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RESEARCH

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^{1}H-MRS reveals abnormal energy metabolism and excitatory-inhibitory imbalance in a chronic migraine-like state induced by nitroglycerin in mice

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Abstract

Background Chronic migraine is closely related to the dysregulation of neurochemical substances in the brain, with metabolic imbalance being one of the proposed causes of chronic migraine. This study aims to evaluate the metabolic changes between energy metabolism and excitatory and inhibitory neurotransmitters in key brain regions of mice with chronic migraine-like state and to uncover the dysfunctional pathways of migraine.

Methods A chronic migraine-like state mouse model was established by repeated administration of nitroglycerin (NTG). We used von Frey filaments to assess the mechanical thresholds of the hind paw and periorbital in wild-type and familial hemiplegic migraine type 2 mice. After the experiments, tissue was collected from five brain regions: the somatosensory cortex (SSP), hippocampus, thalamus (TH), hypothalamus, and the spinal trigeminal nucleus caudalis (TNC). Proton magnetic resonance spectroscopy (^{1}H-MRS) was employed to study the changes in brain metabolites associated with migraine, aiming to explore the mechanisms underlying metabolic imbalance in chronic migraine-like state.

Results In NTG-induced chronic migraine-like state model, we observed a significant reduction in energy metabolism during central sensitization, an increase in excitatory neurotransmitters such as glutamate, and a tendency for inhibitory neurotransmitters like GABA to decrease. The TNC and thalamus were the most affected regions. Furthermore, the consistency of N-acetylaspartate levels highlighted the importance of the TNC-TH-SSP pathway in the ascending nociceptive transmission of migraine.

Conclusion Abnormal energy metabolism and neurotransmitter imbalance in the brain region of NTG-induced chronic migraine-like state model are crucial mechanisms contributing to the chronicity of migraine.

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Keywords Chronic migraine-like state, Energy metabolism, Neuronal excitability, Trigeminovascular system, ^{1}H-MRS metabolomics

Background

Migraine, a common neurovascular disorder, affects over 1 billion people worldwide [1–3]. It is characterized by recurrent, severe headaches often accompanied by nausea, vomiting, and sensitivity to light and sound [4], significantly impacting patients' daily lives [5–7]. Several potential mechanisms for migraine chronification have been proposed, including neurotransmitter imbalance, trigeminovascular dysfunction, and brain network disruptions [8–11].

Nitroglycerin (NTG), a highly lipophilic organic nitrate, has been shown to induce migraine-like pain in patients [12]. Its mechanism primarily involves the release of nitric oxide (NO), affecting neuronal firing and vasodilation, thereby triggering central sensitization and migraine attacks [13, 14]. Given NTG's known ability to induce central sensitization, we employed an NTG-injected mouse model to explore the potential pathophysiological and metabolic mechanisms underlying migraine attacks. On the other hand, familial hemiplegic migraine type 2 (FHM2), a classic monogenic migraine model, does not show significant spontaneous migraine behavior [15–17]. Therefore, to achieve more stable experimental results, we chose to induce migraine attacks in FHM2 mice by NTG injection [18]. Mechanical thresholds of hind paw and periorbital area were then assessed as a standard for chronic migraine-like state model determination [19].

By quantifying various metabolites, ^{1}H-MRS provides a unique opportunity to study multiple brain metabolites simultaneously [20–22]. Therefore, this study utilized the high sensitivity of high-field ^{1}H-MRS to analyze metabolites in migraine animal models, including adenosine monophosphate (AMP), nicotinamide adenine dinucleotide (NAD+), lactate (Lac), glycine (Gly), glutamate (Glu), gamma-aminobutyric acid (GABA), N-acetylaspartate (NAA), and taurine (Tau). These metabolic contribute to a deeper understanding of the impact of chronic migraine-like state model on energy metabolism (nicotinamide adenine dinucleotide, lactate), neurotransmitters (glutamate, GABA), and membrane metabolism and neuron-astrocyte interactions (N-acetylaspartate, taurine). Previous studies by Ma and Cao using spectroscopy investigated metabolite changes during acute phases of migraine in animal models [23, 24]. However, to date, no foundational studies have explored ^{1}H-MRS data in chronic migraine-like state model. Hence, this study evaluated ^{1}H-MRS data in chronic migraine-like state mice to reflect classic NTG-induced migraine-related dysfunctional pathways.

Different stages of migraine attacks involve multiple brain regions [25]. The phenomenology of migraine aura suggests the hypothalamus plays a crucial role early in attacks [26]. The cortex, due to cortical spreading depression, also plays a key role during migraine attacks. The core of migraine chronification is associated with the activation of the trigeminovascular system's spinal trigeminal nucleus caudalis (TNC) [27]. The thalamus, as a major sensory center, is involved in transmitting and processing pain signals and has been shown by MRI studies to be activated during the headache phase of migraine attacks [28]. Additionally, genome-wide association studies (GWAS) suggest a possible negative causal relationship between hippocampal morphology and function and increased migraine risk [29]. Current research rarely considers the interactions between different mouse types and brain regions during chronic migraine-like state progression. Therefore, this study used wildtype (WT) and FHM2 mice to analyze metabolic characteristics in five different brain regions (somatosensory cortex (SSP), thalamus (TH), hypothalamus (HY), hippocampus (HIP), and TNC) under chronic migraine-like state conditions to investigate biochemical changes between chronic migraine-like state mice and controls. Particularly, the TNC region, associated with central sensitization and abnormal excitability, plays a key role. When primary afferent fibers (Aδ fibers and C fibers) of the dura mater are stimulated, they transmit signals through the trigeminal nerve to the brainstem, influencing neuronal activity in the TNC region [30]. This signal transmission not only promotes the development of central sensitization but may also lead to increased neuronal excitability, triggering or exacerbating migraine symptoms [31]. Additionally, Wang et al. reported using regional NAA correlation to analyze pathway connectivity in chronic migraine [32]. Thus, we analyzed the interactions of NAA metabolic changes between different regions, aiding in understanding region-specific metabolic changes and pain pathway network connections in chronic migraine.

Under chronic migraine conditions, key brain regions may experience persistent metabolic imbalance, leading to hyperactivity and sensitization of the trigeminovascular system [33]. To determine whether chronic migraine-like state mouse model is associated with abnormal metabolites and energy changes, we constructed the model of chronic migraine-like state by injecting NTG. Subsequently, we extracted tissue from five brain regions for ^{1}H-MRS detection and performed NAA concentration connectivity analysis to assess migraine-related

dysfunctional pathways, thereby enhancing our understanding of central sensitization.

Materials and methods

Animals

All animal experiments were approved by the Animal Ethics Committee of ShanghaiTech University and all procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The sample size for this study was determined based on previous research [19, 27]. All animals were housed at the Experimental Animal Center of ShanghaiTech University under a 12-hour light-dark cycle (22.7±0.2℃, humidity 49±10%) with free access to food and water. The G→A mutation in exon 8 encoding the FHM2-associated G301R-mutation was introduced by homologous recombination and resulted in α2+/G301R knock-in mice (Supplementary Fig. 1a). This mutation causes a substitution of glycine to arginine at amino acid position 301 of the α2 subunit, which is linked to familial hemiplegic migraine type 2 (FHM2). This mutation at the site can disrupt Na and K channels, leading to cortical spreading depression (CSD), a key mechanism in migraine. Genotypes were confirmed by sanger sequencing before the experiments (Supplementary Fig. 1b). The study utilized 30 mice; however, due to decreased appetite and weight loss in 2 mice during the experiment, these were excluded from the analysis. Ultimately, 14 male FHM2 mice (8 weeks old, weight=18–24 g) and 14 age-matched male WT mice (weight=18–24 g) were used for the experiments. Mice were randomly assigned to different experimental groups. Prior to the start of all experiments, the mice were given one week to acclimate to the experimental environment.

Experimental design

The experimental design included four groups: WT, WT+NTG, FHM2, and FHM2+NTG. Mice in the WT and FHM2 groups received intraperitoneal (i.p.) injections of saline (1 ml/100 g) every other day for 9 days. Mice in the WT+NTG and FHM2+NTG groups received i.p. injections of NTG (10 mg/kg) every other day for 9 days. NTG was administered to induce central sensitization and establish a chronic migraine-like state, as NTG-induced central sensitization is a well-established migraine model in rodents and humans [12]. Inclusion criteria comprised male mice aged 8 weeks with body weights between 18 and 24 g, chosen to ensure maturity and physiological consistency. Genotypes were confirmed via Sanger sequencing for the presence or absence of the G301R mutation in exon 8 of the FHM2 gene. Only mice with confirmed genotypes and normal initial health and behavior were included. Exclusion criteria encompassed mice exhibiting poor health indicators like significant weight loss or abnormal behavior. Those not compliant with experimental procedures or with inconsistent genotyping results were also excluded to maintain result accuracy and experimental integrity. Experimental procedures were performed in a controlled environment, and the flowchart describes the overall process of model construction and data analysis (Fig. 1).

Behavioral testing

Behavioral tests were conducted between 9:00 AM and 3:00 PM. Prior to the experiment, mice of the same strain were housed together in a uniform environment for one week to acclimate. Subsequently, mice were randomly assigned to experimental and control groups using random numbering. Testing equipment was cleaned with 75% ethanol and feces were removed before each session. Mice were allowed to acclimate to the testing room for 30 min two days before the behavioral tests. The behavioral tests on the mice were conducted during the light (inactive) phase. Previous studies have shown that the increase in mechanical sensitivity induced by NTG is most significant 2 h after injection. Therefore, mechanical thresholds were measured before and 2 h after each NTG or saline injection on each injection day. Before the start of the tests, preliminary baseline measurements were conducted to determine the mechanical threshold of each mouse. This was achieved by applying gradually increasing pressure to the plantar surface of the mouse's paw using von Frey filaments, while recording the animal's responses to the stimuli, such as paw withdrawal or escape behavior. The procedure is detailed as follows: for the periorbital mechanical threshold test, mice were placed in 4-ounce cups and allowed to acclimate for 15 min. A series of Von Frey filaments (0.008–2 g) were applied perpendicularly to the periorbital area to assess the threshold [34]. A positive response was defined as a rapid head withdrawal or scratching the face with the ipsilateral paw due to stimulation [35]. For the hind paw mechanical threshold test, mice were placed on a wire grid floor in transparent acrylic chambers (10×7×7 cm) and allowed to acclimate for 30 min. A positive response was defined as withdrawal, shaking, or licking of the paw.

Sample collection

On the 10th day after NTG or saline injections, chronic migraine-like state mouse models were established, and brain tissues were collected. The sampling time for our samples ranged from 9:00 AM to 3:00 PM. Mice from all experimental groups were randomly sampled within this time frame. Mice were fully anesthetized with intraperitoneal injections of 3% tribromoethanol (dose: 50 mg/100 g, volume: 0.5 ml/100 g) and then decapitated. The brains were quickly removed and dissected into the SSP, HIP, TH, HY, and TNC according to anatomical

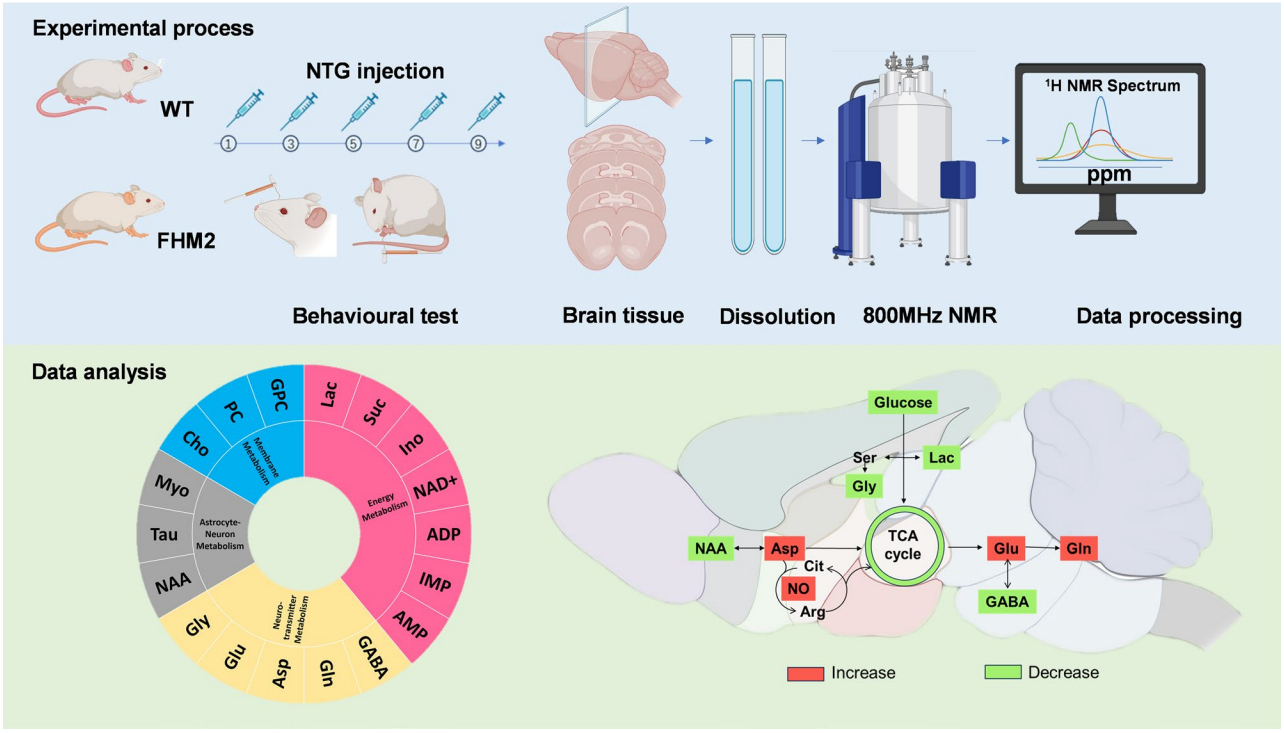

Fig. 1 Experimental Workflow and Metabolite Analysis Overview. The upper half of the figure depicts the experimental workflow, while the lower half illustrates the data analysis process. (Created with BioRender)

boundaries. All brain tissues were immediately frozen in liquid nitrogen and stored at -80 °C until use. Tissue extraction followed the literature method [36]. Frozen tissues were weighed in Eppendorf tubes, ground in 500 µl ice-cold saline for 2 min, and 400 µl of the homogenate was transferred to 5 ml tubes. A mixture of 1875 µl (99% methanol, 98% chloroform, and 36% HCl, 40:20:1) was added and vortexed for 1 min. Then, 625 µl of chloroform and 625 µl of distilled water were added and vortexed for another minute. After standing on ice for 15 min, the mixture was centrifuged at 10,000 g for 15 min at 4 °C. The upper water/methanol phase containing various polar metabolites was then collected. The supernatant was transferred to new Eppendorf tubes, freeze-dried for 24 h, and stored at -80 °C until analysis.

^{1}H-MRS analysis

For analysis, freeze-dried samples were dissolved in 500 µl of heavy water containing 0.05% trimethylsilane propionate (TSP) and transferred to 5 mm Nuclear Magnetic Resonance (NMR) tubes. ^{1}H-MRS were recorded using a Bruker AVANCE III 800 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 25 °C with the NoesyPR1D sequence. Data were acquired as partial echoes and processed by Fourier transformation to generate spectra. High-field spectra of water included significant resonances such as myo-inositol, glycine, taurine, choline, aspartate, N-acetylaspartate, and lactate, which are involved in cellular metabolism and are specific to the central nervous system. These peaks were assigned based on resonances identified in the Human Metabolome Database [37] and are labeled in the representative spectrum. We excluded metabolites with chemical shifts greater than 10 ppm and those located at the same ppm position, as these conditions could interfere with the stability of metabolite concentrations and make it challenging to distinguish and quantify individual signals. Key acquisition parameters were: 128 scans, acquisition time of 2.55 s per scan, and 64 K data points. All free induction decays (FIDs) of ^{1}H-MRS spectra were processed with spectral analysis software MestRe Nova and phase-corrected, baseline-corrected using Topspin software (v3.65, Bruker BioSpin, Germany). A double-blind design was employed throughout the study. the data analysis was performed by an independent observer who was unaware of the experimental group labels. NMR signals were labeled according to the literature [38]. Metabolite quantification was performed by manually integrating peak areas using Topspin software. Metabolite concentrations were calculated as CM=AM/ATSP*fP*CTSP*MWM/WM, and metabolite to tCr ratios were considered relative concentrations. (CM represents the metabolite concentration; AM is the integrated peak area of the metabolite; ATSP is the integrated peak area of the internal standard (TSP); fP is the calibration factor for the metabolite; CTSP is the concentration of TSP; MWM is

the molecular weight of the metabolite; WM is the weight of the metabolite.) To examine inter-regional NAA correlation for each group, Pearson correlation matrices were generated for the five regions of interest.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (version 9.0). Two-way ANOVA was employed to compare behavioral outcomes across experimental groups. Error bars are depicted as mean ± standard deviation (SD). To ensure data normality, the Shapiro-Wilk normality test was applied. For comparisons involving multiple tissues and metabolites, we mitigated the risk of type I errors by employing multiple comparison correction methods. Specifically, Tukey's Honestly Significant Difference (HSD) test was used post-ANOVA to evaluate mean differences among groups. In cases where data did not conform to normal distribution, non-parametric tests were utilized. Pearson correlations were utilized to explore relationships between metabolites across different brain regions. A significance threshold of $p < 0.05$ was applied for all statistical tests.

Results

Repeated NTG administration induces mechanical hypersensitivity in the hind paw and periorbital area

Mechanical thresholds in the hind paw and periorbital areas were assessed using Von Frey filaments (Fig. 2). The results of changes in mechanical thresholds among the four groups of mice within baseline and 2 h after injection are shown in Fig. 2A and B. Consistent with previous studies, we observed that compared to the WT group, mice in the WT + NTG and FHM2 + NTG groups exhibited significantly decreased mechanical thresholds in the hind paw (Fig. 2A) and periorbital area (Fig. 2B) at acute (2 h post-treatment) and chronic states (days 3, 5, 7, and 9) (Supplementary Material Tables 1-4). During the baseline assessment on Day 1, the pain threshold trend of FHM2 mice was slightly lower than that of WT mice. This difference may be attributed to genetic variations between WT and FHM mice strains, and it also suggests that FHM2 mice are more sensitive to pain thresholds compared to WT mice. Overall, our results indicate that NTG successfully induced mechanical hypersensitivity in the hind paw and periorbital area.

Brain metabolism overview in mice

During chronic migraine, structural changes and impaired metabolic function occur in the brain. Recently, MRI studies have revealed changes in the underlying brain functional networks and blood perfusion [39, 40]. However, how chronic migraine affects brain metabolism is still not fully understood. Figure 3A and B illustrate typical ^{1}H-MRS extracted from the TNC region of WT mice brains (Fig. 3A and B). From the NMR-based metabolic profiles, 19 brain metabolites were identified,

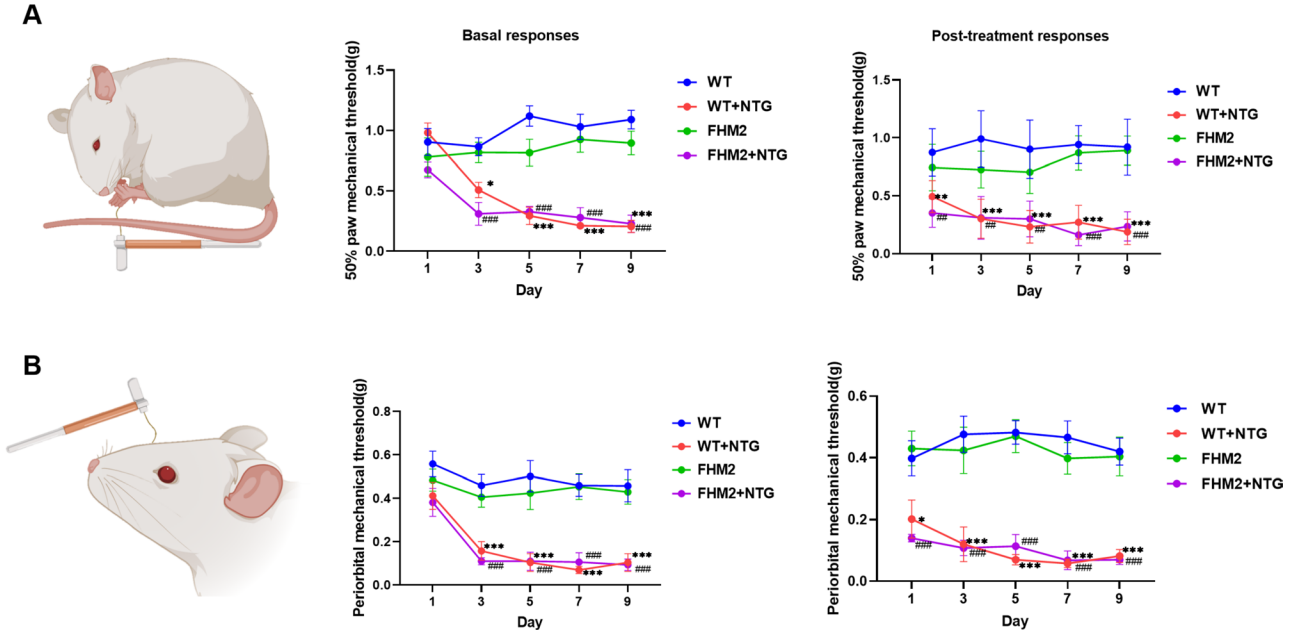

Fig. 2 Repeated NTG Administration Induces Mechanical Hypersensitivity. NTG injection significantly reduced the mechanical thresholds in the hind paw (**A**) and periorbital area (**B**). (**A**) and (**B**) display the baseline thresholds and changes in hypersensitivity thresholds 2 hours after NTG injection in the hind paw and periorbital area of the migraine model mice. Data are presented as mean ± SD. Statistical analysis was performed using two-way ANOVA: (*$p < 0.05$;**$p < 0.01$;***$p < 0.001$) indicate significant differences between WT and WT+NTG groups. (#$p < 0.05$;##$p < 0.01$;###$p < 0.001$) indicate significant differences between FHM2 and FHM2+NTG groups. n=7/group

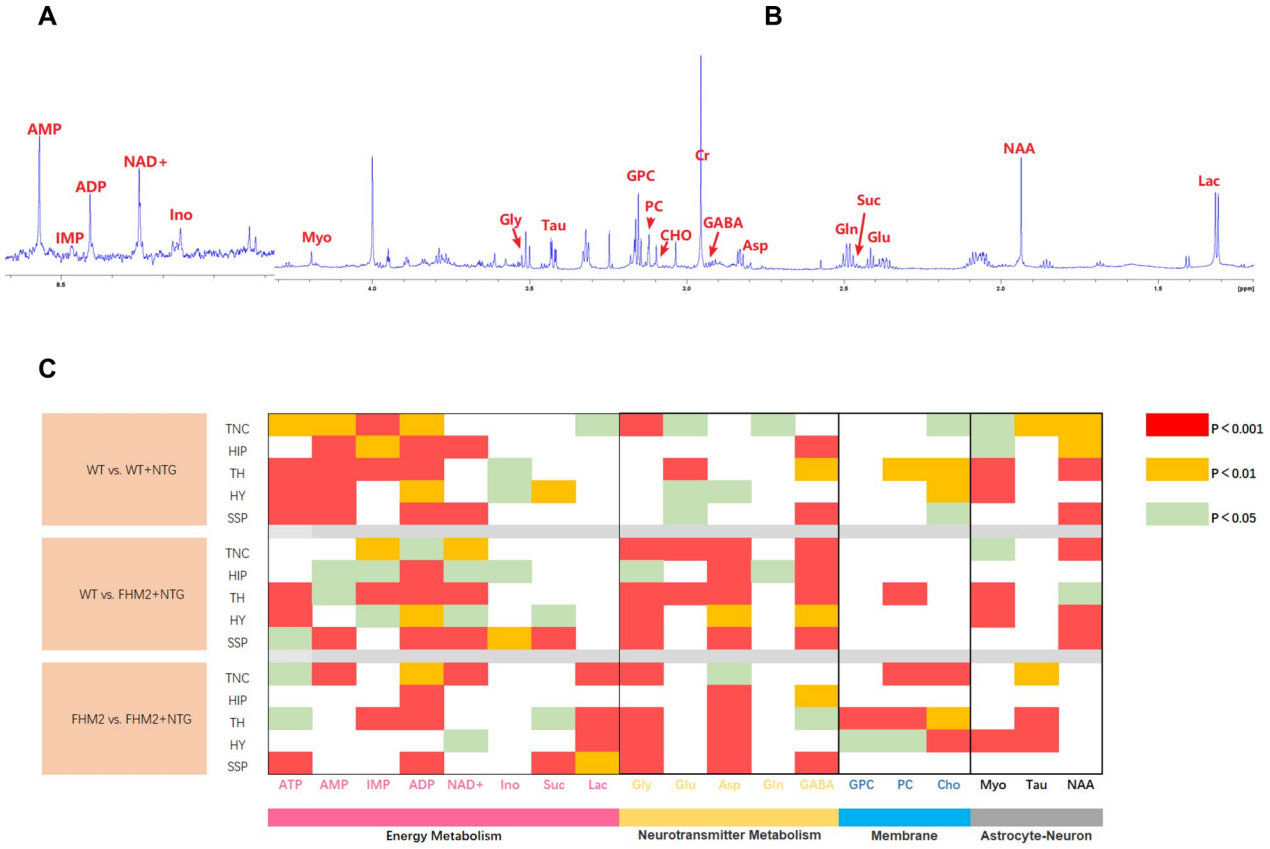

Fig. 3 NMR-Based Metabolomics Analysis. (A-B) Representative 1H-MRS spectra obtained from the TNC region of WT mice. **(A)** Spectral region from 8.0 to 8.6 ppm. **(B)** Spectral region from 1.2 to 4.3 ppm. **(C)** Analysis of variance (ANOVA) results showing the interaction of metabolic changes across five brain regions in mice. Significance levels are indicated as follows: green, $p < 0.05$; yellow, $p < 0.01$; red, $p < 0.001$

which are involved in the following metabolic processes: Energy Metabolism: AMP (adenosine monophosphate), IMP (inosine monophosphate), ADP (adenosine diphosphate), NAD+ (nicotinamide adenine dinucleotide), Ino (inosine), Suc (succinate), Lac (lactate), ATP(adenosine triphosphate); Neurotransmitter Metabolism: Gly (glycine), Glu (glutamate), Asp (aspartate), Gln (glutamine), GABA (gamma-aminobutyric acid); Membrane Metabolism: GPC (glycerophosphocholine), PC (phosphocholine), Cho (choline); Astrocyte-Neuron Metabolism: Myo (myo-inositol), NAA (N-acetylaspartate), Tau (taurine). The brain regions of both WT+NTG and FHM2+NTG mice showed similar metabolic characteristics, with TNC and TH brain regions being the main brain regions of metabolic abnormalities in chronic migraine-like state mice (Fig. 3C). The identification of these metabolites provides valuable insights into the changes in brain metabolism associated with chronic migraine-like state mouse model (Supplemental Data Set and Supplementary Material).

Decreased brain energy metabolism in NTG-induced chronic migraine-like state models

An imbalance between the brain's energy supply and demand is believed to contribute to the onset of migraine [41]. Numerous studies have shown fundamental mitochondrial dysfunction in migraine patients [42–45]. Clinical trials have targeted mitochondrial dysfunction using nutritional supplements to support abnormal energy metabolism [46]. To visualize the differences in energy metabolism, we plotted the changes in energy metabolism-related metabolites AMP (adenosine monophosphate), IMP (inosine monophosphate), ADP (adenosine diphosphate), NAD+ (nicotinamide adenine dinucleotide), Ino (inosine), Suc (succinate), and Lac (lactate), in different brain regions before and after NTG injection in WT and FHM2 mice (Fig. 4). The sunburst chart shows metabolites related to energy metabolism (Fig. 4A). Compared to the WT and FHM2 groups, concentrations of AMP(Fig. 4B), IMP(Fig. 4C), ADP(Fig. 4E), NAD+(Fig. 4F), Ino (Fig. 4G) and ATP(Fig. 4I) significantly decreased in the NTG-induced chronic migraine-like state model groups (WT+NTG and FHM2+NTG), with similar trends observed. However, Lac concentration increased in the TNC region of the WT+NTG

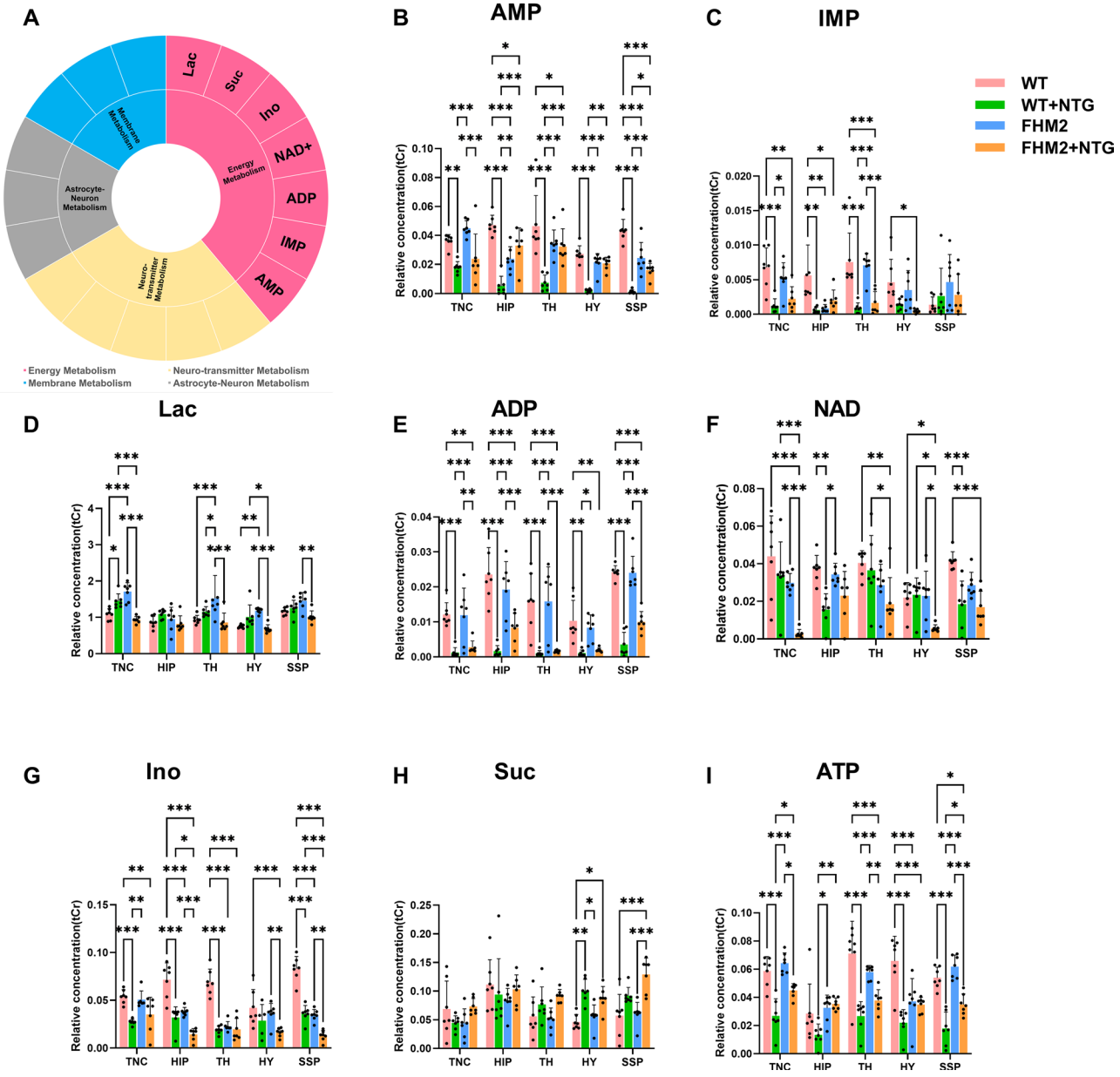

Fig. 4 Decreased Brain Energy Metabolism in NTG-Induced Chronic Migraine-Like State Models. (**A**) Sunburst chart of energy metabolism-related metabolites. (**B**) AMP (adenosine monophosphate); (**C**) IMP (inosine monophosphate); (**D**) ADP (adenosine diphosphate); (**E**) NAD+ (nicotinamide adenine dinucleotide); (**F**) Ino (inosine); (**G**) Suc (succinate); (**H**) Lac (lactate); (**I**) ATP (adenosine triphosphate). Data are presented as mean ± SD. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$. Mouse types: WT, wild-type mice ($n = 7$/group); FHM2, familial hemiplegic migraine type 2 mice ($n = 7$/group). NTG: nitroglycerin. Brain regions: TNC, trigeminal nucleus caudalis; HIP, hippocampus; TH, thalamus; HY, hypothalamus; SSP, somatosensory cortex

group (Fig. 4D), while changes in other brain regions were not significant compared to the WT group. In the TH, HY, and SSP regions, Lac concentration significantly decreased in the FHM2+NTG group compared to the FHM2 group. This phenomenon may be related to the genetic background of the FHM2 mice, particularly the G301R mutation, which affects neuronal responses to external stimuli. The decrease in lactate levels in FHM2 mice may indicate that the energy metabolism in these regions has adapted to a prolonged chronic migraine-like state. These results indicate that the NTG-induced migraine model significantly impacted brain energy metabolism in mice, with notable changes in the concentrations of energy metabolites in specific brain regions.

Imbalance in excitatory-inhibitory neurotransmitter metabolism in chronic migraine-like state model

Next, we investigated whether neurotransmitter metabolism in the brains of mice could provide insights into the process of central sensitization in chronic migraine-like

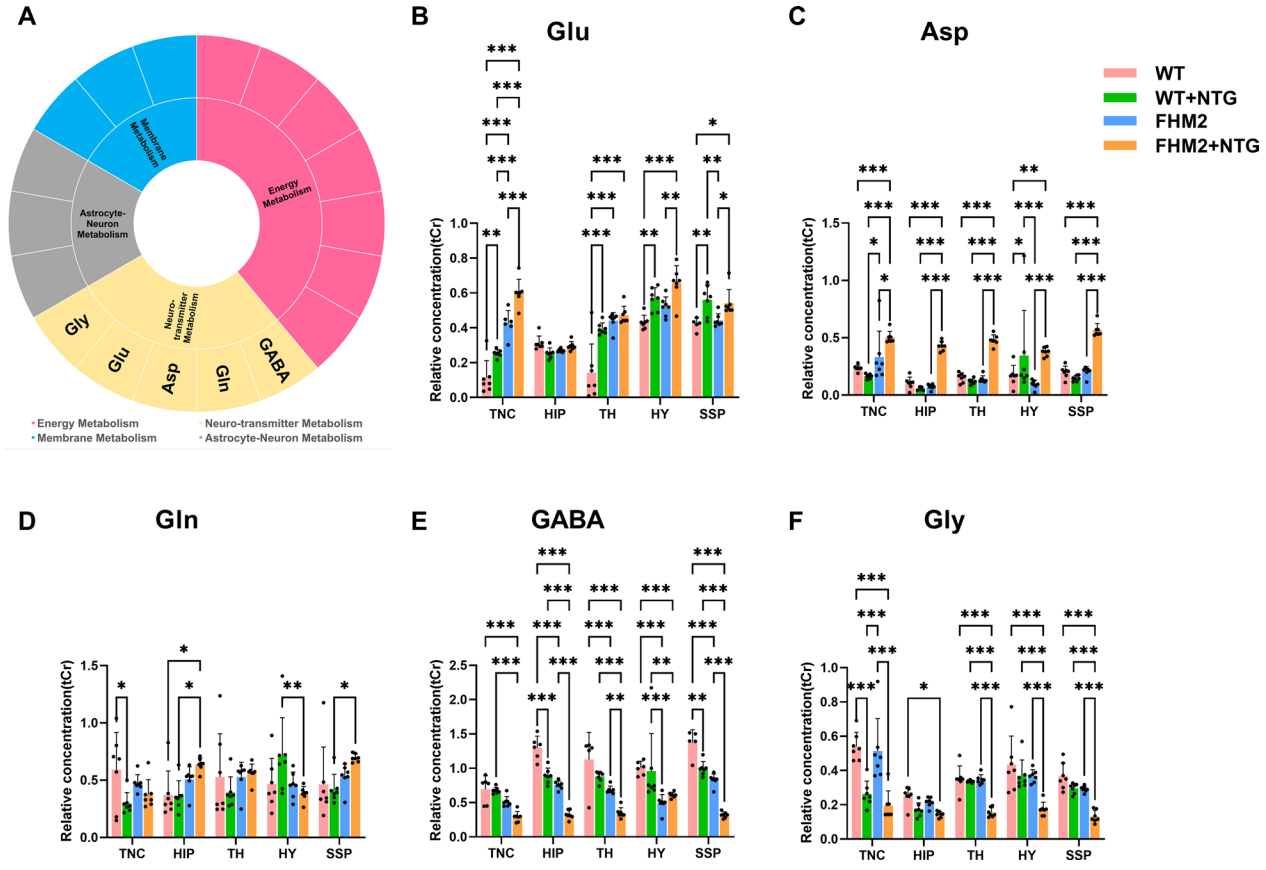

Fig. 5 Imbalance in Excitatory and Inhibitory Neurotransmitter Metabolism in the Chronic Migraine-Like State Model. (**A**) Neurotransmitter metabolism-related metabolites; (**B**) Glu (glutamate); (**C**) Asp (aspartate); (**D**) Gln (glutamine); (**E**) GABA (gamma-aminobutyric acid); (**F**) Gly (glycine). Data are presented as mean ± SD. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$

state mouse mode (Fig. 5). The sunburst chart shows metabolites related to neurotransmitter metabolism (Fig. 5A). We found that, compared to paired WT and FHM2 mice, glutamate significantly increased (Fig. 5B) while GABA decreased (Fig. 5E) in the WT+NTG and FHM2+NTG groups after NTG injection. Additionally, aspartate was significantly higher in FHM2+NTG mice compared to FHM2 mice (Fig. 5C). Glutamine concentrations were higher in the HIP, TH, and SSP brain regions, with no significant differences in the TNC and HY regions (Fig. 5D). Interestingly, there were significant differences in the basal state of glutamate between WT and FHM2 (without NTG injection), especially in the TNC and TH brain regions. Overall, there was an increase in excitatory neurotransmitters like glutamate and a decrease in inhibitory neurotransmitters like GABA, resulting in a state of hyperexcitability in the brain. This finding explains the central sensitization observed in mice exhibiting a chronic migraine-like state. These results are consistent with previous research, which has demonstrated a characteristic pattern of increased excitability and reduced inhibition in migraine, indicating an excitatory-inhibitory imbalance [47].

Chronic migraine involves more than neuronal overactivation: membrane metabolism and astrocyte-neuron interactions

Previous studies have shown that chronic migraine is not solely due to neuronal overactivation but also involves membrane metabolism and astrocyte-neuron interactions [48]. We analyzed the changes in membrane-related metabolites and astrocyte-neuron metabolism-related metabolites in migraine-like mice (Fig. 6). The sunburst chart shows metabolites related to membrane-related metabolites (glycerophosphocholine, phosphocholine, choline) and astrocyte-neuron metabolism-related metabolites (myo-inositol, N-acetylaspartate, taurine) (Fig. 6A). The results indicate that, compared to paired FHM2 mice, glycerophosphocholine significantly decreased in the TH and HY brain regions in the FHM2+NTG group (Fig. 6B), and phosphocholine significantly decreased in the TNC, TH, and HY brain regions, with no differences observed in other regions (Fig. 6C). In the WT+NTG group, choline increased in the TNC, TH, HY, and SSP brain regions, whereas in the FHM2+NTG group, choline decreased in the TNC, TH, and HY brain regions (Fig. 6D). The abnormal changes

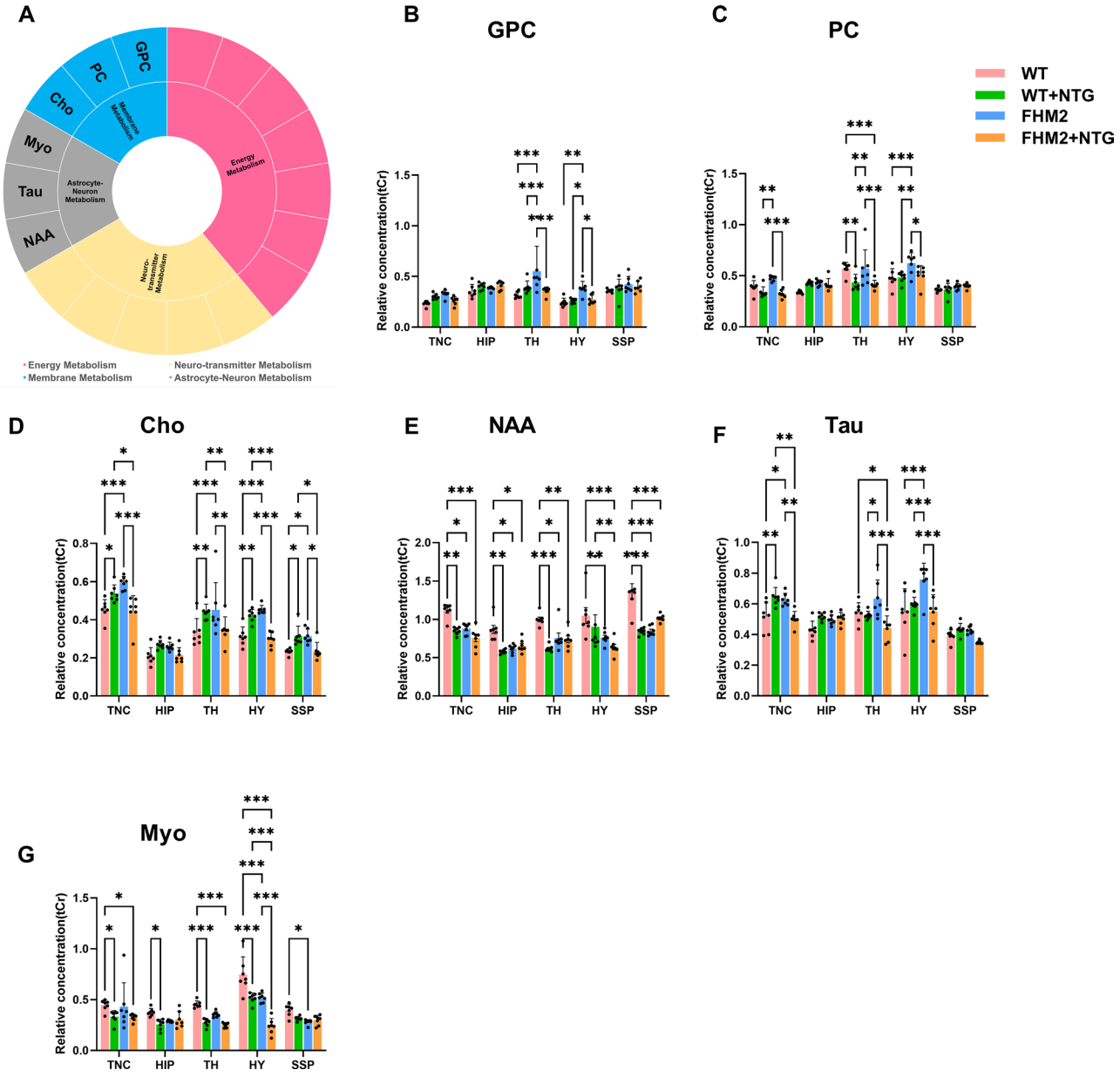

Fig. 6 Changes in Membrane and Astrocyte-Neuron Metabolism-Related Metabolites in Different Brain Regions of Chronic Migraine-Like State Models. (**A**) Changes in membrane metabolism-related metabolites; (**B**) GPC (glycerophosphocholine); (**C**) PC (phosphocholine); (**D**) Cho (choline); (**E**) NAA (N-acetylaspartate); (**F**) Tau (taurine); (**G**) Myo (myo-inositol). Data are presented as mean ± SD. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$

in membrane metabolites may reveal impaired neuronal membrane function, affecting the regulation of pain signals and exacerbating migraine symptoms.

In FHM2 mice, Na+/K+-ATPase dysfunction leads to the inability of astrocytes to effectively clear glutamate from the synaptic cleft, resulting in elevated glutamate levels. Excessive glutamate overactivates glutamate receptors on neurons, particularly NMDA receptors, leading to calcium overload, oxidative stress, and membrane damage, causing neuronal injury [18]. Concurrently, neuronal overactivation and injury trigger overactivation of the trigeminovascular system, exacerbating migraine symptoms.

Inter-regional NAA correlation

To explore the inter-regional NAA (N-acetylaspartate) correlation, we calculated the correlation matrices between different brain regions (Fig. 7). No significant inter-regional correlations were found in the WT group (Fig. 7A). However, in the WT + NTG group, a significant positive correlation was observed between SSP and TH (Fig. 7B). In the FHM2 group, a positive correlation was

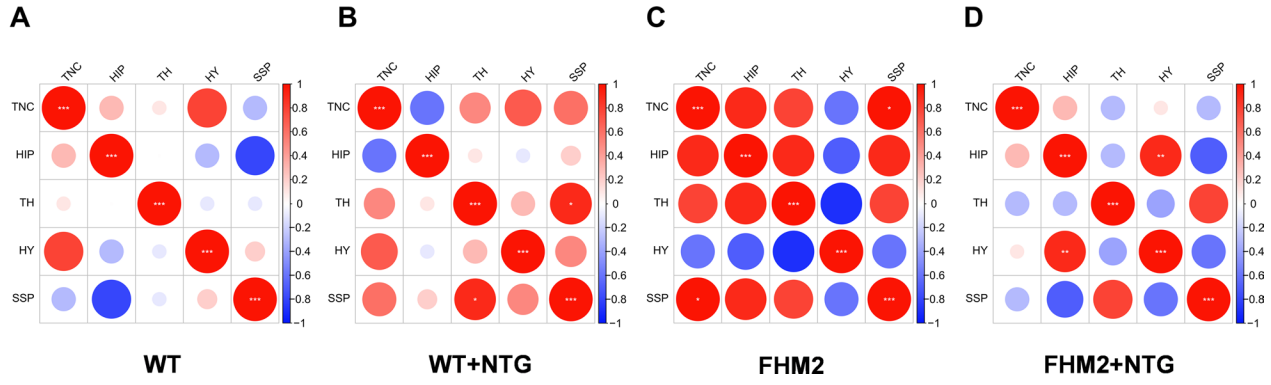

Fig. 7 Inter-Regional NAA Correlation in Four Groups of Mice. (**A**, **B**, **C**, and **D**) Correlation matrices between brain regions calculated using NAA concentrations. Positive correlations are shown in red, and negative correlations are shown in blue. Significant inter-regional correlations are marked with asterisks (*)

noted between SSP and TNC. Notably, although there was no significant difference in HY compared to other brain regions, it consistently showed a negative correlation trend (Fig. 7C). In the FHM2+NTG group, a positive correlation was found between HY and HIP (Fig. 7D). Through these analyses, we revealed the importance of the TNC-TH-SSP pathway in ascending pain transduction in migraine.

We demonstrated the changes in major metabolites in different brain regions following chronic migraine-like state mice. A considerable number of metabolites were upregulated or downregulated synchronously across various brain regions, consistent with global metabolic remodeling and widespread regulation of neural networks during chronic migraine. For instance, NAA decreases with the onset of migraine, and these changes are synchronous across four brain regions. Glutamate increases in multiple brain regions, while GABA continuously decreases in almost all brain regions. This result supports the hypothesis that central sensitization in chronic migraine-like state model of mice is driven by these metabolic changes. We hypothesize that during the transition from acute to chronic migraine-like state, reduced energy metabolism and Glu/GABA imbalance increase the brain's sensitivity to external stimuli, further enhancing the development of central sensitization (Fig. 8).

Discussion

Migraine is a neurovascular disease associated with metabolic alterations, including changes in neurotransmitter metabolites [49]. In this study, we used ^{1}H-MRS-based metabolomics to examine the metabolic changes in five different brain regions (TNC, HIP, TH, HY, and SSP) of WT and FHM2 mice following NTG-induced chronic migraine-like states. We also investigated the connectivity of related pathways based on interregional NAA correlation.

The results demonstrated significant metabolic changes in the brain tissues of WT+NTG and FHM2+NTG mice compared to their respective WT and FHM2 counterparts following the induction of a chronic migraine-like state. These changes encompassed energy metabolism, neurotransmitter metabolism, membrane metabolism, and astrocyte-neuron metabolism. Furthermore, these metabolic alterations were closely related to the TNC-TH-SSP pathways, as indicated by interregional NAA correlation.

Migraine and energy metabolism: pathophysiological links from neuronal activity to the trigeminal vascular system

Energy metabolism plays a critical role in maintaining normal brain function, and deficits in brain energy metabolin can lead to adaptations such as increased ATP synthesis under anaerobic conditions, often accompanied by elevated lactate levels [24]. Additionally, increased glutamate levels may lead to elevated lactate to protect against excitotoxicity.

In this study, significant reductions in energy metabolites such as AMP (adenosine monophosphate), IMP (inosine monophosphate), ADP (adenosine diphosphate), NAD+ (nicotinamide adenine dinucleotide), and Ino (inosine) in HIP and SSP were observed in WT+NTG and FHM2+NTG mice compared to WT and FHM2 mice. These findings suggest insufficient cellular energy availability. Consistently, various studies using MRS methods have reported decreased levels of energy metabolites in migraine with and without aura [50–54]. Therefore, it is hypothesized that decreased energy metabolism is a metabolic feature in the pathophysiology of chronic migraine-like state mouse model.

Furthermore, lactate concentrations increased in the TNC of WT+NTG mice but decreased in FHM2+NTG mice. Neuronal activity-induced lactate increases rapidly and transiently in response to migraine triggers such as hypoxia or intense light stimulation, suggesting increased

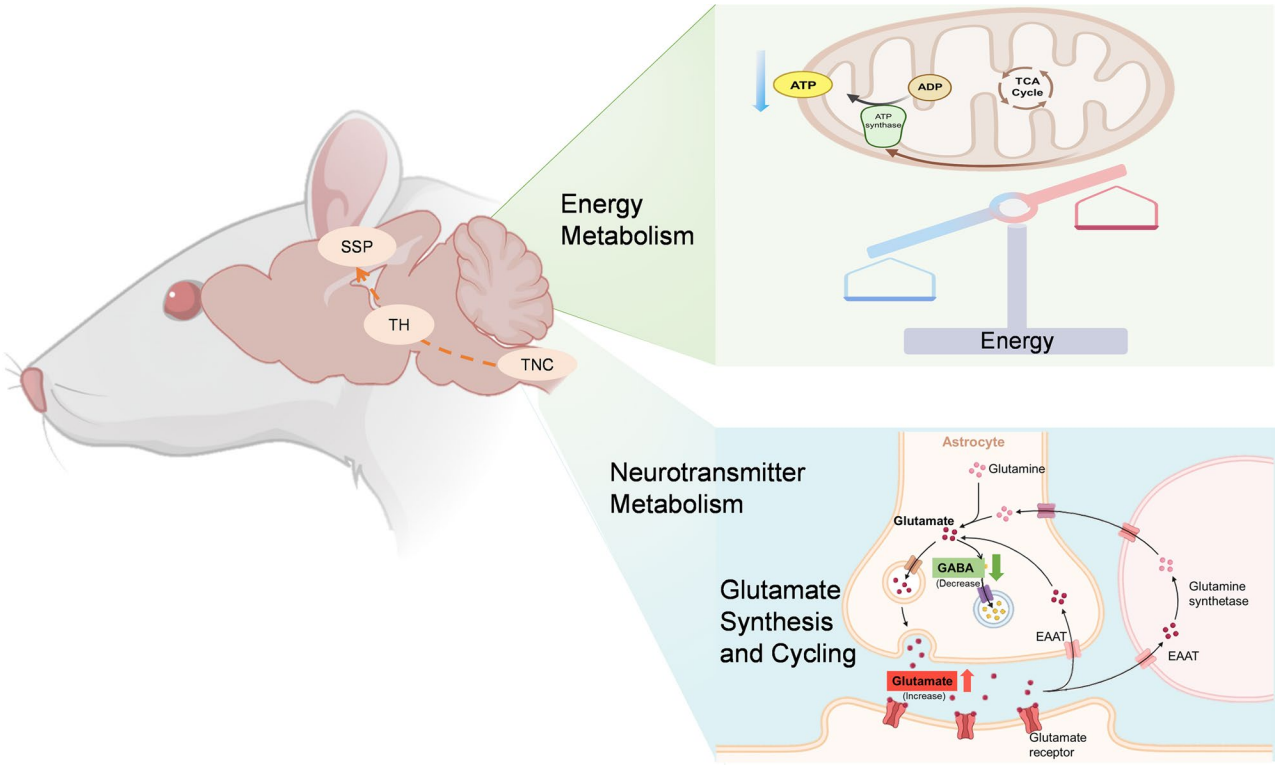

Fig. 8 Mechanisms of Central Sensitization in Chronic Migraine Involving Energy Metabolism and Glu. In the NTG-induced chronic migraine-like state mouse model, impaired energy metabolism and Glu/GABA imbalance are key mechanisms leading to neuronal hyperexcitability and central sensitization. Impaired energy metabolism reduces neuronal energy supply, increasing their vulnerability. Increased Glu and decreased GABA result in an excitation-inhibition imbalance, creating a state of hyperexcitability. These mechanisms collectively contribute to central sensitization and persistent pain in chronic migraine. (Created with BioRender)

brain energy demands and lowered thresholds for migraine onset [22]. TNC is a critical structure in nociceptive processing. Therefore, when pain stimuli transmit to TNC, neuronal activity increases, leading to increased lactate in both sides of the TNC, indicating bilateral activation of TNC neurons [55]. Interestingly, the relative decrease in lactate in the FHM2 group prompts further investigation into whether this phenomenon promotes or inhibits pain.

Studies by Harman et al. using MRS have found lower ATP levels in migraine patients with the highest attack frequency [56], suggesting an association between decreased brain metabolism and migraine susceptibility. Research by Ashina et al. highlighted ATP-sensitive potassium channels as a crucial pathway in migraine pathogenesis [57]. These channels are widespread in the trigeminal vascular system and play a role in its activation and metabolic stress response. The activity of ATP-sensitive potassium channels is regulated by intracellular ATP/ADP ratios and cAMP levels, supporting the hypothesis of mitochondrial energy metabolism's critical role in migraine pathogenesis [58]. Riboflavin and coenzyme Q10 are key components in regulating the mitochondrial respiratory chain and are recommended as effective prophylactic treatments for migraine [59]. In conclusion, changes in energy metabolism represent potential remodeling of metabolic processes in a chronic migraine-like state model of mice under central sensitization. Therefore, energy metabolism in migraine should be a focus of future research efforts.

Neurotransmitter imbalance in chronic migraine: the battle between excitatory and inhibitory activities

Neurotransmitter metabolic disorders can also impact normal brain function [33]. One potential mechanism for the altered neuronal excitability observed in migraine involves the abnormal release of excitatory neurotransmitters, which may either predispose to headache attacks or be a consequence of chronic migraine [47]. Neurotransmission is primarily regulated by excitatory amino acids (glutamate, aspartate) and inhibitory amino acids (GABA, glycine, and taurine).

In this study, we found that excitatory neurotransmitters (glutamate, aspartate, glutamine) increased across most brain regions in mice with a chronic migraine-like state, while inhibitory neurotransmitters (GABA, glycine, and taurine) tended to decrease. Glutamine (Gln), a metabolic product of the excitatory neurotransmitter

glutamate, is specifically produced in astrocytes under the catalysis of glutamine synthetase [60]. The increase in glutamine observed in our study may result from the elevated glutamate levels in FHM2+NTG mice, as glutamate increase is a key factor in the overexcitability due to the mutated glutamate transporter-1 receptor in FHM2 mice. We observed that FHM2 mice had higher glutamate levels in brain regions such as TNC and TH compared to WT mice. These data support the mechanism of coupling between the TNC and TH regions and the glutamate system in FHM2, and are associated with a reduced glutamate clearance rate in the synaptic cleft by astrocytes. The reduced glutamate clearance rate could explain the increased susceptibility of FHM2 mice to experimentally induced CSD, resulting in central sensitization [61, 62]. Additionally, previous studies have reported a phenomenon of glutamate plume in FHM2 mice during migraine, characterized by excessive glutamate accumulation [63], indicating that the excessive glutamatergic transmission and the dysfunction in the regulation of excitatory/inhibitory balance may be a common feature of the FHM2 brain [62].

Based on these findings, we hypothesize that WT+NTG and FHM2+NTG mice exhibit heightened excitatory neurotransmitter metabolism and reduced inhibitory neurotransmitter levels during a chronic migraine-like state, leading to an excitatory-inhibitory imbalance in the brain [64]. When pain stimuli are transmitted to the TNC, increased Glu and decreased GABA lower the pain threshold, enhancing TNC excitability, thereby accelerating the activation of the trigeminal vascular pain pathway and its upward transmission. Similarly, ^{1}H-MRS studies in migraine have reported increased cortical glutamate and decreased GABA levels, indicating heightened sensitivity to excitatory input and reduced inhibitory mechanisms in chronic migraine. Thus, we propose that mice in a chronic migraine-like state exhibit a significant excitatory-inhibitory imbalance in neurotransmitter activities, particularly characterized by elevated Glu and reduced GABA [65, 66], contributing to the pathophysiology of migraine central sensitization.

Abnormal membrane metabolism and astrocyte dysfunction in chronic migraine-like state models

Glycerophosphocholine (GPC), phosphocholine (PC), and choline (Cho) metabolism play crucial roles in the synthesis of cell membrane phospholipids. Boska et al. reported that abnormal brain phospholipids might represent an underlying pathogenic mechanism of migraine [67]. In this study, we found that chronic migraine-like state model is associated with abnormal Cho metabolism in the brains of WT+NTG and FHM2+NTG mice. In our study, we observed a correlation between changes in Cho levels and the concentration of N-acetylaspartate (NAA) in certain brain regions, such as the TNC and TH. NAA is typically regarded as a marker of neuronal health and function, with decreased concentrations often associated with impaired neuronal function or reduced neuronal density. Therefore, the observed correlation between Cho levels and NAA concentration may further support the link between membrane integrity alterations and neuronal damage. Specifically, in the TNC region, we found an increase in Cho levels accompanied by a decrease in NAA, which could indicate damage to the neuronal membrane structure in this area, subsequently affecting neuronal health. Myo-inositol and Tau are considered markers of astrocytes and play key roles in brain osmoregulation [68–70]. In FHM2+NTG mice, myo-inositol levels were significantly reduced in the TNC, TH, and HY regions. In WT+NTG mice, myo-inositol concentrations were significantly lower in the TNC, HIP, TH, and HY regions. These findings suggest astrocyte impairment, consistent with Capuani and Patrick's reports of glutamate clearance defects in FHM2 transgenic mice [62, 63]. Additionally, MRS studies have shown decreased myo-inositol levels in the brains of migraine patients [51]. Interestingly, taurine levels in the cerebrospinal fluid of migraine patients were elevated compared to controls [71, 72], indicating a close link between migraine pathology and myo-inositol and taurine regulation. These results highlight the potential importance of myo-inositol and taurine in migraine pathology and underscore the need for further research into their regulation in migraine models.

Reduced NAA concentration reveals neuronal function and energy metabolism in migraine pathophysiology

Our study also found that NAA (N-acetylaspartate) levels were decreased in several brain regions of WT+NTG mice, indicating potential neuronal dysfunction and mitochondrial impairment following migraine induction. NAA is widely considered a marker of neuronal and axonal integrity and indicates mitochondrial function since it is synthesized in neuronal mitochondria [73, 74]. The synthesis rate of NAA is highly linearly correlated with the ATP synthesis rate, suggesting its close relationship with metabolic energy [75]. The reduction in NAA concentration may be caused by various factors, including decreased synthesis, increased catabolism, neuronal damage, and reduced mitochondrial density [76]. Previous studies have also reported lower NAA levels in the brains of migraine patients, suggesting that NAA could serve as a biomarker for migraine [49, 77]. The TNC is a critical region for nociception, and with repeated stimulation in chronic pain, neurons become damaged, leading to metabolic energy disorders. This results in a decrease in NAA levels in the chronic migraine-like state model. These findings suggest that changes in NAA may be

an important indicator of neuronal health and energy metabolism in the migraine pathological process. Therefore, further investigation into the role and mechanisms of NAA in migraine could enhance our understanding of migraine pathophysiology and potentially provide new therapeutic targets.

Regional NAA correlation reveals dysfunctional SSP-TH-TNC pathway in chronic migraine

In chronic migraine, the interaction of regional NAA correlation networks might be regulated through specific neurotransmitters affecting NAA metabolism [32]. Studies have found that the thalamus is structurally and functionally connected to the somatosensory cortex (SSP), and functional connectivity involving these regions is altered in migraine [78]. The observed NAA correlation pathways between the TNC-TH-SSP further support the notion of neuronal dysfunction and reorganization of pain pathways in mice with a chronic migraine-like state. Eising et al. identified specific brain regions and pathways potentially involved in migraine pathophysiology by integrating migraine genome-wide association study data with gene expression data from the Allen Human Brain Atlas [79]. Their findings suggested that migraine-related genes are involved in the energy supply of the cortex and thalamus, aligning with our results. This supports the idea that reduced NAA metabolism and altered regional NAA correlations indicate a dysfunctional TNC-TH-SSP pathway, playing a significant role in the chronic migraine-like state mouse model. Overall, the reduction in NAA metabolism and changes in regional NAA correlation in chronic migraine-like state mouse model support the involvement of a dysfunctional TNC-TH-SSP pathway in the chronicity of migraine, emphasizing the importance of neuronal energy metabolism and connectivity in the pathophysiology of this condition.

Metabolic changes in brain regions and their association with trigeminal vascular activation

In our study, we focused on brain regions relevant to different stages of migraine attacks, particularly the TNC and thalamus [80, 81]. According to current theories on trigeminovascular activation in migraine, activation of the trigeminal nucleus in the brainstem leads to vasodilation and release of vasoactive neuropeptides, transmitting central pain signals up to the thalamus [82] Metabolic disruptions in energy metabolism and increased neuronal excitability in the TNC enhance the transmission of pain signals and central sensitization [4, 81, 83]. Thus, besides serving as a relay point to the thalamus, the TNC plays a significant facilitating role in transmitting pain signals to the thalamus. Our findings highlight TNC and thalamus as primary brain regions where metabolic changes reflect alterations in neuronal biochemical responses during central sensitization. These metabolic changes in these regions closely correlate with different stages of migraine attacks, further supporting the trigeminovascular theory of migraine [25, 33]. Furthermore, the FHM2 G301R mutation affects the function of sodium-potassium pump, resulting in changes in neuronal excitability, which plays a critical role in the pathogenesis of migraines. Lieke Kros and colleagues demonstrated that G301R mutant mice exhibit heightened susceptibility to cortical spreading depression (CSD) [84]. Utilizing the FHM2 model allows us to gain deeper insights into the neurobiological underpinnings of migraine. Future studies could choose multiple time points and brain regions for measuring absolute concentrations of metabolites [85, 86]. By using more detailed time sequences and spatial resolutions, these studies could investigate the causal relationships between neural activity and metabolic dysregulation more deeply, aiming to understand the lasting effects of metabolic changes on central sensitization following the chronicity of migraine.

We acknowledge certain limitations in this research. First, the experiments were conducted exclusively with male mice, excluding females. Given that migraine is more prevalent in women, including female mice in future studies may provide a more comprehensive understanding of gender differences. Additionally, the absence of a vehicle control group could impact the overall comprehensiveness and reliability of the experimental results. Therefore, including a vehicle control group in future studies will help us more thoroughly assess the pathophysiological mechanisms of migraine.

Conclusion

This study utilized ^{1}H-MRS metabolomics to investigate region-specific metabolic changes in the brains of chronic migraine-like state model during the process of central sensitization, revealing the potential physiological effects of chronic migraine. Our results indicate that the trigeminal nucleus caudalis (TNC) and thalamus are the most significantly affected regions, and changes in NAA levels may serve as potential indicators of migraine onset and progression. The consistency of NAA suggests that the TNC-TH-SSP pathway is an important ascending nociceptive transmission route in migraine. The decline in energy metabolism and the imbalance between excitatory and inhibitory neurons involving glutamate and GABA may be key factors in the chronic progression of migraine.

Abbreviations

ATP	adenosine triphosphate
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
Cho	Choline
FHM2	Familial hemiplegic migraine type 2
GABA	Gamma-aminobutyric acid

Glu	Glutamate
Gly	Glycine
GPC	Glycerophosphocholine
HIP	Hippocampus
HY	Hypothalamus
IMP	Inosine monophosphate
Ino	Inosine
Lac	Lactate
Myo	Myo-inositol
NAA	N-acetylaspartate
NAD+	Nicotinamide adenine dinucleotide
NTG	Nitroglycerin
MRS	magnetic resonance spectroscopy
PC	Phosphocholine
SSP	Somatosensory cortex
Suc	Succinate
Tau	Taurine
TH	Thalamus
TNC	Trigeminal nucleus caudalis
WT	Wild-type

Supplementary Information

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Supplementary Material 1

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Author contributions
This study was designed by YGW, JSG, and JGG. JGG and CLZ participated in behavioral testing and Sample Collection. JW and DW performed the statistical analysis. JGG, ZCL, and DW write the manuscript. XR, TXW, KBZ, YHX and CP revised the manuscript. XR and YGW provided supervision and a final check. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments performed in this study were approved by the Animal Ethics Committee of ShanghaiTech University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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