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1 **Migraine Chronification Reshapes Brain-Wide Neurovascular Activity During**
2 **Acute Attacks**

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12 **Abstract**

13 **Background**

14 Migraine attacks represent dynamic transitions between distinct brain states, involving
15 coordinated changes in neuronal activity, trigeminovascular signaling, and
16 cerebrovascular regulation. Although migraine chronification is associated with
17 persistent sensory sensitization and altered brain network organization, whether
18 chronic migraine reshapes the brain-wide neurovascular dynamics underlying acute
19 attacks remains unknown. Nitroglycerin (NTG) is a clinically relevant translational
20 model that reproduces migraine-like attacks and sensitization; however, previous
21 studies have primarily examined regional cortical, meningeal, or large-vessel
22 responses, leaving the temporal organization of whole-brain hemodynamic responses

unresolved. In this study, we investigated whether migraine chronification alters the dynamics and network organization of acute attack-related neurovascular responses.

Methods

A repeated NTG administration paradigm was used to establish a chronic migraine (CM) model, with vehicle-pretreated mice serving as the vehicle (Veh) group.

Migraine-related behaviors were evaluated using the mechanical withdrawal threshold, light–dark box testing, and phenol red thread assessment. Separate cohorts underwent continuous whole-brain four-dimensional functional ultrasound (4D fUS) imaging or electroencephalographic (EEG) recordings. Following a 15-min baseline period, an acute NTG challenge was administered, followed by 45 min of continuous recording. Whole-brain cerebral blood volume (CBV) dynamics, hemodynamic functional connectivity, and the temporal relationship between CBV and cortical gamma band activity were analyzed.

Results

Repeated NTG exposure induces mechanical hypersensitivity, light avoidance, and increased light-evoked lacrimation. Four-dimensional functional ultrasonography revealed that migraine chronification altered both cerebral hemodynamic recovery and brain-wide network organization during acute NTG-evoked attacks. CM mice show attenuated hyperemia and prolonged recovery from hypoperfusion-dominant CBV changes. Functional connectivity analysis revealed that the resting-state network was remodelled in CM mice. Early attack-related network changes in EM-like mice partially resembled the baseline network state in CM mice, suggesting a transition

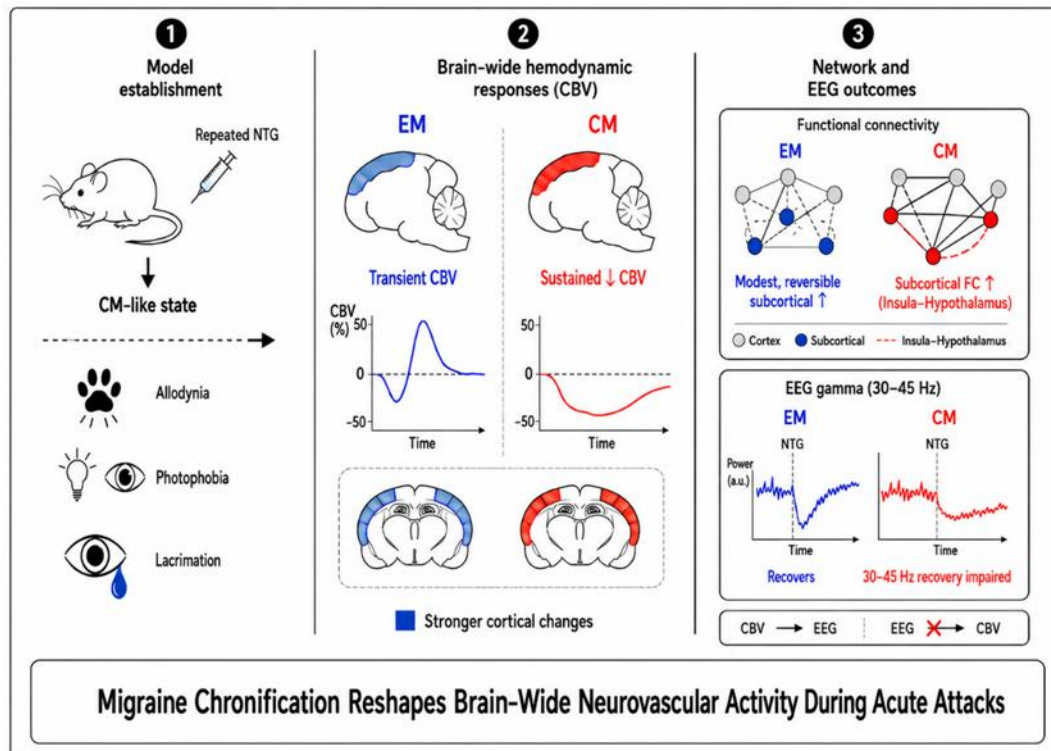
from transient ictal network perturbation to stable baseline remodeling. During attack evolution, CM mice display phase-specific reorganization of selected sensory-autonomic, thalamic-subcortical, and affective/mnemonic connections. Parallel EEG-ECG recordings showed transient cardiac slowing, reduced HRV, and delayed cortical γ -band recovery. These findings highlight impaired neurovascular recovery and phase-resolved network reconfiguration as the central features of migraine chronification.

Conclusions

Migraine chronification reshapes the brain-wide response to acute NTG challenge, which is characterized by a prolonged cortex-predominant CBV reduction, selective functional connectivity reorganization, and delayed cortical electrophysiological recovery. These findings identify impaired neurovascular recovery and altered hemodynamic-neuronal coordination as key features of migraine chronification, providing a dynamic brain-state framework for understanding the pathophysiology of chronic migraine.

Keywords: chronic migraine; functional ultrasound imaging; cerebral hemodynamics; functional connectivity; electroencephalography; neurovascular dynamics.

Graphic Abstract



BACKGROUND

Migraine is a prevalent and disabling neurological disorder characterized by recurrent attacks involving dynamic interactions between neuronal activity, trigeminovascular signaling, and cerebrovascular regulation. The Global Burden of Disease Study 2023 estimated that migraines affect approximately 14.1% of the global population and are the leading contributors to headache-related disability worldwide(1). Chronic migraine (CM), affecting approximately 1–2% of the population, represents a particularly disabling disease state in which episodic migraine (EM) progressively transforms into a condition characterized by frequent attacks, persistent sensory abnormalities, and reduced responsiveness to conventional therapies(2). Historically, migraine has been considered primarily a vascular disorder. However, substantial evidence has shifted this concept toward a disorder of distributed neural networks involving the trigeminovascular system, hypothalamus, brainstem,

thalamus, and cortex(3–5). Nevertheless, the cerebral circulation remains an essential component of migraine biology because neuronal activity, metabolic demand, vascular tone, and cerebral blood volume (CBV) are dynamically coupled through neurovascular mechanisms(6). Thus, migraine attacks should not be viewed solely as transient episodes of nociceptive activation but rather as dynamic transitions between distinct brain states involving coordinated neural and vascular responses.

The progression from EM to CM represents more than an increase in the attack frequency. Repeated attacks may induce persistent alterations in trigeminovascular excitability, sensory processing, autonomic regulation, and vascular responsiveness(7,8). However, the physiological changes that occur when an acute migraine-like attack is superimposed on the chronically sensitized brain are poorly understood. Specifically, it remains unknown whether migraine chronification alters the magnitude, spatial organization, temporal evolution, or recovery capacity of brain-wide neurovascular responses during acute attacks.

Nitroglycerin (NTG), a nitric oxide (NO) donor, has been used as a clinically relevant experimental model of migraine for more than three decades. In humans, NTG induces an immediate nonspecific headache and, particularly in individuals with migraine susceptibility, can trigger a delayed migraine-like attack accompanied by characteristic symptoms, including photophobia, nausea, and autonomic manifestations(9,10). Therefore, NTG is not merely a nociceptive stimulation paradigm, but also a translational model that probes the mechanisms underlying migraine attack initiation and susceptibility.

The historical interpretation of NTG-induced migraine responses was initially influenced by vascular theories, because NO is a potent vasodilator. However, clinical studies have challenged the hypothesis that migraine attacks are caused by excessive cerebral vasodilation. Transcranial Doppler and single-photon emission computed tomography studies have demonstrated that NTG reduces middle cerebral artery flow velocity without consistent alterations in regional cerebral blood flow (CBF), indicating that large-vessel flow velocity does not directly reflect tissue perfusion(11). Positron emission tomography further revealed heterogeneous regional perfusion responses following NTG administration, including increased perfusion in the anterior cingulate cortex and decreased perfusion in the occipital cortex(12). Moreover, magnetic resonance angiography demonstrated that NTG-induced intracranial and extracranial arterial dilation resolved before the development of a delayed migraine-like headache(13).

These findings fundamentally change the interpretation of NTG responses, shifting the field from a simple vascular dilation model to a framework in which migraines involve dynamic interactions between neuronal excitability and vascular regulation. Therefore, the NTG model provides an opportunity not only to investigate pain-related mechanisms but also to examine how the brain transitions into and recovers from a migraine-like attack state.

Preclinical studies have demonstrated that NTG-induced vascular responses are highly heterogeneous and depend on species, vascular compartment, brain region, and observation period. In rodents, NTG produces distinct responses in the meningeal and

cortical circulation, including transient reductions, followed by increases in dural blood flow and delayed cortical hyperemia(14). Two-photon imaging further revealed opposing responses between the meningeal and cortical microvessels after NTG administration, emphasizing that vascular responses cannot be interpreted without considering spatial and temporal context(15).

Repeated intermittent NTG administration has established a widely used migraine chronification model. This paradigm induces progressive basal mechanical hypersensitivity and enhances responsiveness to subsequent NTG challenges, providing an experimental framework for investigating the mechanisms underlying the transition from episodic to chronic migraine-like sensitization(16). Subsequent studies have demonstrated that NTG responses vary substantially according to experimental conditions and biological compartment examined(17,18).

Recent investigations have used advanced imaging and electrophysiological approaches to characterize NTG-induced alterations, including laser speckle contrast imaging of cortical blood flow, optical imaging of vascular responses, and local field potential recordings of cortical activity(19–21). However, these approaches largely focused on isolated cortical regions, meninges, and major cerebral arteries.

Consequently, a fundamental question remains unresolved: Does migraine chronification reshape the entire brain-wide neurovascular response pattern during an acute attack and, if so, which component of the response is altered? In particular, it remains unknown whether chronification enhances the initial attack response or disrupts the subsequent recovery process to restore brain homeostasis.

Addressing this question requires a method capable of simultaneously capturing the spatially distributed and temporally evolving hemodynamic responses throughout the brain. Functional ultrasound imaging (fUS) provides such an opportunity by enabling high-resolution measurement of cerebral blood volume dynamics across large-scale brain networks without exogenous contrast agents. Compared to conventional neuroimaging approaches, four-dimensional functional ultrasound (4D fUS) allows continuous volumetric acquisition of whole-brain hemodynamic signals, overcoming temporal mismatches caused by sequential scanning approaches(22–24). The combination of whole-brain coverage, high temporal resolution, microvascular sensitivity, and long-duration recording makes 4D fUS particularly suitable for studying dynamic brain-state transitions. Although fUS measures hemodynamic signals directly rather than neuronal activity, quantitative relationships between fUS signals and neuronal calcium activity have been established, supporting its application in investigating neurovascular dynamics(25). Combining fUS with electrophysiological measurements provides complementary information on the vascular and neuronal components of migraine-like attacks.

In this study, we used whole-brain 4D fUS imaging combined with EEG to determine how migraine chronification reshapes neurovascular dynamics during an acute NTG challenge. We hypothesized that chronification would not simply amplify the magnitude of the vascular response, but would also impair the coordinated recovery of brain-wide neurovascular states following an acute migraine-like attack.

METHODS

Animals

The sample size and sex of the animals were determined on the basis of previous studies using NTG-induced migraine models. Female mice were selected because migraine disproportionately affects women, and repeated NTG administration has been shown to induce robust and sustained migraine-like hypersensitivity in female C57BL/6 mice(16,26). Specific pathogen-free female C57BL/6 mice (8–10 weeks old, weighing 20–25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed under controlled conditions at a temperature of 22–26°C and relative humidity of 40–60%, with a 12-h light/dark cycle and ad libitum access to food and water. Prior to the experiments, the mice were allowed to acclimatize to the housing environment for 1 week. Eight mice per group were included in the behavioral experiments, consistent with previous NTG model studies that assessed cephalic and hind paw mechanical hypersensitivity(27,28). Mice were randomly assigned to experimental groups using random numbers generated in Microsoft Excel, and behavioral assessments were conducted by two investigators blinded to group allocation. All animal procedures were approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, and were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals(Y2024-10-08-02).

Experimental protocol and groups

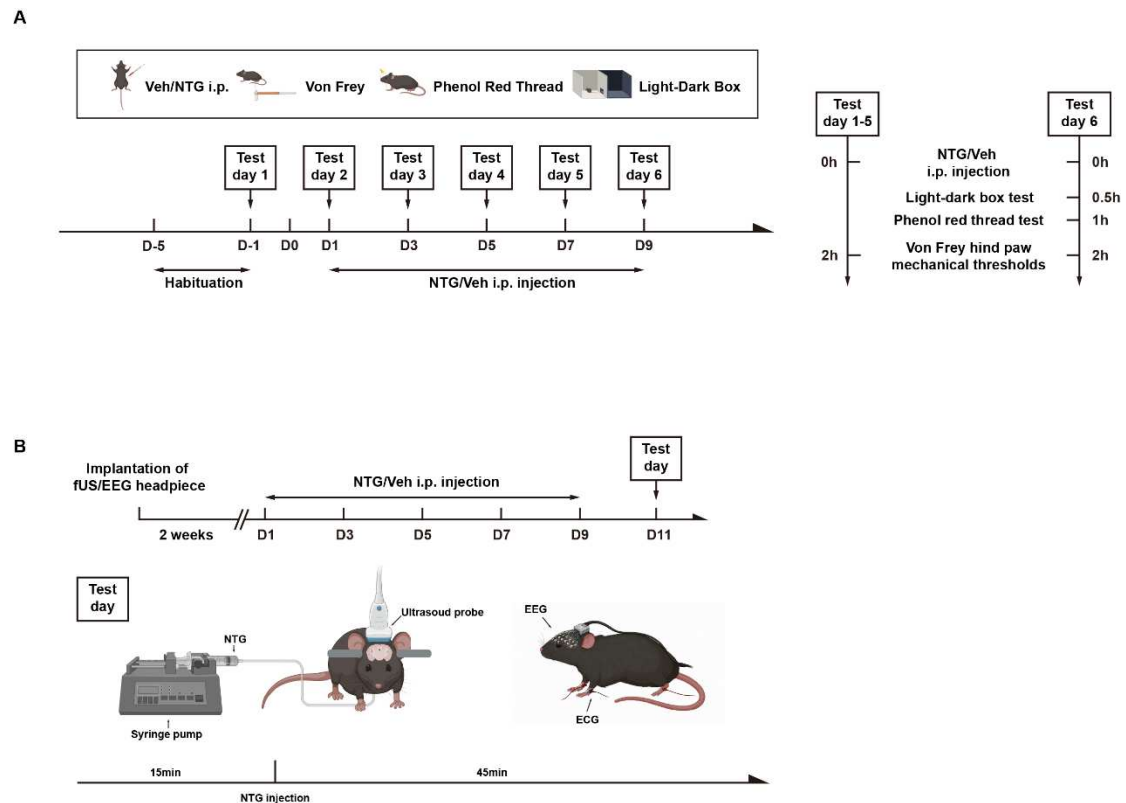


Figure 1. Experimental design for behavioral assessment, fUS and EEG recording.

(A) Experimental timeline for repeated nitroglycerin (NTG) administration and behavioral testing. After habituation from D-5 to D-1, baseline behavioral measurements were obtained on D-1. Mice subsequently received intraperitoneal injections of NTG or Veh every other day on D1, D3, D5, D7, and D9. Hind-paw mechanical withdrawal thresholds were assessed using the von Frey test 2 h after each injection. Following the final injection, photophobia-like behavior was evaluated using the light–dark box test at 0.5 h, lacrimation was measured using the phenol red thread test at 1 h, and mechanical withdrawal thresholds were assessed at 2 h.

(B) Experimental timeline for fUS imaging and electroencephalography recording.

The corresponding fUS or EEG headpiece was implanted 2 weeks before initiation of

the NTG/Veh regimen. On D11, vehicle-pretreated mice representing the episodic migraine-like state and repeatedly NTG-treated mice representing the chronic migraine-like state received an acute NTG challenge. After a 15-min baseline period, NTG was administered intraperitoneally, followed by 45 min of recording. fUS imaging (n = 5 per group) and synchronous EEG/ECG recording (n = 6 per group) were performed in separate cohorts. NTG, nitroglycerin; Veh, vehicle; i.p., intraperitoneal; fUS, functional ultrasound; EEG, electroencephalography; ECG, electrocardiography.

Part I

Mice (n = 8 per group) were randomized into chronic migraine (CM) and vehicle (Veh) groups. The CM group received intraperitoneal injections of NTG on days 1, 3, 5, 7, and 9, whereas the vehicle group received an equivalent volume of vehicle (VEH). Hindpaw mechanical withdrawal thresholds were assessed using the von Frey test at baseline (day -1) and on each injection day. On day 9 after modeling was completed, all mice underwent the light-dark box test (LDT) and phenol red thread test (PRT) to evaluate photophobia and lacrimation, respectively (Figure 1A).

Part II

Two independent cohorts of mice were used for whole-brain 4D functional ultrasound imaging (n = 5 per group) and EEG (n = 6 per group). Within each cohort, the mice were randomized into the CM and Veh groups. The respective fUS or EEG headpieces were implanted two weeks before modeling. The CM group received intraperitoneal

injections of NTG on days 1, 3, 5, 7, and 9, whereas the vehicle group received an equivalent volume of VEH. On day 11, mice in the two independent cohorts underwent either whole-brain 4D fUS imaging or synchronous EEG/ECG recording, respectively. After a 15-min baseline recording, all mice received an acute NTG challenge during continuous acquisition, and fUS or EEG/ECG recording was continued for an additional 45 min (Figure 1B). Both groups received an acute intraperitoneal NTG challenge at 10 mg/kg using the same 1 mg/mL preparation, corresponding to an injection volume of 10 mL/kg. NTG was administered using a syringe pump at 0.2 mL/s.

Establishment of the chronic migraine model

The CM model was established as described previously¹⁶. A commercial nitroglycerin injection (NTG; 5 mg/mL; Beijing Yimin Pharmaceutical Co., Ltd., Beijing, China) containing nitroglycerin, anhydrous ethanol, propylene glycol, and water was freshly diluted with sterile 0.9% saline to a final concentration of 1 mg/mL. NTG was administered intraperitoneally at 10 mg/kg every other day for 9 days, with injections performed on days 1, 3, 5, 7, and 9. The vehicle mice received an equivalent volume of 0.9% saline via the same route and schedule(29).

Behavioral tests

Hindpaw mechanical withdrawal threshold

The 50% hind paw mechanical withdrawal threshold was determined using calibrated von Frey filaments and the up–down method. Before testing, mice were individually placed in transparent Plexiglas chambers on an elevated wire mesh platform and

allowed to acclimatize for 30 min. Two hours after each intraperitoneal injection of NTG or VEH, a von Frey filament was applied perpendicular to the central plantar surface of the hind paw, avoiding the footpads, until the filament bent slightly and was maintained for 3–5 s. Rapid paw withdrawal, shaking, or licking was considered a positive response. Subsequently, a filament with a lower force was applied following a positive response, whereas a filament with a higher force was applied following a negative response. After the first response reversal, four additional stimulations were performed in the same sequence. The 50% hindpaw mechanical withdrawal threshold was calculated using a response pattern.

Light-dark box test (LDT)

The light–dark box test was used to assess migraine-associated light-aversion behavior, a behavioral surrogate of photophobia in mice. The apparatus consisted of equally sized interconnected light and dark compartments. The light compartment was illuminated by a white LED panel, providing an illuminance of 1000 lx at floor level, whereas the black-enclosed dark compartment was maintained at less than 5 lx. Thirty minutes after the intraperitoneal injection of NTG or VEH on the test day, each mouse was gently placed in the center of the light compartment, facing away from the entrance to the dark compartment, and allowed to explore both compartments freely for 10 min. An automated video-tracking system was used to record the time spent in each compartment, latency to first entry into the dark compartment, number of transitions between compartments, total distance travelled, and immobility time. The time spent in the light compartment and percentage of the total test duration were

defined as the primary outcomes, with lower values indicating greater light aversion. Eye closure, reduced locomotion, and rest in the corner of the dark compartment were recorded as supportive behavioral observations. After each trial, the apparatus was cleaned with 75% ethanol and allowed to dry completely prior to testing the next mouse(30).

Phenol red thread test

A modified phenol red thread test was used to assess the light-evoked lacrimation. Mice were tested under light inhalational anesthesia maintained with 0.5–1% isoflurane. A 10-mm segment of phenol red thread (Tianjin Jingming New Technological Development Co., Ltd., Tianjin, China) was gently placed in the lateral one-third of the lower conjunctival fornix of the tested eye to avoid contact with the cornea. A handheld clinical examination penlight, routinely used for funduscopy examination, was used as the light source, and the pupil of the tested eye was illuminated for 15 s at a fixed distance and angle. The thread was immediately removed and the length of the wetted segment, which changed from yellow to red, was measured in millimeters as a relative index of light-evoked tear secretion.

Functional ultrasound imaging

Two weeks before the first NTG/VEH injection, mice underwent implantation of an fUS-compatible headplate and were allowed to recover. On day 11, the mice (n = 5 per group) were maintained under light inhalation anesthesia with 1–1.5% isoflurane and secured using the implanted headplate. Whole-brain fUS data were acquired using the Iconeus One system equipped with an IcoPrime-4D multi-array probe (Iconeus,

Paris, France). The probe was acoustically coupled to the cranial window with ultrasound gel and positioned using a four-axis motorized platform. Data acquisition was controlled using the IcoScan software. Ultrafast tilted-plane waves were compounded to generate Power Doppler volumes, and motorized scanning enabled repeated volumetric coverage of the mouse brain. After a 15-min baseline recording, NTG was administered intraperitoneally using a syringe pump without interrupting the acquisition. Whole-brain fUS imaging was continued for 45 min. The isoflurane concentration was maintained within the same range throughout the recording to minimize anesthesia-related variations in cerebral hemodynamics(23).

Image processing

Raw ultrasound data were reconstructed and processed using the Iconeus software suite. Tissue- and motion-related clutter were separated from blood-flow signals using spatiotemporal singular-value decomposition filtering, and the filtered Doppler energy was integrated to generate Power Doppler volumes. The data acquired by the four subarrays at successive motor positions were combined into a four-dimensional whole-brain Power Doppler time series. Volumes affected by marked motion or the loss of acoustic coupling were excluded. The time-averaged vascular volume was automatically registered to the Allen Mouse Brain Common Coordinate Framework using the Brain Positioning System in IcoStudio, followed by visual inspection and manual adjustment when required. Registered datasets were processed in batches using IcoLab(23).

Brain-wide CBV analysis

Power Doppler intensity was used to measure the local cerebral blood volume (CBV). For each atlas-defined brain region, the mean Power Doppler signal was extracted at every time point. The mean signal recorded during the 15-min preinjection period was used as the baseline, and the relative CBV changes were calculated as follows:

$$\Delta CBV(t) = \frac{CBV(t) - CBV_{baseline}}{CBV_{baseline}} \times 100\%.$$

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Brain-wide analyses were performed across atlas-defined cortical and subcortical regions, followed by focused analyses of the primary motor cortex (MOp), primary somatosensory cortex (SSp), auditory cortex (AUD), visual cortex (VIS), posterior parietal association cortex (PTLp), anterior cingulate cortex (ACA), and agranular insular cortex (AI). The magnitude and latency of the post-injection CBV nadir, subsequent peak response, nadir-to-peak interval, duration of CBV below baseline, negative area under the curve, and time required to recover 90% of the baseline were calculated for each regional CBV time course.

Hemodynamic functional connectivity analysis

For each mouse, the raw functional ultrasound data were processed using IcoLab. Functional connectivity matrices were generated for 108 atlas-defined brain regions and for 19 bilateral regions of interest (ROIs), which were anatomically defined by merging the corresponding left- and right-hemisphere regions according to the registered brain atlas. Connectivity matrices were calculated for four prespecified 15-min periods: baseline (0–15 min), early (15–30 min), middle (30–45 min), and late

(45–60 min). For the hemodynamic correlation analysis, Pearson correlation coefficients between ROIs were transformed using Fisher's *r*-to-*z* transformation to improve normality before group-level statistical testing. Between-group differences in Fisher-*z*-transformed connectivity values were assessed using two-sided independent-samples *t*-tests, whereas NTG-induced changes relative to the corresponding baseline resting state were assessed within each group using two-sided paired-samples *t*-tests. The individual mouse was considered the experimental unit(31).

Electroencephalographic (EEG) signal acquisition

Two weeks before the first NTG/VEH injection, the mice were implanted with a 16-channel cortical electroencephalographic/electrocorticographic array (Kedou, China). The array contained 16 cortical recording contacts, an independent reference electrode, and a ground electrode. Reference and ground electrodes were positioned over the right and left posterior skulls, respectively. The electrode array was terminated with a Blackrock-compatible 16-channel Omnetics connector and connected to an Omnetics headstage and a Blackrock neural signal acquisition system (CerePlex Direct; Blackrock Neurotech, Salt Lake City, UT, USA). On day 11, EEG signals were continuously recorded from light inhalation anesthesia with 1–1.5% isoflurane mice using Central Software Suite (Blackrock Neurotech). After a 15-min baseline recording, NTG was administered intraperitoneally without interrupting data acquisition, and EEG recording was continued for an additional 45 min. Signals from all 16 channels were acquired simultaneously at a sampling rate of 1000 Hz. The injection time was defined as 900 s from the beginning of recording. Continuous

signals were stored in the native Blackrock NS2 format and exported as European Data Format files for subsequent offline analysis. Electrode impedance and signal quality were checked before each recording, and the same reference configuration was maintained throughout the experiment.

Continuous EEG signals were processed offline in MATLAB R2023a using EEGLAB (Delorme and Makeig, 2004). Recordings were analyzed at the original sampling rate of 1,000 Hz. Flat or persistently noisy channels and segments containing prominent nonphysiological artifacts were identified and removed using the EEGLAB procedure, after which the retained channels were re-referenced to their common average. Power-line interference was attenuated using a zero-phase finite impulse response band-stop filter at 49–51 Hz. The cleaned signals were subsequently filtered at 30–45 Hz using the EEGLAB function to obtain gamma-band activity. Gamma-band power was calculated as the squared magnitude of the Hilbert-derived analytic signal, smoothed using a 2-s moving-average window, and normalized using the mean and standard deviation of the 0–900-s preinjection baseline. The normalized time series were averaged into 0.6-s bins for subsequent statistical and hemodynamic–electrophysiological analyses. EEGLAB preprocessing and FIR filtering procedures were implemented according to established methodological recommendations(32).

Electrocardiogram (ECG) Recording

On day 11, electrocardiographic (ECG) signals were continuously recorded from light inhalation anesthesia with 1–1.5% isoflurane mice concurrently with EEG acquisition.

Three surface electrodes were attached to each mouse, with the positive electrode

placed on the left hindlimb, the negative electrode on the right forelimb, and the ground electrode on the right hindlimb. ECG signals were acquired using an Animal Bio Amp system and LabChart software (version 8.0; AD Instruments, New South Wales, Australia). After a 15-min baseline recording, NTG was administered intraperitoneally without interrupting data acquisition, and ECG recording (filter reference: high-pass, 100 Hz; low-pass, 1 kHz; sampling frequency, 4 kHz/s) was continued for an additional 45 min. The injection time was defined as 900 s from the beginning of recording. The ECG analysis module automatically identified stable cardiac cycles, which were aligned based on the QRS peaks and averaged to generate a representative waveform. The heart rate was calculated from the RR intervals, whereas the PR intervals and QRS durations were determined from the averaged ECG waveform. All HRV-related data were calculated using LabChart's HRV module based on the aforementioned parameters.

Time-lagged prediction of cortical gamma-band activity from regional CBV dynamics

Regional CBV–EEG temporal relationships were evaluated separately in the Veh and CM groups using a custom Python time-lagged prediction pipeline. Because fUS and EEG recordings were obtained from independent cohorts, regional CBV and 30–45 Hz EEG power were first averaged across animals within each group. The resulting group-mean time series were aligned by nearest-neighbor timestamp matching at a common temporal resolution of approximately 0.6 s. Each of the 19 regional CBV time series was then analyzed separately as a predictor of subsequent group-averaged

397 EEG activity.

398 Candidate source-leading lags ranging from 0.6 to 120 s and history windows of 0.6,

399 1.2, 3, 6, 12, 30, and 60 s were evaluated. A positive lag indicated that the regional

400 CBV signal preceded the EEG value being predicted. For each history window, four

401 features were extracted from the CBV signal: the terminal value, mean, standard

402 deviation, and first-to-last slope. Equivalent features derived from the preceding EEG

403 signal were used to construct an autoregressive reference model.

404 Three ridge-regression models were compared for each ROI–lag–window

405 combination: a regional CBV-only model, an EEG-history-only autoregressive model,

406 and a combined model containing both CBV and EEG-history features. Ridge

407 regression was selected to stabilize coefficient estimation in the presence of correlated

408 temporal predictors(33). Predictor standardization and model fitting were performed

409 independently within each training fold. Predictive performance was evaluated using

410 fivefold forward-chaining time-series cross-validation, with a temporal gap

411 corresponding to the lag and history-window duration inserted between the training

412 and test segments to reduce temporal overlap and information leakage(34).

413 Model performance was assessed from pooled out-of-fold predictions using the

414 coefficient of determination (R^2), Pearson correlation coefficient, mean absolute error,

415 and root-mean-square error. Individual ROIs were primarily ranked according to their

416 maximum CBV-only cross-validated R^2 . The additional predictive information

417 provided by CBV beyond the EEG signal’s own history was quantified as:

$$418 \Delta R^2_{\text{vsAR}} = R^2_{\text{combined}} - R^2_{\text{AR-only}}$$

Statistical significance was evaluated using a circular-shift surrogate procedure that preserved the internal temporal structure and autocorrelation of the regional CBV signal while disrupting its temporal correspondence with EEG(35). For each permutation, the complete CBV time series was circularly shifted by at least 300 s, and the full feature extraction, model fitting, and cross-validation procedure was repeated. Permutation-derived P values across the 19 ROIs were corrected using the Benjamini–Hochberg false-discovery-rate procedure, with an FDR-adjusted $q < 0.05$ considered statistically significant. Because fUS and EEG were recorded in independent animals, these results were interpreted as group-level temporal prediction rather than within-animal neurovascular coupling or evidence of causality.

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM). Normality and homogeneity of variance were assessed using Shapiro–Wilk and Brown–Forsythe tests, respectively. Comparisons between the two independent groups were performed using a two-tailed unpaired Student’s *t*-test. Comparisons involving multiple groups were performed using a one-way analysis of variance (ANOVA), whereas data collected repeatedly over time were analyzed using a two-way repeated-measures ANOVA, with group and time as factors. Significant ANOVA effects were followed by Šídák, Tukey, or Bonferroni multiple comparison tests, as appropriate. When parametric assumptions were not satisfied, the Mann–Whitney *U* test or Kruskal–Wallis test followed by Dunn’s multiple comparison test was used. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. Sample sizes

and statistical tests are specified in the corresponding figure legends.

RESULTS

Repeated NTG exposure establishes a sensitized migraine-like state with multimodal behavioral alterations

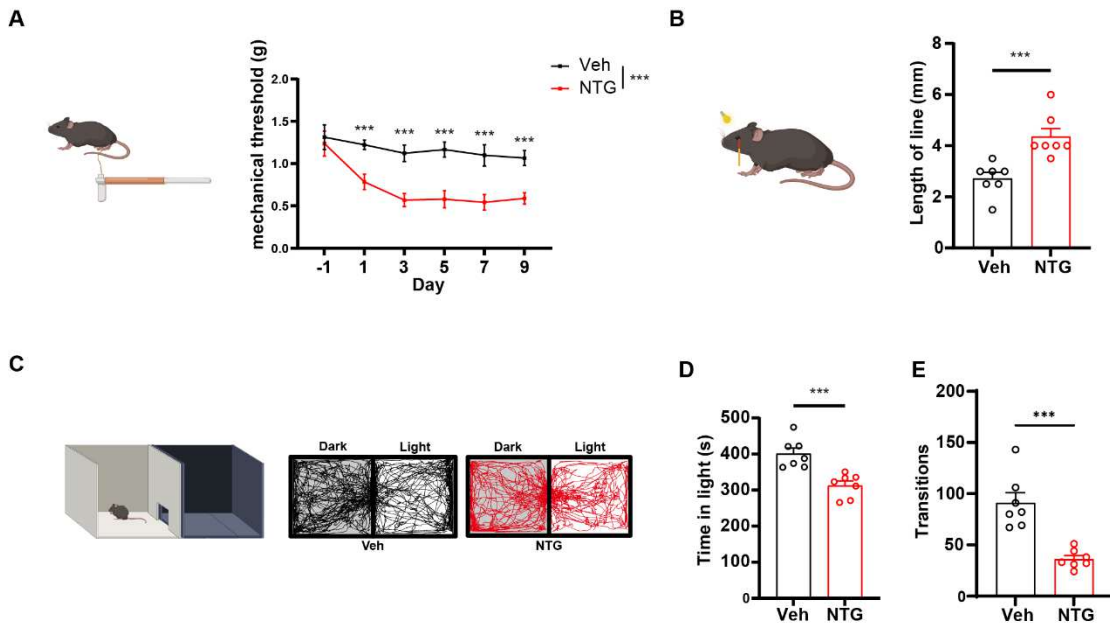


Figure 2. Repeated nitroglycerin administration induces persistent mechanical hypersensitivity, increased lacrimation, and light-averse behavior.

(A) Hind-paw mechanical withdrawal thresholds measured using the von Frey test from day -1 (D-1) to day 9 (D9) in vehicle-treated (Veh) and nitroglycerin-treated (NTG) mice. Repeated NTG administration produced a progressive and sustained reduction in mechanical withdrawal threshold. (B) Lacrimation assessed on D9 using the phenol red thread test. NTG-treated mice showed a greater wetted thread length than Veh-treated mice. (C) Schematic illustration of the light-dark box test and representative movement trajectories of Veh- and NTG-treated mice. (D, E) Quantification of the time spent in the light compartment (D) and the number of

transitions between the light and dark compartments (E) on D9. NTG-treated mice spent less time in the light compartment and exhibited fewer transitions than Veh-treated mice, indicating increased light aversion and reduced exploratory activity. Data are presented as mean $\pm$ SEM; individual circles represent individual mice. *** $P < 0.001$ versus the Veh group. Veh, $n = 8$; CM, $n = 8$.

To investigate how migraine chronification modifies the brain's response to acute migraine-like attacks, we first established a chronic migraine (CM) model using repeated intermittent NTG administration as the CM group. Mice received NTG injections every other day for nine days, whereas vehicle-treated mice served as the vehicle (Veh) control group (Figure 1A). Repeated NTG exposure induced mechanical hypersensitivity, as reflected by a significant reduction in hindpaw withdrawal thresholds on days 1, 3, 5, 7, and 9, compared to vehicle-treated mice ($n = 8$ per group; all $P < 0.001$; Figure 2A). In addition to nociceptive sensitization, CM mice exhibited behavioral alterations relevant to migraine-associated sensory and autonomic symptoms. Specifically, the CM mice spent less time in the illuminated compartment and showed fewer light–dark transitions, indicating enhanced light-avoidance behavior (Figure 2C–E). Furthermore, light stimulation induced greater tear secretion in the CM mice (Figure 2B). This lacrimation may reflect NTG-induced parasympathetic activation, which was supported by our electrocardiographic findings (Figure S2). This suggests enhanced trigeminal-autonomic responsiveness.

Acute NTG challenge induces distinct whole-brain neurovascular trajectories in episodic and chronic migraine

To determine whether migraine chronification alters brain-wide hemodynamic responses during an acute attack, we performed continuous four-dimensional functional ultrasound (4D fUS) imaging following an acute nitroglycerin (NTG) challenge. After 15 min of baseline acquisition, the mice were administered NTG and monitored continuously for an additional 45 min. Whole-brain power Doppler angiograms were registered in a mouse brain atlas, enabling the extraction of regional cerebral blood volume (CBV) dynamics across cortical and subcortical structures (Figure 3A–C).

In the Veh group, acute NTG administration elicited a transient reduction in CBV shortly after the injection, followed by a brief increase. The CBV then declined again to a sustained hypoperfused state before gradually returning to baseline. During the late recording period, the CBV transiently exceeded baseline levels in multiple cortical regions, suggesting vascular recovery and adaptive compensatory responses following the initial hemodynamic perturbation (Figure 3D, E).

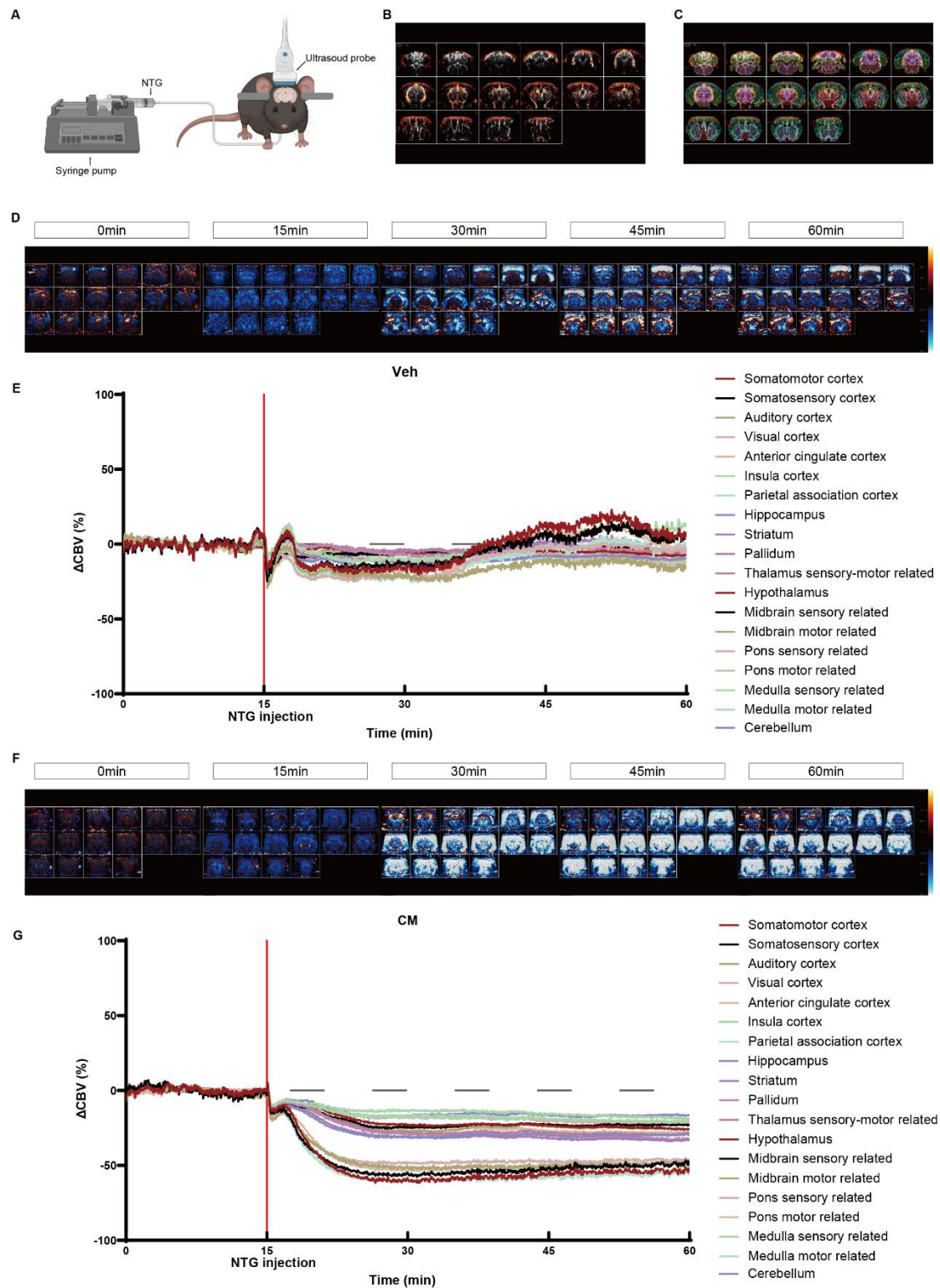


Figure 3. Functional ultrasound imaging reveals distinct brain-wide cerebrovascular responses to nitroglycerin in two groups.

(A) Schematic of the functional ultrasound (fUS) imaging experiment. (B)

Registration of the original ultrasound images to a mouse brain vascular template. (C)

Co-registration with the Allen Mouse Brain Atlas to anatomically localize brain regions and define regions of interest (ROIs). (D) Representative fUS-derived relative cerebral blood volume (Δ CBV) maps from vehicle-treated (Veh) mice at 0, 15, 30, 45, and 60 min. (E) Time courses of relative CBV changes in the selected cortical and subcortical ROIs in the Veh group. The red vertical line indicates NTG injection at 15 min. (F) Representative Δ CBV maps from chronic migraine (CM) mice at the corresponding time points. (G) Time courses of relative CBV changes in the selected ROIs in the CM group. Veh mice showed an initial widespread reduction in CBV followed by gradual recovery and hyperemic responses in several regions, whereas CM mice exhibited a sustained and widespread reduction in CBV after NTG administration. Lines represent group means. Veh, n = 5; CM, n = 5.

In contrast, the CM group exhibited a distinct hemodynamic trajectory following the same acute NTG challenge. Although the initial decline in CBV was broadly similar, CM mice failed to enter the recovery phase, as observed in the Veh mice. Instead, the CBV remained persistently reduced across widespread cortical and subcortical regions throughout the observation period (Figure 3F, G).

These findings indicate that migraine chronification did not amplify the magnitude of the initial NTG-induced reduction in cerebral perfusion. Rather, it transforms a transient and reversible neurovascular response into a prolonged hypoperfused state characterized by the impaired recovery of cerebral blood flow.

Migraine Chronification Impairs Post-Attack Cortical Hemodynamic Recovery

Without Altering the Initial Vascular Response

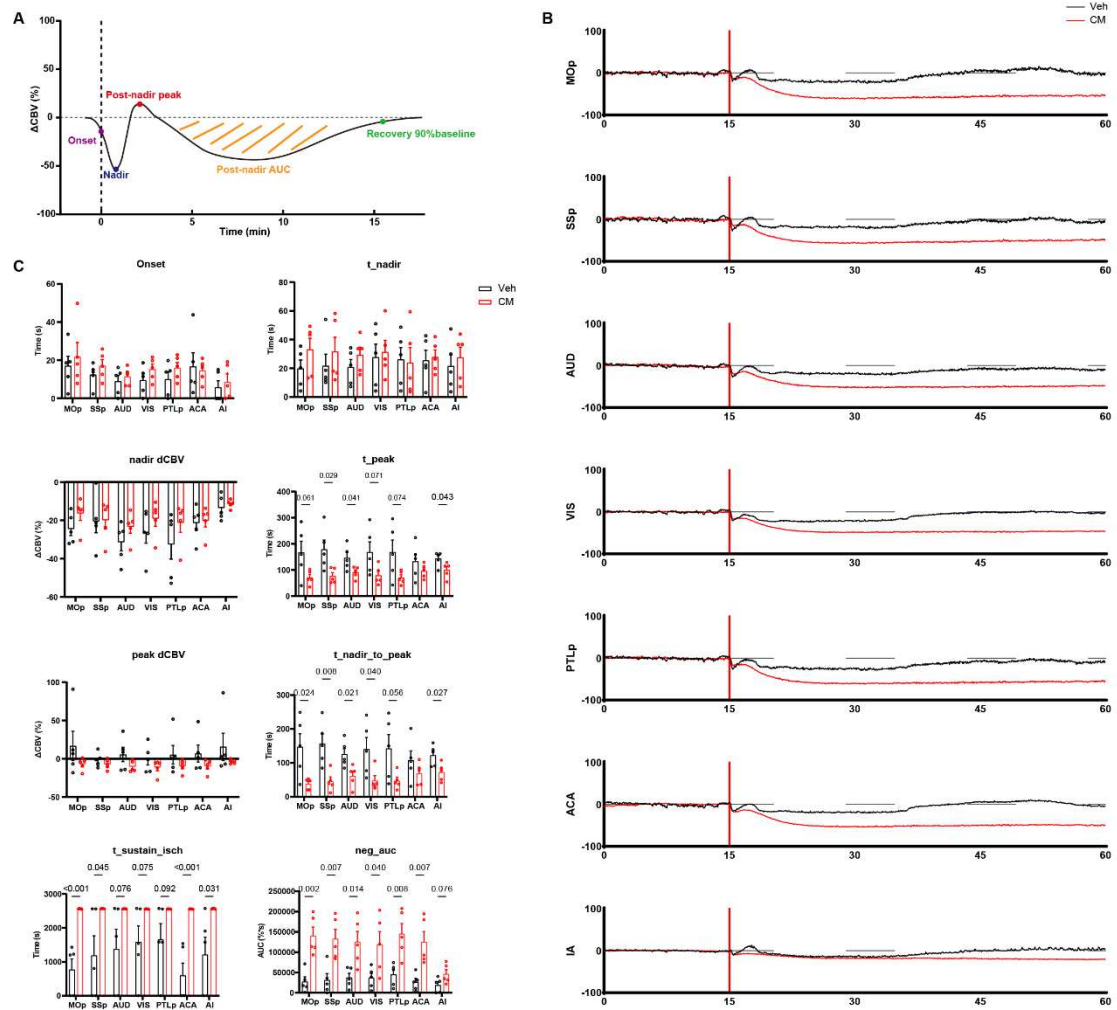


Figure 4. Chronic migraine alters the temporal dynamics and magnitude of cortical hemodynamic responses to nitroglycerin.

(A) Schematic illustration of the parameters extracted from the fUS-derived relative cerebral blood volume (Δ CBV) curves, including response onset, nadir, post-nadir peak, post-nadir negative area under the curve (AUC), and recovery to 90% of baseline. (B) Time courses of Δ CBV in representative cortical regions in vehicle-treated (Veh, black) and chronic migraine (CM, red) mice. NTG was administered at 15 min, as indicated by the red vertical line. Traces are shown for the primary motor cortex (MOp), primary somatosensory cortex (SSp), auditory cortex (AUD), visual cortex (VIS), posterior temporal lobe (PTLp), anterior cingulate cortex (ACA), and inferior association cortex (IA).

cortex (VIS), posterior parietal association cortex (PTLp), anterior cingulate cortex (ACA), and agranular insular cortex (AI). (C) Quantitative comparison of cortical hemodynamic parameters between Veh and CM mice, including response onset, time to nadir (t_{nadir}), nadir ΔCBV , time to the post-nadir peak (t_{peak}), peak ΔCBV , interval from nadir to peak, duration of sustained ischemic response ($t_{\text{sustain_isch}}$), and negative AUC. CM mice generally exhibited impaired post-nadir recovery, prolonged cortical hypoperfusion, and a greater cumulative reduction in CBV after NTG administration. Bars represent mean $\pm$ SEM, and individual points represent individual mice. Exact P values are shown above the corresponding comparisons. Veh, $n = 5$; CM, $n = 5$.

To determine which components of the hemodynamic response were altered by chronification, we extracted multiple temporal parameters from the regional CBV trajectories, including response onset, nadir amplitude, post-nadir peak, recovery duration, and cumulative CBV reduction (Figure 4A). Among the regions showing significant group differences (MOp, SSp, AUD, VIS, PTLp, ACA, and IA), the onset of the acute vascular response, time to nadir, and nadir amplitude were broadly comparable between Veh and CM mice. These findings indicated that migraine chronification did not substantially alter the onset or initial magnitude of the acute vascular response (Figure 4B). In contrast, significant differences were observed during the recovery phase. All seven brain regions examined in CM mice exhibited significantly attenuated hyperemic responses and prolonged hypoperfusion. Moreover, the cumulative burden of the

reduced blood volume, quantified as the area under the curve (AUC), was significantly greater in the MOp, SSp, AUD, VIS, PTLp, and ACA groups (Figure 4C).

Importantly, these alterations did not manifest as an amplification of the initial vascular response, but rather as a loss of recovery capacity. Thus, migraine chronification primarily affects the recovery phase of the acute neurovascular response, suggesting that chronic migraines are associated with impaired restoration of cortical hemodynamics following an attack.

Migraine chronification reshapes functional connectivity network

At baseline, functional connectivity was significantly stronger in the CM mice than in the Veh mice for the HIP–MYmot, STR–MYmot, PAL–CB, HY–MBmot, HY–MYsen, HY–MYmot, and MYmot–MBsen connections (Figure 5A). This baseline configuration indicates that repeated NTG exposure altered the intrinsic network state, resulting in tighter coupling among the subcortical systems before the NTG-evoked attack.

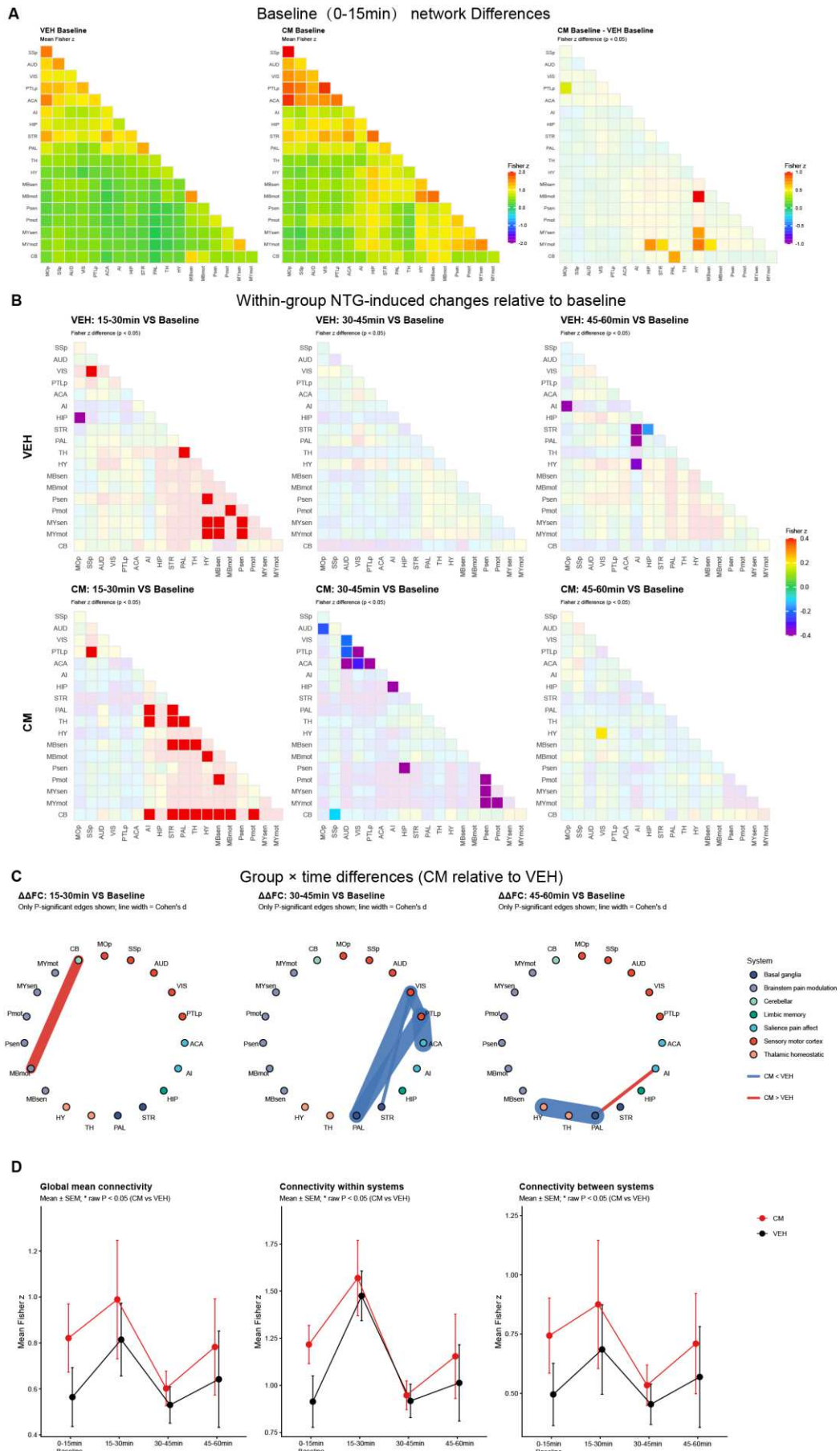


Figure 5. Resting-state functional connectivity abnormalities and nitroglycerin-induced network reorganization in chronic migraine mice.

(A) Baseline functional connectivity (FC) matrices in vehicle-treated (Veh) and chronic migraine (CM) mice before nitroglycerin (NTG) administration, together with the between-group as CM minus Veh. In the difference matrix, significant connections are displayed at full opacity, whereas nonsignificant connections are faded. (B) Within-group changes in FC relative to baseline during the early, middle, and late post-NTG periods. The upper row shows Veh mice and the lower row shows CM mice. Red and blue indicate increased and decreased connectivity, respectively; connections that did not reach the raw significance threshold were faded. Baseline, early, middle, and late periods correspond to 0–15, 15–30, 30–45, and 45–60 min, respectively. (C) Network representations of group-by-time differences during the early, middle, and late periods, calculated as $\Delta\Delta FC = \Delta F_{CCM} - \Delta F_{CVeh}$. Only connections with raw $P < 0.05$ are shown. Red edges indicate greater NTG-induced connectivity changes in CM mice than in Veh mice, whereas blue edges indicate smaller changes in CM mice. Edge width is proportional to Cohen's d , and node colors indicate the corresponding functional systems. (D) Quantitative comparison of global mean connectivity, within-module connectivity, and between-module connectivity across the four recording periods. Data are presented as mean $\pm$ SEM. Red and black lines represent CM and Veh mice, respectively. * $P < 0.05$ for the indicated between-group comparison. Veh, $n = 5$; CM, $n = 5$.

599 Fifteen minutes after NTG administration, hemodynamic functional connectivity
 600 underwent time-dependent reconfiguration in both groups, with distinct patterns
 601 emerging between the Veh and CM mice. In the Veh mice, the early phase after NTG
 602 administration (15–30 min) was characterized by significantly increased functional
 603 connectivity between SSp–VIS, PAL–TH, HY–Psen, HY–MYsen, HY–MYmot,
 604 MBsen–MYsen, MBsen–MY mot, Pmot–MBmot, Psen–MYsen, and Psen–MYmot,
 605 while decreased between Mop–HIP. In the late phase (45–60 min), significant
 606 decreases were observed in MOp–AI, AI–STR, AI–PAL, AI–TH, AI–HY and HIP–
 607 STR connectivities. Notably, the early phase network alterations observed in the Veh-
 608 pretreated mice after acute NTG challenge partially resembled the baseline network
 609 configuration of the CM mice. This suggests that repeated migraine-like attacks
 610 gradually consolidate transient attack-related network perturbations into a persistent
 611 baseline network architecture.

612 In the CM mice, the early phase (15–30 min) was characterized by significantly
 613 increased connectivity among PTLp–SSp, AI–PAL, AI–TH, AI–CB, STR–PAL, STR–
 614 TH, STR–MBsen, STR–CB, PAL–TH, PAL–MBsen, PAL–CB, TH–MBsen, TH–CB,
 615 HY–MBmot, HY–CB, MBsen–Pmot, MBsen–CB, MBmot–CB, and Pmot–CB.

616 During the middle phase (30–45 min), functional connectivity was significantly
 617 reduced among the Mop–AUD, SSp–CB, AUD–VIS, AUD–PTLp, AUD–ACA, VIS–
 618 PTLp, VIS–ACA, PTLp–ACA, AI–HIP, HIP–Psen, Psen–Pmot, Psen–MYsen, Psen–
 619 MYmot and Pmot–MYmot groups. In the late phase (45–60 min), VIS–HY
 620 connectivity was significantly increased. (Figure 5B). In CM mice with baseline brain

network remodeling, NTG-evoked attacks were further characterized by enhanced functional coupling between emotion- and memory-related regions and thalamic/subcortical networks. This finding suggests that after migraine chronification, migraine-like attacks may no longer be confined to sensory-nociceptive transmission alone, but may expand into a broader network state involving affective pain processing, enhanced pain salience, and pain-related mnemonic processing.

A comparison of baseline-referenced changes between groups across different time windows revealed that, relative to Veh mice, CM mice exhibited significantly increased MBmot–CB functional connectivity during the early phase after NTG administration (15–30 min), reduced VIS–PTLp, VIS–STR, VIS–PAL, PTLp–PAL, PTLp–PAL and PTLp–ACA connectivity during the middle phase (30–45 min), and increased AI–PAL while decreased PAL–HY connectivity during the late phase (45–60 min). However, when assessed relative to their respective baseline states, the direction of NTG-induced connectivity changes in both groups was similar at 15–30 min and 45–60 min, whereas a decrease in functional connectivity both between cortical regions and between the cortex and basal ganglia was observed during the 30–45 min interval (Fig. 5C). At the whole-brain network level, NTG did not produce consistent between-group differences in either global mean connectivity or intersystem connectivity (Figure 5D). These findings suggest that, after controlling for the influence of the baseline brain state, the brain functional changes induced by a single NTG injection are relatively consistent. In contrast, greater pain severity and

the accompanying symptoms observed in chronic migraines appear to be more closely associated with the process of migraine chronification.

Collectively, these results indicate that migraine chronification alters the evolution of functional connectivity in the brain during acute migraine attacks. Chronic sensitization does not simply amplify the acute response to NTG; rather, it reshapes the temporal sequence and regional specificity of network recruitment.

Migraine chronification impairs cortical electrophysiological recovery after acute NTG challenge

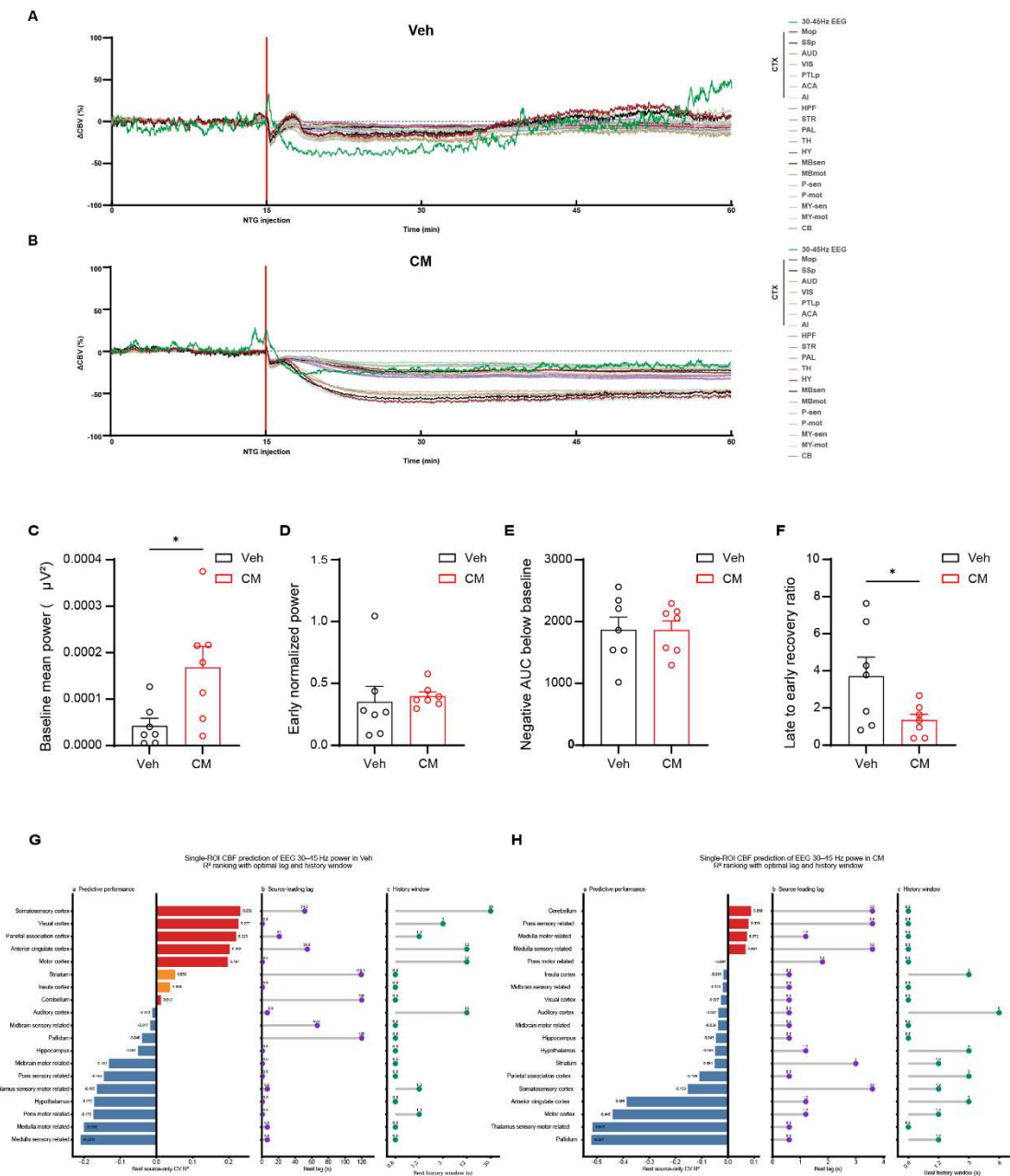
We performed EEG and ECG recordings in an independent cohort following acute NTG administration to determine whether altered hemodynamic recovery was accompanied by changes in cortical functional recovery.

In both groups, NTG induced rapid suppression of cortical 30–45 Hz gamma band activity. In vehicle-treated mice, gamma activity gradually recovered after initial suppression and increased during the late recording period, paralleling the restoration of regional CBV responses. In contrast, the CM mice showed persistent suppression of gamma band activity without a comparable late recovery (Figure 6A, B).

The baseline gamma band power was higher in CM mice than in Veh mice, whereas early post-NTG suppression and cumulative negative gamma response did not differ significantly between the groups. However, the late-to-early recovery ratio was significantly reduced in CM mice, indicating impaired restoration of cortical electrophysiological activity after acute challenge (Figure 6C–F).

These findings indicate that migraine chronification impairs not only vascular

665 recovery during an acute attack but also the restoration of cortical functional activity.



667 **Figure 6. Migraine chronification alters NTG-evoked hemodynamic and**

668 **electrophysiological dynamics and regional CBV-to-EEG predictive**

669 **relationships.**

670 (A, B) Temporally aligned group-mean changes in functional ultrasound-derived

671 cerebral blood volume (Δ CBV) across 19 brain regions and cortical 30–45 Hz EEG

672 power in vehicle-pretreated (Veh; a) and chronically NTG-treated (CM; b) mice. The

red vertical line indicates acute NTG administration at 15 min, following a 15-min baseline period. Recordings continued for 45 min after injection. fUS and EEG data were obtained from independent cohorts and were aligned according to the common experimental timeline rather than recorded simultaneously in the same animals. (C) Baseline mean 30–45 Hz EEG power during 0–15 min. (D) Early normalized EEG power during the first 15-min period after NTG administration (15–30 min), expressed relative to baseline. (E) Negative area under the curve (AUC) below baseline during the post-injection period. (F) Late-to-early recovery ratio, calculated as normalized EEG power during 45–60 min divided by that during 15–30 min. Individual circles represent individual animals, and bars and error bars indicate the mean $\pm$ SEM. * $P < 0.05$; nonsignificant comparisons are not indicated. (G, H) Single-region CBV prediction of 30–45 Hz EEG power in the Veh (G) and CM (H) groups. Brain regions were ranked according to the best source-only cross-validated coefficient of determination (CV R^2 ; left). Positive R^2 values indicate predictive performance above the test-set mean, whereas negative values indicate poor out-of-sample prediction. The corresponding optimal source-leading lag, representing the interval by which the regional hemodynamic signal preceded EEG power (middle), and the optimal CBV history window used for prediction (right) are shown for each region. Veh, $n = 6$; CM, $n = 6$.

Chronic migraine alters the relationship between hemodynamic dynamics and cortical functional recovery

Given the impairment of CBV and gamma band recovery, we examined whether the temporal relationship between ROIs' CBV of the ROIs and EEG was altered by chronification. Using a time-lagged prediction model, CBV signals from individual ROIs were used to predict subsequent gamma band activity. In the Veh mice, the cortical regions involved in sensory processing and pain integration, including the SSp, VIS, PTLp, ACA, and MOp, showed the strongest predictive performance for subsequent gamma band activity (Figure 6G).

In contrast, none of the ROIs in the CM mice exhibited appreciable predictive performance ($R^2 < 0.1$; Figure 6H). Furthermore, the optimal lag times and history window durations differed between the two groups, suggesting that migraine chronification alters the temporal relationship between hemodynamic and electrophysiological activities.

DISCUSSION

In this study, repeated NTG exposure induced mechanical hypersensitivity, light-avoidance behavior, and increased light-evoked lacrimation, supporting the establishment of a chronic migraine-like state. We examined how migraine chronification reshaped the brain-wide response to subsequent acute NTG-evoked attacks. Using whole-brain four-dimensional functional ultrasound imaging, together with parallel electrophysiological and electrocardiographic recordings, we found that repeated NTG pretreatment altered the temporal evolution of attack-related brain responses. Specifically, chronification was associated with an attenuated hyperemic

response capacity, prolonged recovery from hypoperfusion-dominant hemodynamic changes, remodeling of both baseline- and attack-related hemodynamic networks, delayed recovery of cortical gamma band activity, and altered cardiac autonomic responses. Together, these findings indicate that migraine chronification not only amplifies the response to an acute trigger but also modifies the regulation of attack-related brain state transitions.

NTG, a NO donor, has been widely used as a translational migraine model because it can induce migraine-like attacks in susceptible individuals and activate key components of the trigeminovascular system in experimental animals(9,36). Early interpretations of NTG-induced responses were strongly influenced by vascular theories of migraine because nitric oxide is a potent vasodilator. However, subsequent clinical and experimental studies have shown that migraines cannot be explained solely by cerebral vasodilation. Changes in the arterial diameter, cerebral perfusion, and headache development were not necessarily temporally or spatially coupled, indicating that vascular alterations represent only one component of a broader neurovascular process(11–13). Current concepts place the NTG within the framework of nitric oxide-dependent trigeminovascular activation, which involves trigeminal afferent activation, neuropeptide release, and central sensitization(5,37).

Repeated intermittent NTG administration has established a widely used model for studying migraine chronification-like states characterized by persistent basal hypersensitivity and enhanced responsiveness to subsequent NTG exposure(16–18). Previous studies using this model have mainly focused on behavioral sensitization,

trigeminal activation, inflammatory signaling, and molecular mechanisms underlying chronicity. However, it remains unclear whether prior sensitization alters the spatial and temporal organization of the brain-wide response to subsequent acute attacks. Our findings extend the application of the NTG model from a model of pain hypersensitivity toward a model for investigating dynamic neurovascular and brain-state responses during acute migraine-like attacks.

An important finding of this study is that repeated NTG pretreatment did not markedly alter the initial phase of the attack-related CBV response. Instead, principal differences emerged in the capacity to generate a hyperemic response and in the subsequent recovery from hypoperfusion-dominant hemodynamic alterations. These observations provide a new perspective on migraine chronification. Existing models have emphasized enhanced excitability of the trigeminovascular system, increased nociceptive transmission, and central sensitization as the major mechanisms driving the transition toward chronic migraine-like states(4,7). Our findings do not contradict these mechanisms but suggest an additional dimension: chronification may also impair the ability of the brain to regulate physiological recovery after acute neurovascular perturbation.

This interpretation is consistent with the heterogeneous and time-dependent cerebrovascular responses reported in human and animal studies following NTG administration. NTG can induce large-artery dilation and reduced flow velocity without corresponding increases in regional cerebral blood flow. Cortical and meningeal vascular responses may differ in both direction and timing(11–

15,19,20,38–43)(Table1). Thus, NTG-evoked hemodynamic responses are region-specific, phase-dependent, and partially dissociable from systemic vascular effects. Accordingly, the NTG model is better understood as a dynamic neurovascular perturbation model than a simple vasodilation model.

Table1. Previous studies on cerebral hemodynamics in NTG-induced migraine models

Year	Journal	Subjects /animals	NTG administration	Test methods	Hemodynamic variable	Tested regions or vessels	Main NTG-induced changes
1989 Dahl et al.	<i>Stroke</i>	Humans; n = 10	Sublingual NTG, 1 mg	TCD and SP ECT	MCA blood-flow velocity; rCBF	MCA and its perfusion territory	Mean MCA blood-flow velocity ↓, whereas rCBF ↔; findings suggested dilation of basal intracranial arteries. Global CBF showed a slight nonsignificant ↑; significant regional CBF ↑ occurred predominantly in the occipital regions. Cortical blood flow ↑ within approximately 5 min after infusion onset and remained elevated during the 80-min observation period; the in
1999 Oliver and Dormehl	<i>Arzneimittelforschung</i>	Non-human primates; n = 31	Intravenous NTG, 0.25 µg/kg/min for 10 min	^{99m} Tc-HMPAO SPECT	Global and regional CBF	Whole brain and cortical regions	
1999 Read et al.	<i>Brain Research</i>	Rats; n = 31	Intravenous NTG, 2 µg/kg/min for 30 min	Laser Doppler flowmetry	Local cortical blood flow	Cerebral cortex	

							crease was prevented by sumatriptan.
2000 White et al.	<i>Journal of the Neurological Sciences</i>	Humans; n = 8	Intravenous NTG, 0.1, 0.4 and 1.0 µg/kg/min	H ₂ ¹⁵ O PET, TCD and carotid color-flow imaging	Global/rCBF; MCA velocity; carotid flow	Whole brain, MCA, common and internal carotid arteries	Global and regional CBF ↔ and carotid flow ↔, but MCA velocity decreased dose-dependently, indicating dilation of basal cerebral arteries. Global CBF ↑ by approximately 23%, whereas MCA velocity ↓ by approximately 12–21%; occipital rCBF ↓. Anterior-cingulate perfusion ↑ was detected later during continued GTN after sumatriptan administration.
2002 Bednarczyk et al.	<i>Cephalalgia</i>	Humans; n = 12	Intravenous NTG, 0.125, 0.25 and 0.5 µg/kg/min for 60 min	H ₂ ¹⁵ O PET and TCD	Global/rCBF; bilateral MCA velocity	Whole brain, brainstem vascular regions, anterior cingulate and occipital cortex	CBV ↑ by approximately 13%; rCBF ↔; MCA velocity ↓ by approximately 20% during infusion and remained approximately
2008 Iversen et al.	<i>The Journal of Headache and Pain</i>	Humans; n = 6	Intravenous NTG, 0.5 µg/kg/min for 20 min	SPECT and TCD	rCBF; CBV; MCA blood-flow velocity	Whole brain and right MCA	

							ely 15% below baseline at 60 min.
2008 Schoonman et al.	<i>Brain</i>	Humans with migraine; NTG n = 27 and placebo n = 5	Intravenous NTG, 0.5 µg/kg/min for 20 min	3-T MRA and phase-contrast flow imaging	Vessel diameter; arterial blood flow	Middle meningeal, external carotid, internal carotid, MCA, basilar and posterior cerebral arteries	All examined vessels transiently dilated by 6.7–30.3% during infusion. Diameters returned to baseline during delayed migraine-like attacks; ICA and basilar artery flow ↔ throughout. GTN induced transient dural blood-flow ↑ accompanied by dilation of relatively large dural and pial arteries.
2008 Gozalov et al.	<i>Headache</i>	Rats; n = 6	Intravenous NTG bolus, 20 µg/kg	Intravital microscopy and laser Doppler flowmetry	Arterial diameter; dural blood flow	Large dural and pial arteries	Dural blood flow showed a biphasic response: initial ↓ at approximately 30 min followed by ↑ from 90–150 min, reaching approximately 30% above baseline. Cortical blood flow showed only a d
2011 Greco et al.	<i>Cephalalgia</i>	Rats; NTG, n = 7; Veh, n = 5	NTG, 10 mg/kg i.p.	Laser Doppler flowmetry	Regional blood flow	Dura mater and cerebral cortex	

								elayed ↑ from 90–150 min.
2014 Pryazhnikov et al.	<i>PLOS ONE</i>	Rats; n = 27	NTG, 10 mg/kg i.p.	In vivo two-photon microscopy	Microvessel diameter	Meningeal and cortical arterioles and venules		Meningeal arterioles transiently constricted, whereas cortical arterioles dilated; changes in small venules were not significant. MCA cross-sectional area ↑ and blood velocity ↓, but calculated total MCA blood flow ↔, directly confirming that velocity reduction does not necessarily indicate reduced flow.
2018 Schulz et al.	<i>Experimental Physiology</i>	Humans; n = 10	Sublingual NTG spray, 0.4 mg	7-T MRI and TCD	MCA cross-sectional area, velocity and total flow	Right MCA		Migraine-like head-scratching behavior was accompanied by cortical CBF ↑. Ultrasound treatment reduced behavioral responses but did not significantly reduce CBF velocity.
2022 Yao et al.	<i>IEEE Transactions on Neural Systems and Rehabilitation Engineering</i>	Rats; Vehicle, n = 7; NTG, n = 7	NTG, 10 mg/kg, single s.c.	Laser-speckle contrast imaging	Relative cortical CBF velocity	Exposed cerebral cortex		

2023		Rats; n	Imaging	Pulsatil	NTG produc
Sokolo		= 25; sal	photop	e-comp	ed bidirectio
v et a		ine-pretre	lethysm	onent a	nal individual
l.	<i>Scientific</i>	ated, n	ograph	mplitud	responses:
	<i>Reports</i>	= 13; su	y with	e as a	pulsatile am
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CBF, cerebral blood flow; rCBF, regional cerebral blood flow; CBV, cerebral blood volume; MCA, middle cerebral artery; ICA, internal carotid artery; TCD, transcranial Doppler; SPECT, single-photon emission computed tomography; PET, positron emission tomography; MRA, magnetic resonance angiography; iPPG, imaging photoplethysmography; ↑, increase; ↓, decrease; ↔, no significant change.

Our functional connectivity results showed that the early phase network alterations observed in Veh-pretreated mice after the NTG challenge partially resembled the baseline network configuration of CM mice. This suggests that repeated migraine-like attacks may progressively transform transient attack-related network perturbations into stable baseline network remodeling. This interpretation is consistent with longitudinal clinical neuroimaging studies showing that resting-state functional connectivity changes dynamically across the migraine cycle, and that experimentally induced migraine attacks can alter large-scale brain network connectivity(44). In parallel, patients with chronic migraines show abnormal interictal connectivity within pain processing, salience, and corticolimbic/subcortical networks(45). These clinical findings support the idea that recurrent attacks drive maladaptive plasticity, whereby transient ictal network states are gradually incorporated into a chronified interictal network phenotype. In this context, the baseline network alterations observed in CM

mice may represent a network signature of prior repeated attack exposure, rather than a static background difference unrelated to attack evolution.

In CM mice with baseline network remodeling, NTG-evoked attacks were accompanied by enhanced functional coupling between the affective/mnemonic regions and thalamic–subcortical networks. This pattern suggests that migraine chronification may transform an acute attack from a predominantly sensory-nociceptive event into a broader network state involving affective pain processing, enhanced pain salience, and pain-related mnemonic processing. This interpretation is supported by chronic migraine imaging studies showing atypical connectivity of affective pain-processing regions with the amygdala, insula, pulvinar, mediodorsal thalamus, temporal cortex, and periaqueductal gray(46). This network-level interpretation is also consistent with broader chronic pain evidence showing that pain chronification can shift brain representations from nociceptive circuits toward emotional and memory-related circuits(47). Thus, enhanced coupling between affective/mnemonic regions and thalamic–subcortical hubs in CM mice may reflect chronification-dependent recruitment of circuits encoding pain salience, affective valuation, contextual association, and pain-related memory traces.

Conventional neuroimaging approaches have primarily examined the static differences between the interictal and ictal states. The dynamic evolution of the whole-brain physiological responses during acute migraine attacks remains poorly understood. By applying four-dimensional functional ultrasound imaging, we addressed an important gap in the characterization of the dynamic transitions of brain

800 network states.

801 These parallel fUS and EEG findings further suggest that migraine chronification
802 alters the temporal coordination between vascular dynamics and cortical functional
803 activity during an attack. After the NTG challenge, repeated NTG-pretreated mice
804 showed delayed recovery of cortical gamma band activity, which paralleled the
805 prolonged CBV abnormalities observed with fUS. Moreover, CBV signals contain
806 predictive information regarding subsequent gamma band activity, whereas EEG
807 signals provide limited predictive information for subsequent CBV changes. These
808 findings suggest that after repeated NTG exposure, the temporal relationship between
809 hemodynamic signals and cortical electrophysiological recovery was altered.

810 However, these findings should not be interpreted as evidence that vascular changes
811 directly drive the neuronal dysfunction. Neurovascular coupling is a bidirectional
812 process that involves interactions among neurons, astrocytes, endothelial cells,
813 vascular smooth muscle cells, and metabolic pathways(6,48). Predictive modeling can
814 identify temporal relationships within complex biological systems; however, it does
815 not establish causality. Therefore, our results are best interpreted as evidence that
816 migraine chronification modifies the coordination between the vascular and neuronal
817 recovery processes during an attack.

818 Current concepts of migraine chronification have largely focused on the mechanisms
819 that increase attack susceptibility, including trigeminovascular sensitization, altered
820 neurotransmission, cortical excitability changes, and neuroimmune interactions(4,7).
821 Our findings suggest an additional dimension: chronic sensitization may alter the

temporal regulation of physiological recovery after an acute attack. In this context, the key distinction between non-sensitized and sensitized states may involve not only how strongly the brain responds to a trigger, but also how effectively it returns to baseline after the perturbation. This recovery-centered perspective may help explain several clinical features of chronic migraine, including prolonged symptoms, incomplete interictal recovery, and increased vulnerability to recurrent attacks. In this model, chronification is not simply a state of increased attack susceptibility but also a state of impaired recovery from attack-related neurovascular and network disturbances. Nevertheless, whether altered hemodynamic recovery represents a cause, consequence, or a parallel manifestation of migraine chronification remains unclear.

Limitations

This study has several limitations. First, fUS primarily measures relative hemodynamic signals related to cerebral blood volume and flow and therefore should not be interpreted as a direct measurement of absolute cerebral blood flow or tissue oxygenation. Secondly, the between-group analysis of functional connectivity employed a t-test with Fisher z-transformation; while this approach aligns with previous fUS testing methods, increasing the sample size and enhancing the stability of the FDR test would yield more reliable results. Third, although fUS and EEG provided convergent information regarding hemodynamic and electrophysiological recovery, recordings were obtained from parallel experimental cohorts. Therefore, the temporal relationship between CBV and EEG should be interpreted at the group and

model levels rather than as direct within-animal causality. Fourth, continuous arterial blood pressure, cerebral oxygenation, and metabolic measurements were not included, limiting our ability to completely separate local neurovascular regulation from systemic cardiovascular effects. Fifth, the absence of DC-coupled electrophysiological recordings prevented definitive identification of canonical cortical spreading depolarization. Finally, the fUS sample size was limited, and larger cohorts are required to validate the network-level findings.

CONCLUSIONS

In conclusion, repeated NTG exposure resulted in mechanical hypersensitivity, light avoidance, and increased light-evoked lacrimation, and substantially altered the brain-wide response to a subsequent NTG challenge. The CM state was characterized by prolonged, cortex-predominant CBV reduction, selective reorganization of functional connectivity, increased baseline γ -band activity, and impaired electrophysiological recovery. These findings suggest that defective recovery of cerebral hemodynamics and its altered relationship with neural activity are important features of migraine chronification. Understanding how chronic migraines affect brain blood flow, brain connectivity, and EEG signals can provide ideas for the treatment of CMs and their complications. This information can help in the development of more precise drug and non-drug treatments.

List of abbreviations

4D fUS, four-dimensional functional ultrasound; ANOVA, analysis of variance; CBF, cerebral blood flow; CBV, cerebral blood volume; CM, chronic migraine; EEG, electroencephalographic; EM, episodic migraine; LDT, Light-dark box test; NO, nitric oxide; NTG, nitroglycerin; Veh, vehicle; AUC, area under the curve; MOp, primary motor area; SSp, primary somatosensory area; AUD, auditory areas; VIS, visual areas; PTLp, posterior parietal association areas; ACA, anterior cingulate area; AI, agranular insular area; HPF, hippocampal formation; STR, striatum; PAL, pallidum; TH, thalamus; HY, hypothalamus; MBsen, sensory- related midbrain; MBmot, motor-related midbrain; P-sen, sensory- related pons; P-mot, motor-related pons; MY-sen, sensory-related medulla; MY-mot, motor-related medulla; CB, cerebellum.

DECLARATIONS

Ethics approval and consent to participate

All animal experimental procedures were reviewed and approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (Y2024-10-08-02). All experiments were conducted in accordance with the relevant institutional guidelines for the care and use of laboratory animals. Consent to participate was not applicable because this study involved animals only. Clinical trial number: not applicable.

Consent for publication

Not applicable.

887 **Availability of data and materials**

888 The datasets used and/or analysed during the current study are available from the
889 corresponding author on reasonable request.

890 **Competing interests**

891 The authors declare no competing interests.

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899 **Authors' contributions**

900 X.R. and T.L. designed and conducted the fUS experiments, collected and processed
901 the data, and drafted the manuscript. Z.Z. provided technical support for the
902 electrophysiological experiments. K.L. contributed to the design and supervision of the
903 behavioral experiments. B.Z. supervised and contributed to the experimental design.
904 Y.L. provided algorithmic support and performed data analysis. X.G. conceived and
905 supervised the overall study, guided manuscript preparation, and synthesized the key
906 scientific questions and conclusions. All authors reviewed and approved the final

907 manuscript.

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910

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