Methods: Using transcriptome method, we sequenced the serum of 3 stroke patients with progenitor TIA and 3 stroke patients without prodromal TIA. We explored the expression profiles of miRNAs and mRNAs in response to IPC, and predicted the regulatory pathway of IPC related genes and their expression in cerebral neurons. The methylation consistent expression of IPC-related gene ATP2B1 in blood and brain and alternative polyadenylate (APA) analysis were used to identify the pathway and molecular mechanism of endogenous neuroprotection of IPC. Results: We found that the brain protective effect of IPC was related to platelet homeostasis and Ca^{2+} concentration. IPC-related gene ATP2B1 was highly expressed in γ-aminobutyric acid (GABA)-containing neurons in the brain. From the mechanism, we speculated that ATP2B1 was representative of the same methylation in blood and brain and was affected by alternative polyadenylation. Conclusion: We speculate that IPC can induce alternative polyadenylation of ATP2B1 and trigger the mechanism of brain endogenous neuroprotection by regulating the decrease of Ca^{2+} concentration in platelet homeostasis pathway and the activation of GABAB receptor.

Keywords: Ischemic preconditioning, neuroprotection, ATP2B1, platelet homeostasis, alternative polyadenylation

Introduction

Ischemic stroke causes a long-term interruption of blood supply to the brain and causes tissue damage. Endogenous neuroprotection is the strongest protective response of the brain to self-injury. At present, clinical studies have shown that patients suffering from mild transient ischemic attack (TIA) before more severe ischemic stroke have a lower degree of damage and a more positive functional prognosis after stroke [1]. After experiencing a short-term sub-threshold ischemic injury, it induces an endogenous protective response in the brain and reduces the brain damage caused by the more serious ischemic injury that occurs later. It is called ischemic preconditioning (IPC), and has been fully demonstrated in heart and brain models [2]. Therefore, it is particularly important to focus on exploring the neuroprotective mechanism of cerebral ischemic preconditioning.

The IPC phenomenon was first discovered in 1986 [3]. Before the circumflex coronary artery is occluded for 40 minutes, a 5-minute transient ischemia experiment can significantly reduce the myocardial infarction area by about 75%. Then further study found that transient ischemia attack has an overall protective effect on arrhythmias and cardiovascular dysfunction, not just the size of infarction [4]. Clinical studies have also proved that IPC can reduce ischemic injury in the brain. Previous ischemic stroke with ipsilateral TIA had better results than patients without TIA [5-10]. Therefore, it provides clear evidence that IPC can effectively reduce human cerebral ischemic injury.

In recent years, a large amount of evidence shows that the brain can activate a strong endogenous protective mechanism against ischemia after transient ischemia attack and other adverse events [11-13], which can improve the prognosis in the face of future injury

caused by fatal ischemia. It is reported that brain IPC is involved in different mechanisms such as the release and inhibition of glutamate, or excitotoxicity, the expression of related apoptotic genes and the initiation of neuroprotective responses through post-translational modification of proteins [14, 15].

However, so far, the molecular mechanism of IPC-mediated neuroprotective effect has not been elucidated [13]. We hope to find the mechanism of improving stroke prognosis by studying TIA-mediated ischemic preconditioning and provide more opportunities for future studies to be included in bedside clinical trials. Since there is almost no data on the molecular changes of IPC in the human brain, in this study, we first analyzed the transcriptome expression profile of patients with IPC, and then we carried out the visual analysis of differentially expressed RNAs, the construction of miRNA-mRNA interaction, and the exploration of potential regulatory pathways. And to explore the endogenous neuroprotective mechanism of ATP2B1 in the transcriptional regulation of ischemic preconditioning by analyzing the methylation consistency of blood and brain DNA of ischemic preconditioning gene ATP2B1 and the event of alternative polyadenylate (APA).

Materials and methods

Patient selection and sample collection selected

We selected patients with anterior circulation cerebral infarction who were hospitalized in the Department of Neurology of the first affiliated Hospital of Guangxi Medical University as the object of study. The inclusion criteria were as follows: experimental group (ischemic stroke patients with prodromal TIA): 1, ipsilateral TIA attacks 2-3 times, lasting 10-20 minutes each time, TIA occurred within one week before cerebral infarction; 2. Head CT or MRI confirmed anterior circulation infarction with related neurological symptoms; 3. TOAST classification was atherosclerosis of large arteries and occlusion of small arteries; 4. Patients with acute cerebral infarction for the first time. Control group (ischemic stroke patients without progenitor TIA): 1. There was no TIA before the disease. 2. Anterior circulation infarction was confirmed by head CT or MRI; 3. TOAST classification was classified as atherosclerosis of large arteries and occlusion of small arteries; 4.

Patients with acute cerebral infarction for the first time. 5. patients aged between 60 ± 15 years old. Elimination criteria: 1. Recurrent cerebral infarction (previous focus of old cerebral infarction); 2. Incomplete image data such as head CT or MRI; 3. Patients with malignant tumor; recurrent stroke; hematological disease; renal or liver failure; mental disorder; history of severe dementia. 4. Patients who do not agree to join the group. According to the diagnostic criteria established by the Cerebrovascular Disease Association, 3 patients of ischemic stroke with prodromal TIA (treatment group) were compared with 3 patients without prodromal TIA (control group). All patients were admitted to hospital within 24 hours after onset, and peripheral blood samples were drawn from patients on the first day of admission for RNA preparation. The research scheme was approved by the Ethics Committee of the first affiliated Hospital of Guangxi Medical University (2020-KY-E-109), and the project process was carried out in accordance with the relevant guidelines.

RNA sequencing and expression profile analysis

Whole blood RNA was prepared by Trizol reagent method (experimental group = 3; control group = 3). The concentration of RNA was detected by NanoDrop, and the quality of all samples met the testing requirements for further sequencing. Before the formal chip hybridization experiment, we used Agilent 2100 bioanalyzer for quality control, and its RIN integrity values ranged from 6.5 to 7.6. We use non-contact ultra-micro sampling core technology and square array gene chip high-speed mass production technology (GUANG ZHOU RIBOBIOCO, LTD) to synthesize microarray (Rio Bo) for miRNA analysis. Secondly, we use LOWESS filtering to normalize the signal strength and subtract the background value to eliminate the systematic error caused by inconsistent sample content, fluorescence label deviation and other factors. We uploaded the data to the FigShare website at https://doi.org/10.6084/m9.figshare.13291868. In the process of differential gene and miRNA screening, the signal ratio of the two samples was obtained by calculating the Fold Change value and converting its expression value into a logarithm based on $\log_2|\text{Fold Change}|$. At the same time, the t-test statistical method was used to calculate the P value. We filter according to the criteria of sig-

nal ratio |log$_2$FC| > 1 and P < 0.05. We use TBtool software to construct differential expression RNAs visual heat map [16].

Construction of regulatory network

The DIANA-microT-CDS database is a comprehensive data concentration point that provides many sets of predicted and experimentally verified target gene interactions [17]. We submit the differentially expressed miRNAs (DEMs) screened by sequencing to the DIANA database to predict the target. Then, we combine the target genes predicted by DEMs with the sequenced mRNAs (DEGs) to obtain the intersection of mRNAs. When the target genes of DEMs share the same mRNA as DEGs, they may exist in similar regulatory pathways. Generally speaking, miRNA is negatively correlated with the expression of target genes, so the data of up-regulated/down-regulated miRNA expressed in sequencing results corresponding to down-regulated/up-regulated mRNA are screened. Cytoscape3.7.0 is used to visualize the miRNA-mRNA regulatory network, describing the interaction between miRNA and potential mRNA in ischemic preconditioning.

Pathway enrichment analysis

NetworkAnalyst database is used to analyze the pathway enrichment of reactome pathway in DEGs to identify the important pathways involved in genes [18]. Reactome is a biological functional relationship database written, created, verified and annotated by domain experts [19]. It is a manual and peer-reviewed pathway database, which represents the consensus in this field. NetworkAnalyst database can provide enrichment and analysis of reactome database. It can expand our in-depth research and exploration of the disease process, and reveal the molecular changes and the regulatory mechanism of the pathway.

Analysis of the distribution of excitatory and inhibitory neurons

In brain regions sensitive to ischemic injury, the regulatory interaction of excitatory neurotransmitter glutamate and the release of inhibitory GABA are important events that determine the fate of apoptosis [20]. In order to explore the expression of key genes involved in ischemic preconditioning pathway in excitatory and inhibitory neurons in transcriptology, we used

Allen human brain snRNA-seq database [21], where mononuclear RNA sequencing was used to analyze brain cell types and isolate a highly diverse group of excitatory and inhibitory neurons. A total of 45 inhibitory neurons expressing GABA-Ergic neurons, 24 excitatory neurons expressing glutamate and 6 non-neuronal types were included.

Analysis of the consistency of blood-brain expression of ischemic preconditioning gene

Because it is relatively difficult to obtain human brain tissue for experiments in clinic, many studies have used blood as a substitute for the brain in the study of neurobiological diseases, because of its accessibility, and may be directly related to the disease through a series of regulation such as post-transcriptional translation of the body. However, because of the specific nature of the tissue, in order to explore whether the CpG identified in these studies can replace the brain DNAm to provide information, we combined the "blood-brain epigenetic consistency" (BECon) database [22] to explore blood-based DNAm information in the human brain. At the same time, we found a database of matching DNA methylation data in blood and brain regions on Blood Brain DNA Methylation Comparison Tool [23]. For most genomes, blood-based EWAS can provide information about underlying pathological processes for diseases that target the brain.

APA analysis of ATP2B1

Alternative polyadenylated (APA) is the RNA processing mechanism of post-transcriptional 3'UTR. APA plays an important role in almost every aspect of gene expression, including splicing, mRNA transport, mRNA stability and protein translation [24]. Extensive APA will affect the regulation of post-transcriptional genes in mRNA translation and stability, and produce transcripts with the ability to change proteins and RNA coding. Therefore, we use APAatlas [25] database and APA analysis of genes by Dapars algorithm [26] to explore transcriptional regulatory events after ischemia.

Result

Identify DEMs and DEGs, to construct miRNA-mRNA interaction network

Ischemic preconditioning of the brain can make cells have a reactive protective effect, which
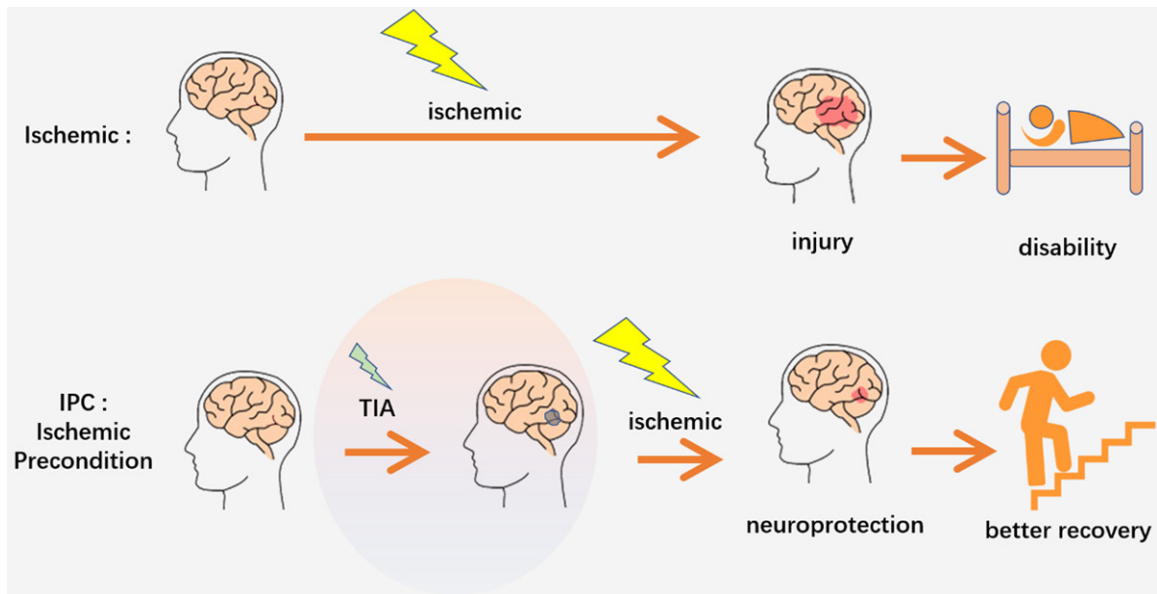
Figure 1. The pattern diagram of Ischemic preconditioning. After transient ischemic attack (TIA), the brain can activate a strong endogenous protective mechanism against ischemia, which can improve the prognosis in the face of more severe ischemic injury.

can reduce the brain damage caused by subsequent ischemic injury. IPC phenomenon helps to discover various neuroprotective mechanisms (**Figure 1**). Compared with the control group, 29 DEMs and 222 DEGs were identified in the experimental group (**Figure 2A**, **2B**). There were significant differences in the expression of miRNA and mRNA in the experimental group. It is suggested that IPC can cause extensive changes in the transcriptome of the body. In order to further understand the regulatory relationship between 29 important DEMs and 222 mRNAs, a miRNA-mRNA regulatory network is established (**Figure 2C**, **2D**). Using DIANA-microT-CDS to predict the target genes of DEMs, there were 11180 target genes, and 93 target genes intersected with DEGs. Furthermore, the up-regulated/down-regulated miRNA, corresponding to down-regulated/up-regulated mRNA, were selected to construct miRNA-mRNA interaction networks.

Pathway enrichment analysis

In order to understand the potential biological function of DEMs, the pathway enrichment analysis of DEGs was carried out by using the reactome database of NetworkAnalyst. Pathway analysis showed that DEGs could significantly enrich multiple pathways related to ischemic preconditioning, the most related pathways were platelet homeostasis (P = 0.00156),

others including activation of G protein gated potassium channel (P = 0.00232), inhibition of voltage gated Ca^{2+} channel through Gbeta/γ subunit (P = 0.00232), activation of GABAB receptor (P = 0.00504), neuronal system (P = 0.0415) and so on (**Figure 3**). As we all know, ischemic stroke is not only related to Ca^{2+} overload [27, 28], but also related to platelet homeostasis [29, 30], and is closely related to GABA [31, 32]. These pathways contain ischemia-related genes ATP2B1, PPP2CA, PDE11A, KCNJ5, KCNJ6, VAMP2, which are indirectly or directly involved in the mechanism of ischemic preconditioning. We select these genes for further analysis. Among them, ATP2B1 may play a role in maintaining intracellular Ca^{2+} homeostasis in platelet homeostasis pathway [33-35]. Platelets have complex regulatory mechanisms. There are more than six pathways of platelet homeostasis, Ca^{2+} which is the central and common second messengers downstream of most signaling pathways in platelets. Therefore, the regulation of Ca^{2+} signal may be an interesting target for maintaining platelet homeostasis.

Distribution of genes in excitatory and inhibitory neurons

We can see that the expression of ATP2B1 in GABAergic neurons and glutamatergic neurons is abnormal, which is obviously different from
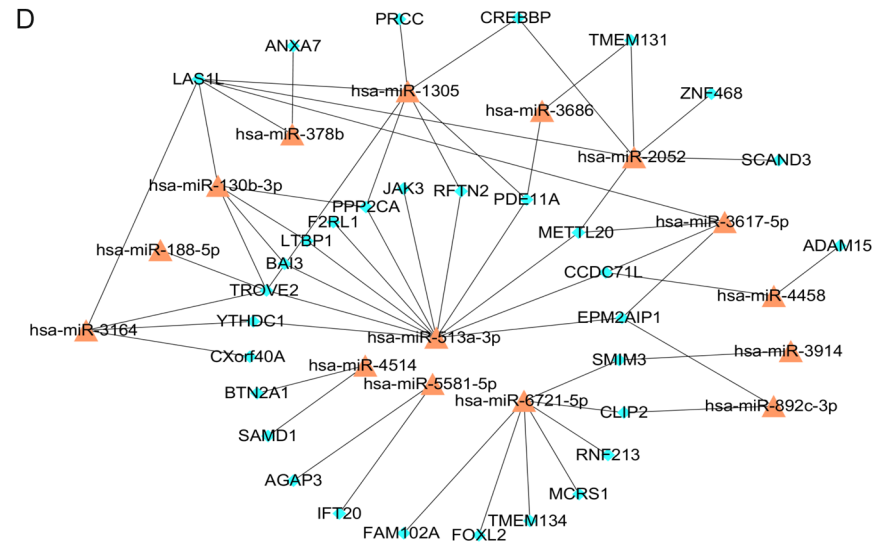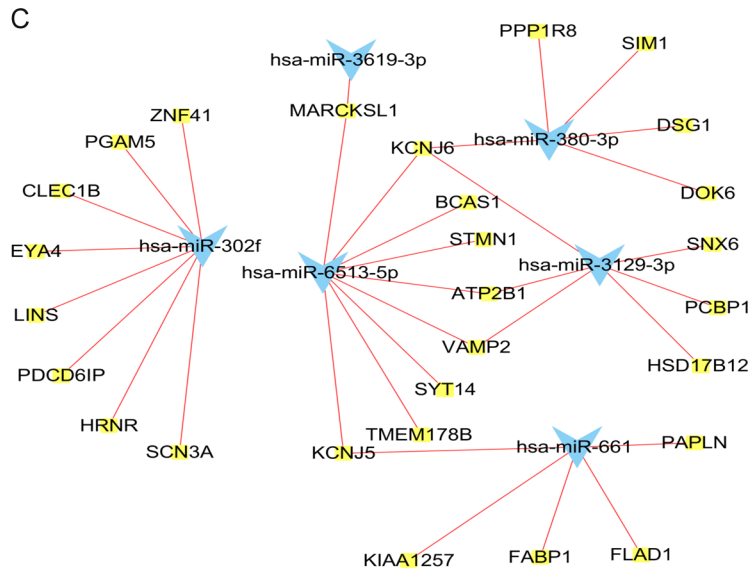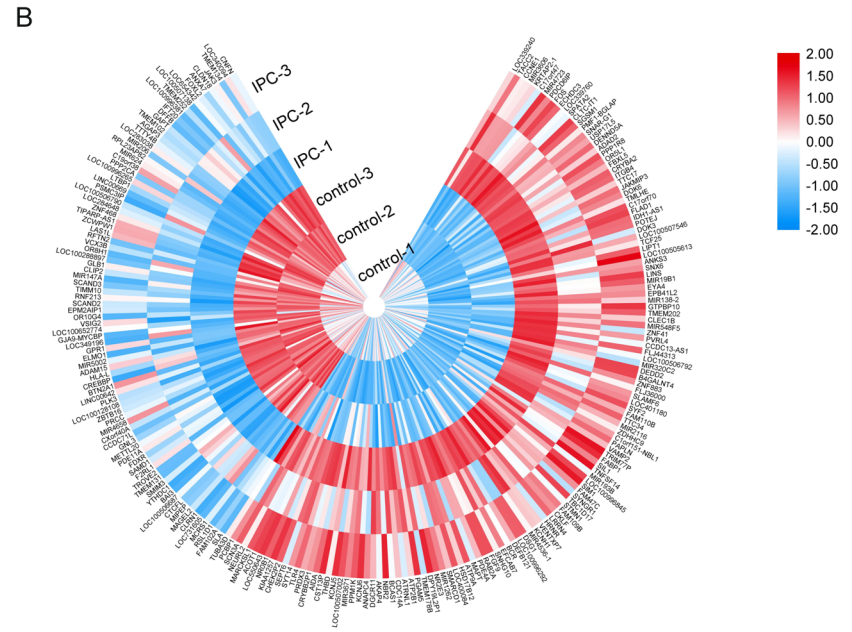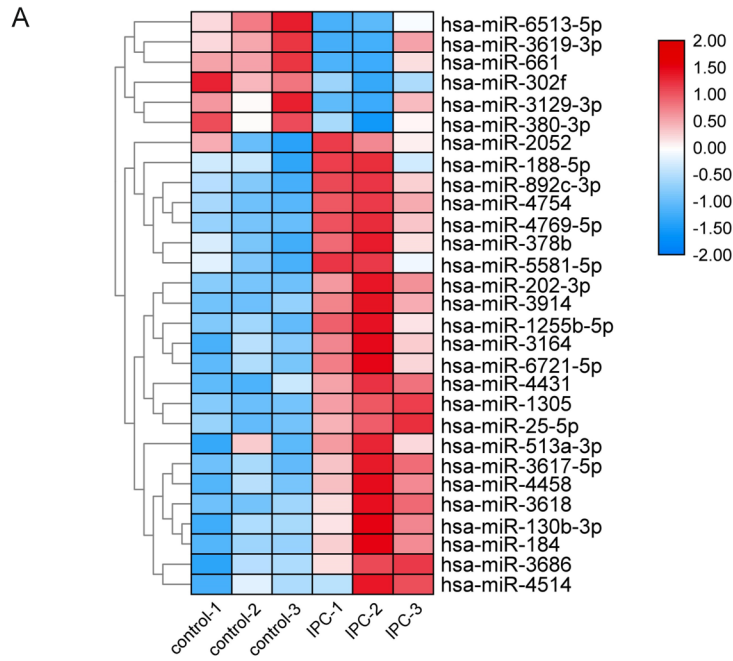
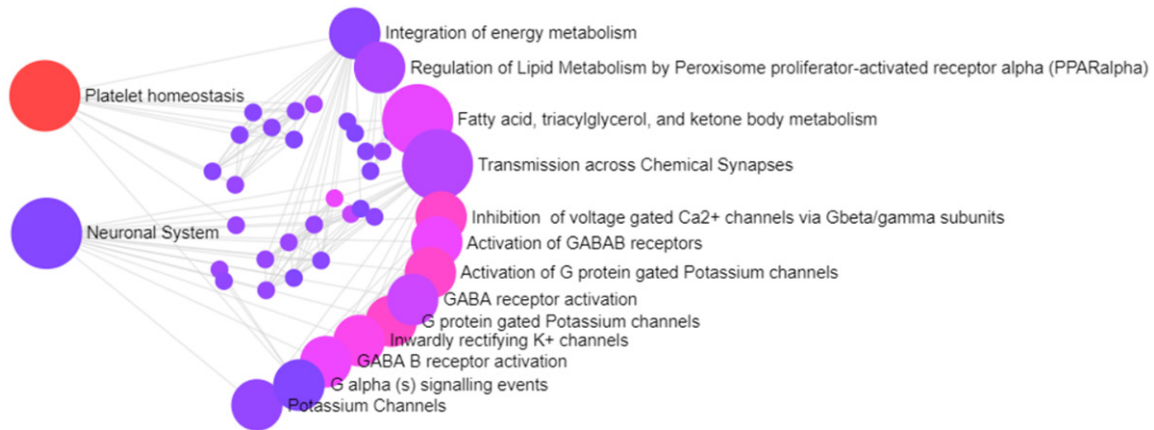# Neuroprotective mechanism of ATP2B1

Figure 3. Enrichment analysis of kegg pathway of ischemic preconditioning differentially expressed genes, which is mainly enriched in neuronal system, platelet homeostasis and GABA pathway. The view of path enrichment represents each path as a node, which is surrounded by concentric node rings. The node is scaled proportionally to the number of events it contains. An arc edge represents a partial relationship between a path and a sub path.

other genes (**Figure 4A**). At the same time, ATP2B1 is displayed visually in the cell cluster diagram (**Figure 4B**) and expression diagram (**Figure 4C**). Ischemic preconditioning can promote the release of GABA or the expression of its receptor [36]. However, the brain also has a coordinated and balanced protective mechanism. After hypoxic-ischemic injury, glutamate will also be over-released and its receptor will be over-stimulated, which will trigger postsynaptic depolarization and lead to a large influx of Ca^{2+} [37, 38], which can depolarize the neuronal membrane potential and inhibit the effect of GABA in an antagonistic way, so as to achieve the balance between the two and promote the survival of neurons. Similarly, some experiments have proved that ischemia leads to the release of neurotransmitter amino acids, which depends on the influx activation of Ca^{2+} [39], so ATP2B1 plays an important role in ischemic preconditioning as a stable regulator of cellular Ca^{2+} level.

Analysis of blood-brain consistency of ATP2B1

Before explaining the results based on blood samples, we examine the consistency of DNA methylation (DNAm) between the blood and the brain. We found that there were data on the

relationship between blood and brain in seven CpG, all of which showed a consistent CpG between blood and brain (**Figure 5A**, the red and blue lines score close to 1, and the trend is the same). At the same time, the degree of methylation in blood and brain was the same, and in the cg01156747 probe, ATP2B1 had the same correlation in the four brain regions (prefrontal cortex, entorhinal cortex, superior temporal gyrus and cerebellum) matched by DNA methylation in blood ($P < 0.05$) (**Figure 5B**). These results suggest the feasibility of using blood-based DNAm to identify biomarkers of disease phenotypes in the brain. Although there is tissue-specific DNAm, between blood and brain, the information of blood-brain consistency suggests that ATP2B1 is also equivalent in blood and can be used as a substitute for brain (**Figure 5C**).

APA analysis of ATP2B1

It is easy to stimulate the changes of transcriptional regulation in vivo after ischemia [40-43]. Post-transcriptional regulatory events are considered to regulate the core characteristics of mRNA transcripts to create an environment suitable for promoting the survival and recovery of brain nerve tissue, thus promoting the
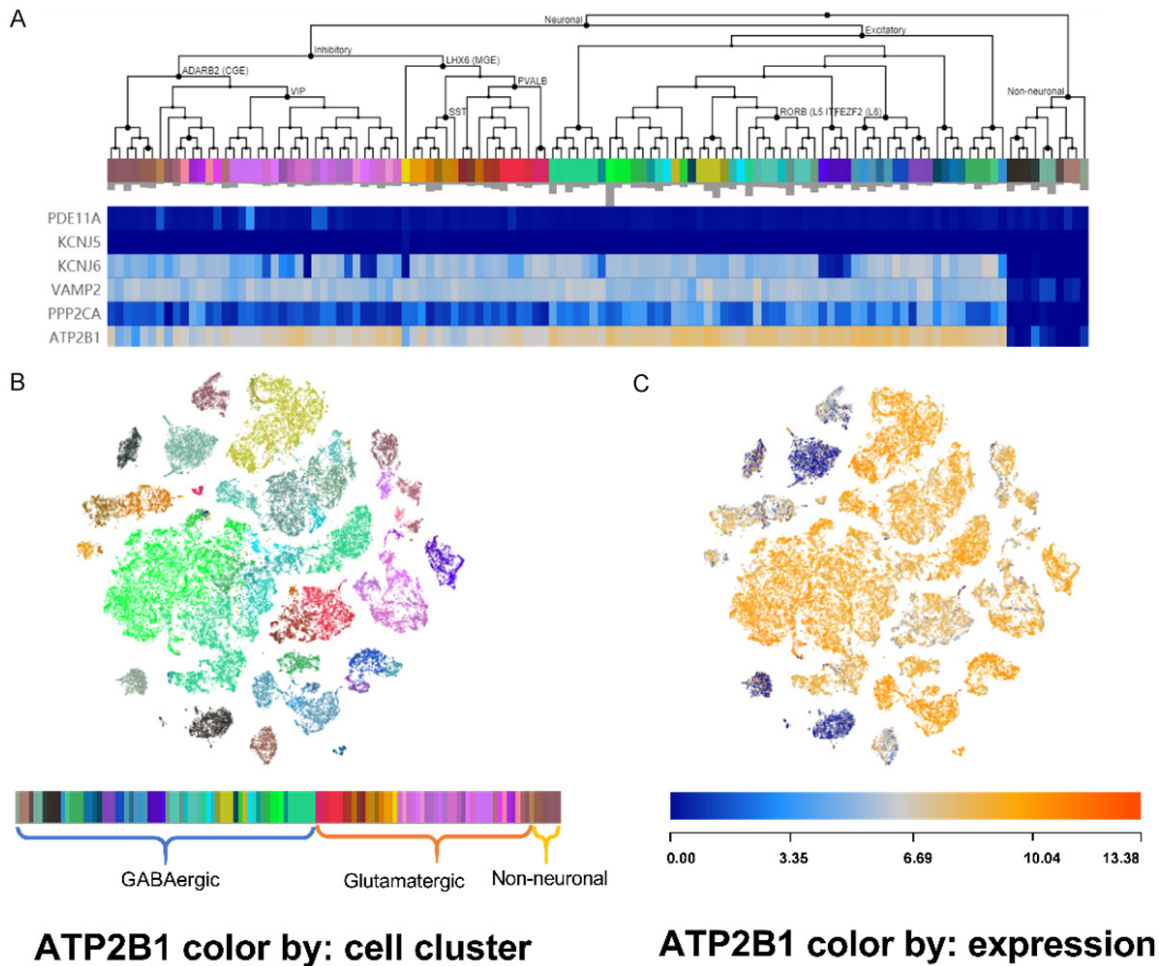
Figure 4. Gene expression map in brain tissue. A. Gene expression heat map. B. t-distributed stochastic neighbour embedding (t-SNE) plots based on cell clustering sequencing. C. t-SNE plots based on ATP2B1 expression. (Diagram: Based on the taxonomy of 69 kinds of neurons expressed in the median cluster (45 inhibitory neurons expressing GABA-Ergic neurons, 24 excitatory neurons expressing glutamatergic neurons) and 6 non-neuronal cell types. The branch is marked with the category of cells).

brain to adapt to various attacks. We used RNA-seq data from genotypic tissue expression to identify APA events of ATP2B1 in blood and brain tissues, and examined the association between ATP2B1 gene expression and APA at different ischemic times.

We found that the APA site of ATP2B1 gene was located in chr12:89983649-89984737 in the first exon 3'-UTR by using DaPars algorithm (**Figure 6A**). At the same time, in all parts of the body, the Percentage of Distal poly A site Usage Index (PDUI) of ATP2B1 gene is significantly different between the brain and other tissues, and the brain tissue is more prone to APA events than other tissues (**Figure 6B**). In whole blood, the probability of APA events of ATP2B1

gene in different tissues varies in different periods of ischemia (**Figure 6C**). In the 0-300 s of ischemia, the probability of APA events in ATP2B1 is higher than that in the later period. After the early ischemic stimulation, the occurrence of APA events tends to be stable in 300-1200 s, indicating that there are obvious APA events in the body at the beginning. In the death classification of Hardy scale, in the cases of using ventilator due to hypoxia, the probability of APA event is the highest, which is higher than that of rapid natural death and chronic death, so we evaluate that ATP2B1 gene is easy to trigger APA events during ischemia and hypoxia. The histogram showed the quantitative distribution of positive and negative correlation with ATP2B1 gene expression, in which
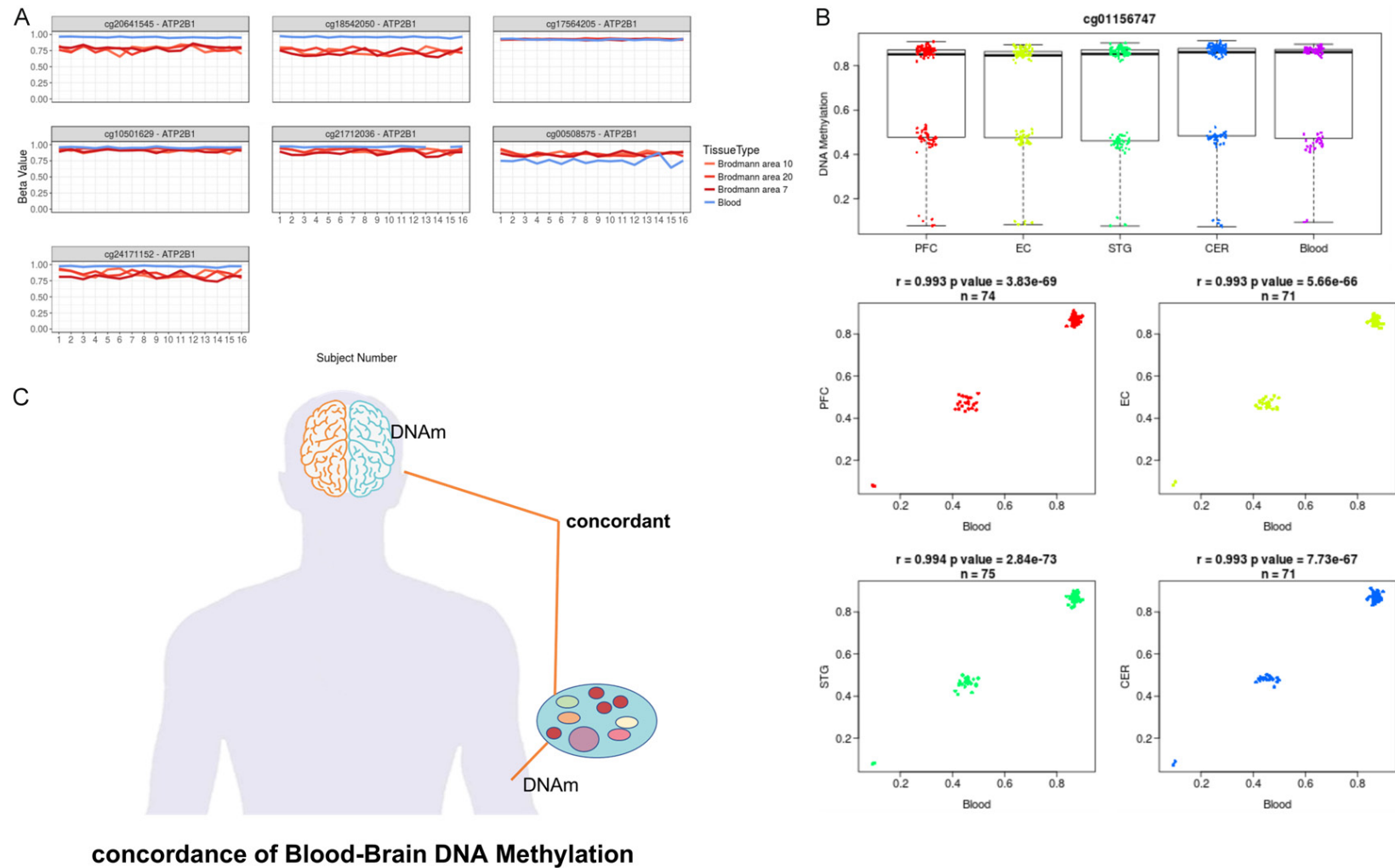
Figure 5. Consistency analysis of DNA methylation data in blood and brain regions. A. In ATP2B1, the trend of DNA methylation was consistent in whole blood and multiple brain tissues (The red lines basically match the blue). B. The distribution of DNA methylation value of ATP2B1 in four brain tissues and the correlation coefficient between blood and each individual value in four brain regions were positive correlation (P < 0.05). C. Visual diagram of DNA methylation consistency in the blood and the brain.
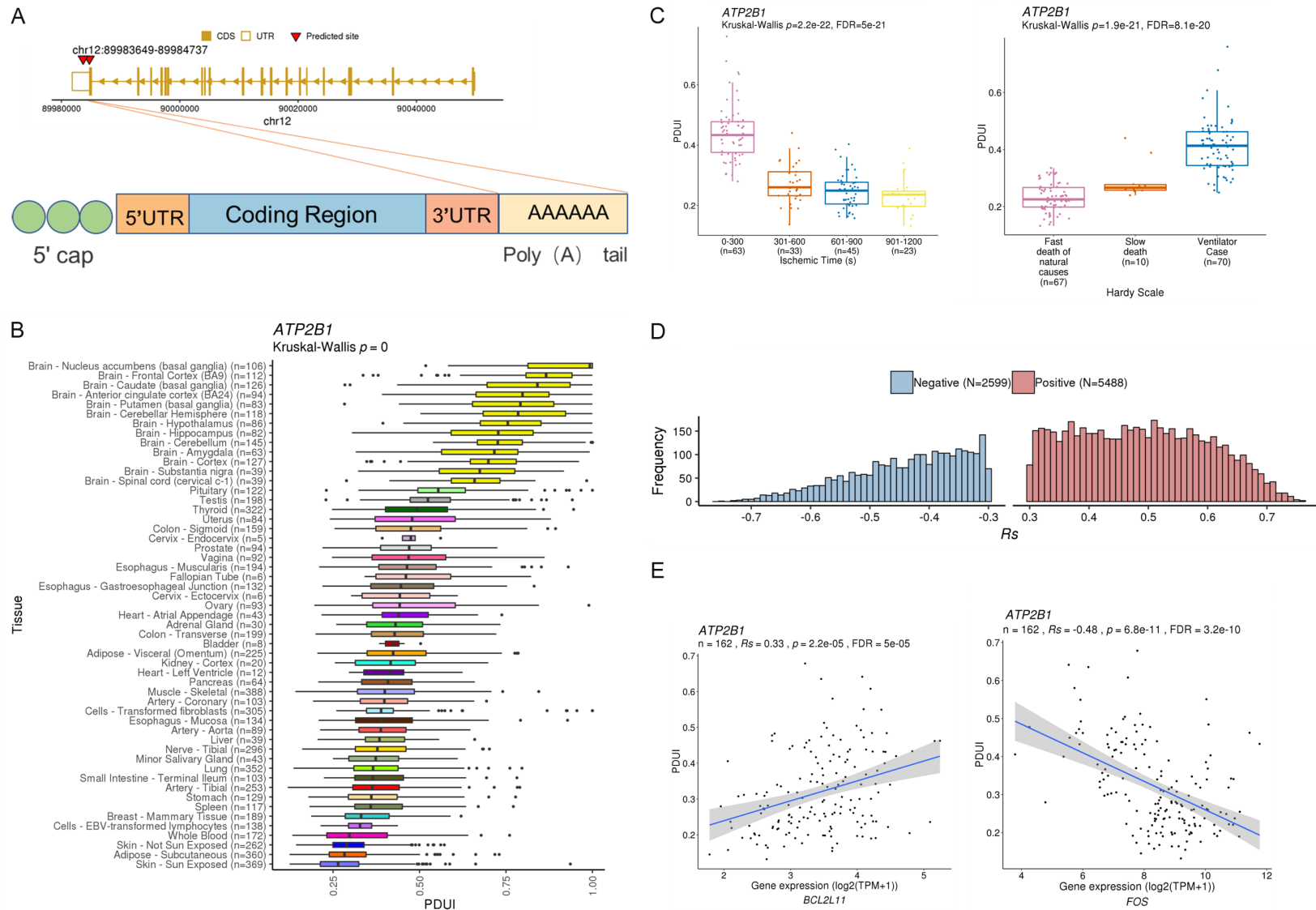
Figure 6. APA event analysis of ATP2B1. A. APA Locus Mapping of ATP2B1 Gene. B. Box map of the changes of ATP2B1 gene APA events in different brain tissues. C. PDUI of ATP2B1 gene in different periods of ischemia and death classification of Hardy scale. D. Distribution of positive and negative related genes in PDUI-related gene expression of ATP2B1 gene. E. The PDUI of ATP2B1 was positively correlated with the expression of anti-apoptotic gene BCL2L11, but negatively correlated with the expression of apoptotic factor FOS. Percentage of Distal poly A site Usage Index (PDUI) is defined as the proportion of transcripts with distal poly A loci (used to quantify APA events). The significant correlation between gene expression level and APA usage was defined as |Rs| > 0.3 and |FDR| < 0.05.

the number of positive correlation genes was more than that of negative correlation (**Figure 6D**). The PDUI of ATP2B1 is positively correlated with the expression of anti-apoptotic gene BCL2L11, but negatively correlated with the expression of apoptotic factor FOS (**Figure 6E**), indicating that the APA event of ATP2B1 also plays an active role in anti-apoptosis during ischemia and hypoxia.

Discussion

Although people are committed to the prevention and treatment of stroke, so far, a variety of drugs have failed in clinical trials [44]. At present, most of the clinical use of thrombolysis-based therapy, but the treatment time window is limited, the beneficiaries are small, only some stroke patients meet the requirements. In view of the fact that ischemic preconditioning can protect the body in many different tissues (such as heart [45, 46], liver [47, 48], kidney [49]), TIA provides another strategy as ischemic preconditioning to stimulate brain protection. Judit studied 2874 patients with acute stroke. It was found that the in-hospital mortality of ischemic stroke patients with prodromal TIA was lower than that of stroke patients without prodromal TIA (P < 0.001) [10]. Sitzer et al found that there was a strong correlation between the occurrence of TIA before stroke and a good prognosis. Previous TIA increased the likelihood of a good prognosis by 1.52 times [50]. The results of Wang et al also support the similar argument that previous transient ischemic attacks have neuroprotective effects on subsequent ischemic stroke [6].

In this study, we investigated transcriptome expression profiles during IPC and determined the changes of miRNA and mRNA in IPC by comparing stroke patients with TIA or without TIA. In view of the fact that a single miRNA can inhibit the production of hundreds of proteins, our miRNA-mediated ischemic protection may involve a variety of ischemia-related regulatory pathways, including platelet homeostasis and activation of GABA B receptors. These pathways indirectly indicate the mechanism of ischemic preconditioning.

This study has some limitations. One of these limitations is the small sample size of patients and the lack of detailed information about previous TIA, such as duration and infarct size of stroke. If we increase the sample size and the information about the pathogenesis of the patient, the conclusion that TIA has a neuroprotective mechanism as an ischemic preconditioning method will be more credible. With the deepening of the study, more and more evidence show that previous hypoxia can reduce the occurrence of cerebrovascular events by stimulating endogenous neuroprotection, such as improving neural results by activating transcription factors and post-translational modification. In order to explore the molecular mechanism of these findings and collect more patient information to further verify this conclusion, it will be helpful to find new stroke prevention strategies.

ATP2B1 is a transport protein that removes Ca^{2+}, from cells and represents the only high-affinity Ca^{2+} transport system on the plasma membrane [51]. Imbalance of platelet homeostasis can lead to thrombosis [52], which can cause ischemic stroke, and then lead to Ca^{2+} overload and brain damage. Besides, the concentration of GABA increases significantly during ischemia, which protects neurons by triggering neuronal membrane hyperpolarization [53]. Ischemic preconditioning in rats can control the plasma membrane proteins of ion homeostasis by mediating the filling of Ca^{2+} into the endoplasmic reticulum, inhibit the up-regulation of caspase-3, and play a neuroprotective role in ischemic stroke [54]. Therefore, platelet homeostasis and GABA B receptor activation pathway, as the regulation of intracellular and extracellular Ca^{2+} concentration, are also one of the important mechanisms of ischemic preconditioning to promote brain protection.

Because human brain samples are not easy to obtain, we need to use substitute tissue blood for indirect analysis when studying neurobiological diseases. DNA methylation is an important gene modification mechanism [55], which changes with the influence of external environment, so the study of DNA methylation can reflect the effect of changing environment on gene interaction to a certain extent [41]. In order to explain the blood-based results in the brain, we analyzed the DNA methylation consistency of the ATP2B1 gene in the blood and the brain. Our results suggest that the ATP2B1 gene is consistent in the DNA methylation of the blood and brain. Therefore, the effect of DNA methylation in ATP2B1 blood on the body can be largely analogous to that in the brain.
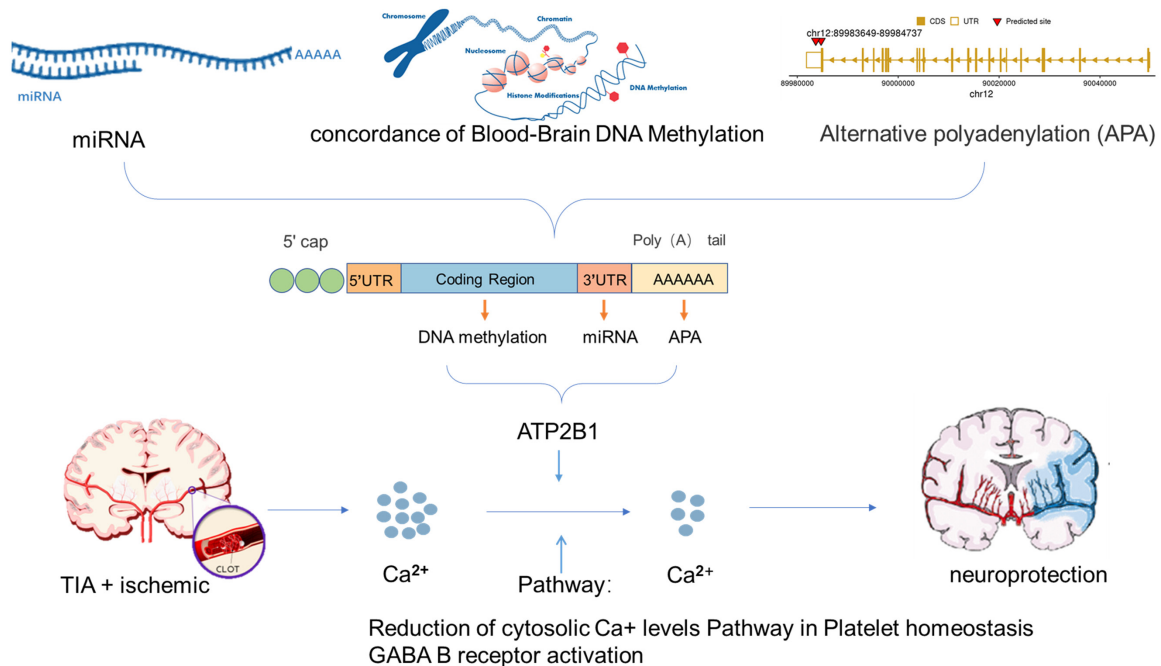
Figure 7. Mechanism pattern diagram. Cerebral ischemic preconditioning stimulates APA events in ATP2B1, which adapts to stress response and plays a neuroprotective role by regulating the level of Ca^{2+} in platelet homeostasis pathway and activating GABAB pathway.

Alternative polyadenylated (APA) affects the transformation of human biological processes, such as cell activation, differentiation and proliferation, immune defense and neuronal activity [56]. Through the analysis of ATP2B1, the results show that the occurrence of APA events is the highest in the brain tissue. In the early stage of ischemia, the probability of APA event is higher than that in the later period, and then it tends to be stable. Similarly, under the condition of hypoxia, the probability of APA event is the highest, so we evaluate that ATP2B1 gene is easy to trigger APA event during ischemia and hypoxia. In the similarity of gene expression, we found that ATP2B1 was positively correlated with the expression of anti-apoptotic genes, but negatively correlated with the expression of apoptotic factors, indicating that ATP2B1 plays a protective role in the regulation of human stress response in APA events.

Shao found that APA regulates gene differential expression by affecting RNA binding proteins in pleomorphic glioblastoma, thus discovering the mechanism of APA-mediated transcriptional regulation [57]. In a study of the contribution of APA to neurological function, the authors analyzed the functional annotation of genes with differential APA between excitatory neurons and inhibitory neurons. The results also showed that these genes were highly enriched in the biological process of positive regulation of neural development [58]. Therefore, we speculate that APA events may occur in ATP2B1 during the occurrence of cerebral ischemic preconditioning, which can adapt to the stress response of the body and play a neuroprotective role by regulating the positive effect of neurogenesis and the concentration of intracellular Ca^{2+} (**Figure 7**).

Conclusion

Our results suggest that ATP2B1 affects posttranscriptional regulation through parallel regulation of microRNA and alternative polyadenylation in ischemic preconditioning. After further analysis, our study for the first time suggests that APA events may occur in ATP2B1 in GABA neurons during ischemic preconditioning, which adapts to the stress response of the body through positive regulation of neurogenesis, and the mechanism of regulating Ca^{2+} concentration in platelet homeostasis and activation of GABA B receptors triggers the brain endogenous neuroprotective mechanism to resist subsequent more severe cerebral ischemic injury.

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