The Journal of Headache and Pain

RESEARCH

Open Access

Mechanistic investigation of HMGB1 in an in vitro model of the trigeminovascular system under migraine-like conditions

Maomei Song[1*], Angelica Barone[2], Suiyi Xu[1], Changxin Li[1], Giuseppe Gigli[3,4,5], Giacomo Frati[6,7], Cherubino Di Lorenzo[6], Gianluca Coppola[6*†] and Barbara Cortese[5,8*†]

Abstract

Background Migraine is a complex neurovascular disorder driven by abnormal activation of the trigeminovascular system (TVS), neurogenic inflammation and excessive release of calcitonin gene-related peptide (CGRP). While CGRP-targeted therapies have demonstrated clinical efficacy, the upstream molecular mechanisms sustaining CGRP dysregulation remain incompletely understood. High-mobility group box 1 (HMGB1), a pro-inflammatory damage-associated molecule implicated in neuroinflammation, has emerged as a potential contributor to migraine pathophysiology. However, its role in neuron-endothelial interactions within the TVS under migraine -relevant conditions remains unclear.

Methods To model inflammatory and hypoxic stress associated with migraine, SH-SY5Y neuron-like cells were stimulated with lipopolysaccharide (LPS) and cobalt chloride ($CoCl_2$). HMGB1 expression was silenced using siRNA to evaluate its role in regulating CGRP and NF-κB signaling. Conditioned medium from stimulated neuronal cells was applied to human umbilical vein endothelial cells (HUVECs) to investigate neuron–endothelial interactions, and CGRP signaling was inhibited using a receptor antagonist. Protein expression of HMGB1, CGRP, and NF-κB pathway components was assessed by Western Blot. In addition, neuronal migration and intracellular reactive oxygen species (ROS) were assessed to evaluate cellular activation under inflammatory stress conditions.

Results Inflammatory and hypoxic stimulation increased HMGB1 expression and activated NF-κB signaling in SH-SY5Y cells, accompanied by elevated CGRP production. HMGB1 knockdown reduced NF-κB activation and attenuated CGRP upregulation, suggesting that HMGB1 contributes to CGRP regulation under these conditions. Conditioned medium enriched in CGRP promoted NF-κB activation and HMGB1 expression in endothelial cells, effects

†Gianluca Coppola and Barbara Cortese share last authorship.

*Correspondence:
Maomei Song
songmm1996@163.com
Gianluca Coppola
gianluca.coppola@uniroma1.it
Barbara Cortese
barbara.cortese@cnr.it

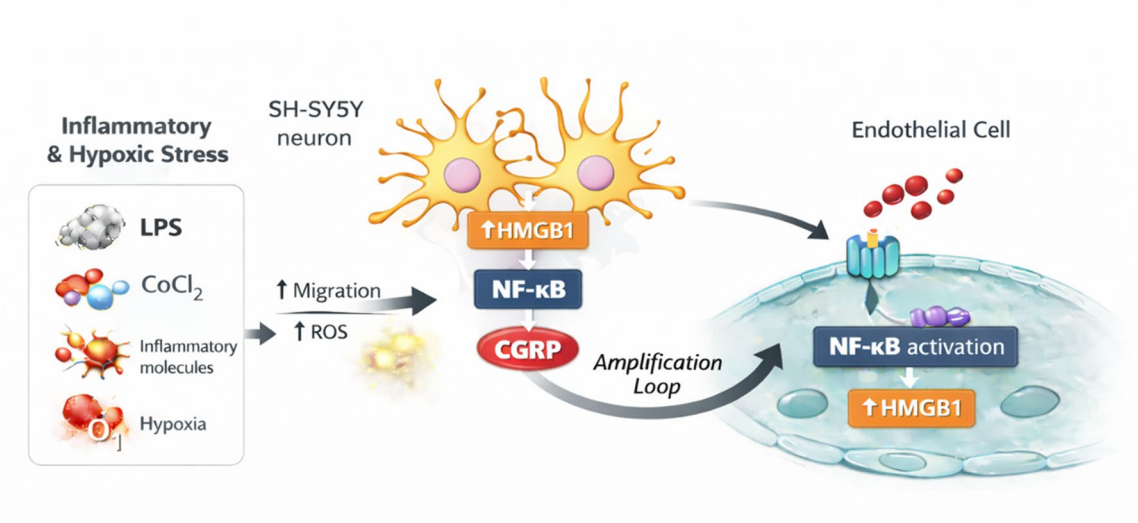
Full list of author information is available at the end of the article

that were attenuated by CGRP receptor blockade. In addition, HMGB1 enhanced neuronal migration and oxidative stress responses in stimulated SH-SY5Y cells.

Conclusion These findings support a model in which HMGB1 and CGRP participate in a positive neuron–endothelial feedback loop under inflammatory and hypoxic stress, potentially amplifying neurovascular signaling relevant to migraine. Although derived from an in vitro system, this study identifies HMGB1 as a potential upstream modulator of CGRP-associated pathways and highlights its possible contribution to peripheral mechanisms involved in trigeminovascular activation.

Keywords Migraine, Trigeminovascular system, Inflammatory, Hypoxia, HMGB1, CGRP, NF-κB, Migration, Oxidative stress

Graphical Abstract



Background

Migraine is a multifactorial neurovascular disorder characterized by recurrent attacks of disabling headache accompanied by sensory, autonomic, and cognitive symptoms [1, 2]. Its pathophysiology involves altered sensory processing, trigeminovascular system (TVS) activation, neurogenic inflammation, metabolic stress, and dysregulated neurovascular coupling [3, 4]. The TVS comprises sensory neurons originating from the trigeminal ganglion and the upper cervical dorsal root ganglia that innervate the dura mater, pia mater vessels, and cerebral blood vessels, thereby contributing to the transmission of cranial pain and the regulation of cerebral blood flow [5, 6]. Upon activation by mechanical, chemical, or inflammatory stimuli, trigeminal nerve terminals are activated and release various neuropeptides. Among these mechanisms, excessive release of calcitonin gene-related peptide (CGRP) from trigeminal sensory neurons represents a central event in migraine pathogenesis and a validated therapeutic target [7, 8]. CGRP is synthesized by sensory neurons in the trigeminal ganglion and is predominantly released from their peripheral terminals during migraine attacks. This release induces meningeal vasodilation, increases vascular permeability, and promotes immune cell recruitment, thereby triggering neurogenic inflammation and enhancing nociceptive signal transmission [9, 10]. Clinical studies have shown that patients with migraine exhibit elevated levels of CGRP in serum, cerebrospinal fluid, and saliva during attacks [11–13]. Moreover, targeted therapies against CGRP or its receptor have demonstrated significant clinical efficacy, further supporting the critical role of peripheral mechanisms in migraine pathophysiology [14, 15]. However, despite the clinical success of CGRP-targeted therapies, the upstream molecular signals sustaining CGRP dysregulation remain incompletely understood.

In recent years, inflammation-related molecules have emerged as important contributors to TVS activation [16, 17]. High mobility group box 1 (HMGB1) is a ubiquitously expressed nuclear protein that can be released into the extracellular space as a damage-associated molecular pattern (DAMP), where it exerts pro-inflammatory effects in response to cellular injury or inflammatory stimulation [18, 19]. HMGB1 has been reported to contribute to neurogenic inflammation by activating Toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE), thereby facilitating interactions among neurons, glial cells, and immune cells [20–22].

Notably, Karatas et al. demonstrated that neuron-derived HMGB1 triggers TVS activation and initiates a glia-mediated inflammatory cascade during migraine-associated cortical spreading depression (CSD) [23]. Studies in familial hemiplegic migraine type 1 (FHM1) animal models have also revealed that CSD induces sustained HMGB1 release [24]. Additionally, recent evidence suggests that HMGB1 plays an important role in both animal models and patients with medication-overuse headache (MOH) [25–27]. However, whether HMGB1 promotes CGRP production within the TVS and contributes to the peripheral mechanisms of migraine remains to be elucidated.

Moreover, migraine is not only a neurovascular disorder but is also associated with enhanced inflammatory responses and metabolic stress. Clinical and experimental studies indicate that migraine attacks are characterized by elevated levels of pro-inflammatory mediators, increased oxidative stress, and mitochondrial dysfunction [28–31]. Inflammatory mediators and relative hypoxia have been implicated in migraine pathophysiology, particularly during cortical spreading depression and chronic migraine states [32, 33]. Lipopolysaccharide (LPS) and cobalt chloride ($CoCl_2$) were therefore used to reproduce a controlled inflammatory-hypoxic microenvironment known to activate NF-κB signaling and stabilize HIF-1α, molecular features that have been reported in migraine-related studies. Although these stimuli are not migraine-specific triggers, they reproduce key molecular features associated with migraine-related neuroinflammatory and metabolic stress responses.

Accordingly, the present study employed neuron-like cells and vascular endothelial cells as in vitro models to investigate HMGB1-mediated neuron-endothelial interactions under inflammatory and hypoxic conditions. Although these cell lines do not fully reproduce the anatomical *scenario* and the functional complexity of the TVS in vivo, they provide a controlled platform to explore molecular mechanisms linking inflammation, hypoxia, and neuropeptide signaling relevant to migraine. We focused on analyzing the reciprocal regulation between HMGB1 and CGRP within the TVS and further evaluated the effects of HMGB1 on neuronal migration and oxidative stress, with the aim of providing novel molecular insights into the peripheral mechanisms of migraine.

Methods

Cell culture

SH-SY5Y cells and HUVECs were purchased from ATCC. SH-SY5Y cells are a human neuroblastoma-derived neuronal cell line widely used to investigate neuroinflammatory signaling, neuropeptide regulation, and NF-κB–dependent mechanisms. Although they do not fully replicate primary trigeminal ganglion neurons, they provide a reproducible human neuronal model suitable for dissecting intracellular signaling pathways relevant to trigeminal sensory activation. SH-SY5Y cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, #31966021, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, #A5256701), 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, #15140122) and 1% amphotericin B (Sigma-Aldrich, #A2942, USA). HUVECs were used as a representative human endothelial model to explore neurovascular signaling mechanisms. While they do not reproduce the specialized phenotype of cerebral microvascular endothelial cells, they are widely employed to investigate endothelial inflammatory responses and neuropeptide-mediated signaling in vitro. HUVECs were cultured in endothelial cell growth medium (Cell Applications, Inc., #211500, USA) supplemented with 1% penicillin/streptomycin. Both cells were incubated at 5% CO_2 and 37 ℃.

SH-SY5Y cells treatment and conditioned medium (CM) preparation

SH-SY5Y cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated overnight until they reached 60–80% confluence. According to previous studies [34, 35], cells were treated with 1 μg/mL LPS (Sigma-Aldrich, #L3012) and 300 μM $CoCl_2$ (Sigma-Aldrich, #C8661) for 48 h. Control cells were cultured in complete medium without LPS or $CoCl_2$. After 48 h of treatment, the culture medium was removed and replaced with fresh complete DMEM containing 300 μM $CoCl_2$. For the control group, cells were incubated with fresh DMEM only. After an additional 24 h, the CM was collected and centrifuged at 1200 g for 5 min and stored at -80 ℃ until further use.

Human umbilical vein endothelial cells (HUVECs) treatment

HUVECs were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated overnight until the cells were 60–80% confluent. Then the cells were treated with CM derived from SH-SY5Y cells for a further 24 h. For the control group, HUVECs were incubated with CM from the control group of SH-SY5Y. For the LPS+$CoCl_2$-CM group, HUVECs were treated with CM from SH-SY5Y exposed to LPS+$CoCl_2$. For the antagonist group, HUVECs were treated with CM from the LPS+$CoCl_2$-treated SH-SY5Y cells in the presence of 100 nM Olcegepant (BIBN4096, MedChemExpress, #HY-10095, USA). After 24 h of incubation, cells were collected for subsequent experiments.

Cell transfection

To inhibit HMGB1 expression in SH-SY5Y cells, small interfering RNA (siRNA) was used for gene silencing. HMGB1-targeting siRNA (siHMGB1) was purchased from Santa Cruz (sc-37982). SH-SY5Y cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated overnight until the cells were 60–80% confluent. Cells were transfected with 200 nM siHMGB1 using Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific, #11668019, USA) in Opti-MEM (Gibco, Thermo Fisher Scientific). After 6 h of transfection, the medium was replaced with complete DMEM. To assess the transfection efficiency, cells were harvested at 24 h for real-time quantitative polymerase chain reaction (RT-qPCR) and at 48 h for Western Blot analysis.

Western blot

After treatment, cells were collected and lysed with RIPA buffer containing 1% protease and 1% phosphatase inhibitor. Protein concentrations were quantified using a RC DC™ Protein Assay Kit (Bio-Rad, #5000121, USA). Equal amounts of protein from each sample were separated by 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% Bovine Serum Albumin (BSA, Sigma-Aldrich, #A3059) for 1 h at room temperature and then incubated with primary antibodies overnight at 4 ℃. The primary antibodies were as follows: anti-HMGB1 antibody (1:5000, Abcam, #ab79823, UK), anti-CGRP antibody (1:1000, Abcam, #ab189786), anti-HIF-1α antibody (1:1000, Abcam, #ab1), anti-NF-κB antibody (1:1000, Abcam, #ab16502), anti-p-NF-κB antibody (1:1000, Abcam, #ab86299), anti-α-Tubulin antibody (1:1000, Sigma-Aldrich, #T6074), anti-β-actin antibody (1:1000, Sigma-Aldrich, #A1978). After washing, membranes were incubated with goat anti-rabbit and goat anti-mouse IgG (H + L)-HRP conjugate secondary antibodies (1:5000, Bio-Rad, #1721019, 1706516) for 1 h at room temperature. The protein bands were detected using Clarity MaxTM Western ECL Substrate (Bio-Rad, #1705062) and imaged with the ChemiDoc imaging system (Bio-Rad).

RT-qPCR

After 24 h of transfection, total RNA was extracted from SH-SY5Y cells using TRIzol Reagent (Sigma-Aldrich, #T9424). RNA concentration and purity were assessed using a NanoDrop spectrophotometer. Total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, #1708891). RT-qPCR was performed with SYBR Green Supermix (Bio-Rad, #1725274) on a ViiA^{TM}7 system (Applied Biosystems). Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with GAPDH as the reference gene.

The forward (F) and reverse (R) primers used for the indicated genes are as follows: human HMGB1 F: 5′-TTT GGG CGA TAC TCA GAG CA-3′, R: 5′-TAT CGC AAA AGC GGA CAA GG-3′; GAPDH F: 5′-CAT CAT CTC TGC CCC CTC T-3′, R: 5′-CAA AGT TGT CAT GGA TGA CCT-3′).

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected and centrifuged at 1500 g for 5 min. CGRP levels were measured using a Human CGRP1 ELISA Kit (Elabscience, #E-EL-H0619, China) according to the manufacturer's instructions. Optical density was measured at 450 nm using a GloMax Discover microplate reader.

Immunofluorescence

After treatment, cells were fixed for 1 h at room temperature and washed with PBS. Cells were permeabilized with 0.1% Triton X-100 and blocked with BSA at room temperature. Cells were then incubated with primary antibody (CXCR4, 1:200, Invitrogen, #35-8800) overnight at 4 ℃. After washing, cells were incubated with the corresponding Alexa Fluor 594-conjugated secondary antibody at room temperature. Nuclei were stained with DAPI and images were acquired using a Zeiss LSM 980 laser scanning confocal microscope equipped with a Plan-Apo 40× oil immersion objective (NA = 1.40).

Cell migration

SH-SY5Y cells were seeded in 35 mm culture dishes at a density of 2×10^5 cells per dish. After 48 h of treatment, cell migration was assessed according to previously reported method [36, 37]. Briefly, cell migration was observed using an Olympus IX73 inverted microscope equipped with a QImaging OptiMOS sCMOS camera (Crisel, QImaging, Surrey, BC, Canada) and MetaVue software (Molecular Devices, Sunnyvale, CA, USA), together with a stage-mounted incubation system providing CO_2 and temperature control (H201; Okolab, Pozzuoli, Italy). Individual cell trajectories were continuously recorded at defined time intervals for at least 3 h using a 20× objective (LUCPlan FLN, NA = 0.45, Ph2). Trajectories were aligned to a common origin for spatial comparison. Cell migration was analyzed using the MTrackJ plugin of ImageJ (NIH, Bethesda, MA, USA) to determine cell displacement and migration velocity.

Reactive oxygen species (ROS) measurement

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to detect the intracellular oxidative stress level in SH-SY5Y cells. Briefly, after 48 h of treatment, cells were incubated with 10 μM H_2DCFDA in PBS at 37 ℃

and 5% CO_2 for 30 min. Cells were then visualized using a Zeiss LSM 980 laser scanning confocal microscope equipped with a Plan-Apo 40× oil objective (NA = 1.40) with an excitation wavelength of 488 nm. Quantification of fluorescence was detected using a microplate reader (GloMax Discover, Promega) with 485 nm excitation and 538 nm emission filters and values were subtracted of blank (cells with medium only) and normalised by protein content (SRB assay) for each well as previously reported [38]. Each experiment was performed in triplicate and at least three biological replicates were carried out.

Statistical analysis

All experiments were performed with at least three independent biological replicates. In this study, n represents independent experiments performed on separate cell cultures, rather than individual cells. For assays involving population-level measurements (Western blot, ELISA, RT-qPCR), quantitative values were obtained from each independent experiment and used for statistical analysis. For assays based on single-cell measurements (cell migration and fluorescence-based ROS analysis), multiple individual cells were analyzed within each biological replicate (typically ~ 30–40 cells per condition). Cell-level measurements were first quantified individually and subsequently averaged to obtain a single representative value for each biological replicate. Statistical analyses were then performed using these biological replicate means to avoid pseudoreplication. Cell-to-cell variability was observed within each replicate and is reflected in the distribution of individual data points shown in the figures. For migration analyses, measurements were derived from individual cell trajectories and summarized for each condition within each experimental day. Each experimental day was considered an independent biological replicate. Because experiments were performed across multiple days with an unbalanced distribution of conditions, statistical analysis was conducted using a linear mixed-effects model with condition as a fixed effect and experimental day as a random effect. Multiple comparisons were performed using the Holm–Šídák method.

For comparisons between two groups, statistical significance was evaluated using the unpaired two-tailed Student's t-test (parametric) or the Mann–Whitney U test (non-parametric). For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (parametric) or the Kruskal–Wallis test followed by Dunn's post hoc test (non-parametric) was applied. For tracked cells, each point represents one independent acquisition. Data were analyzed using a mixed-effects model. All statistical analyses were performed using GraphPad Prism 9. A p-value < 0.05 was statistically significant.

Results

LPS and $CoCl_2$ induce inflammation and hypoxia in SH-SY5Y cells

LPS is commonly used to induce inflammatory responses, whereas $CoCl_2$ serves as a chemical hypoxia mimetic [39, 40]. In this study, SH-SY5Y cells were treated with a combination of LPS and $CoCl_2$ to simultaneously induce inflammation and hypoxic conditions, thereby mimicking the pathophysiological microenvironment of migraine.

Concentrations and exposure times of LPS and $CoCl_2$ were selected based on preliminary experiments evaluating cytotoxic and hypoxia induction in SH-SY5Y cells assessed by Western blot analysis of the hypoxia marker HIF-1α (data not shown), consistent with previous literature reporting minimal cytotoxicity in SH-SY5Y cells under similar experimental conditions [34, 41–43]. In addition, no evident morphological alterations were observed during the experiments. To validate the successful establishment of this in vitro model, the expression levels of HMGB1, CGRP, HIF-1α, NF-κB and phosphorylated NF-κB (p-NF-κB) were examined.

As shown in Fig. 1A, LPS stimulation increased the expression of HMGB1 and CGRP as well as the p-NF-κB/NF-κB ratio in SH-SY5Y cells, indicating activation of inflammatory signaling pathways. The induction of HIF-1α following $CoCl_2$ treatment, shown in Fig. 1B, confirms the establishment of a hypoxic-like cellular environment. Quantitative analyses of p-NF-κB/NF-κB, HMGB1, and CGRP expression (Fig. 1C–E) further demonstrated a significant upregulation of these inflammatory and neuropeptide markers following stimulation. Figure 1F presents the quantitative analysis of HIF-1α expression, supporting the effectiveness of $CoCl_2$ treatment in inducing hypoxia-related signaling in SH-SY5Y cells. These results indicate that the inflammatory-hypoxic model was successfully established and the NF-κB signaling pathway was activated under these conditions.

HMGB1 promotes CGRP production through the NF-κB signaling pathway in SH-SY5Y cells under inflammatory and hypoxic conditions

As in the inflammatory-hypoxic model established by LPS and $CoCl_2$ stimulation, HMGB1 expression was significantly increased in SH-SY5Y cells, to determine whether HMGB1 contributed to the regulation of CGRP under these conditions, siRNA-mediated knockdown of HMGB1 was performed prior to evaluating downstream signaling changes. Efficient suppression of HMGB1 expression was confirmed at both the mRNA and protein levels (Fig. 2). This approach allowed us to assess whether HMGB1 upregulation induced by inflammatory-hypoxic stimulation was functionally involved in CGRP regulation. Representative Western blot results are shown in Fig. 2A, demonstrating reduced HMGB1 protein

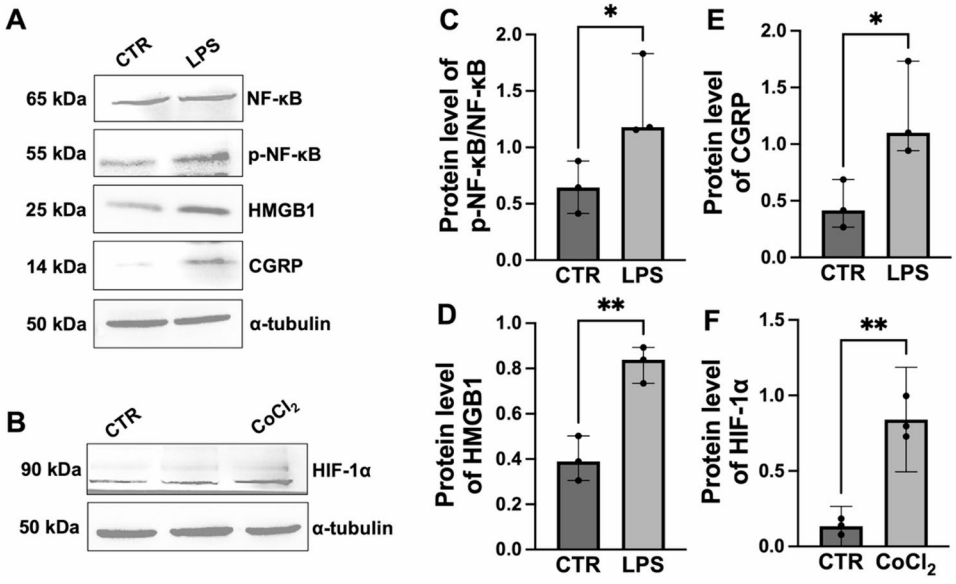


Fig. 1 LPS and $CoCl_2$ induce inflammation and hypoxia in SH-SY5Y cells with the activation of NF-κB pathway. (**A**) Representative Western Blot bands of NF-κB, p-NF-κB, HMGB1 and CGRP in SH-SY5Y cells treated with LPS (1 μg/mL) for 48 h compared with untreated control cells. (**B**) Representative Western Blot bands of HIF-1α in SH-SY5Y cells treated with $CoCl_2$ (300 μM) for 48 h compared with untreated control cells. (**C**-**E**) Quantitative analysis of p-NF-κB/NF-κB (**C**), HMGB1 (**D**) and CGRP (**E**) protein expression in SH-SY5Y cells with and without LPS stimulation. (**F**) Quantitative analysis of HIF-1α protein expression in SH-SY5Y cells with and without $CoCl_2$ stimulation. Data, normalized to α-tubulin expression, were presented as mean values and error bars indicate 95% confidence intervals (CI) from three independent biological replicates ($n = 3$). Each biological replicate represents an independent experiment performed on separate cell cultures. Individual data points represent biological replicates. Statistical analysis was performed on biological replicates using the unpaired two-tailed Student's t-test. *$p < 0.05$; **$p < 0.01$

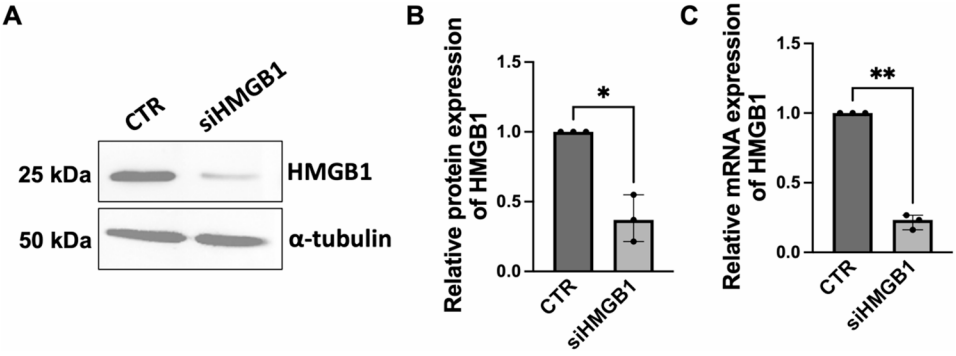


Fig. 2 HMGB1 expression is markedly reduced following siHMGB1 transfection. (**A**) Representative Western Blot band of HMGB1 in SH-SY5Y cells transfected with 200 nM siHMGB1 using Lipofectamine® 2000 for 48 h compared with untreated control cells. (**B**) Quantitative analysis of HMGB1 knockdown efficiency at the protein level. Data were normalized to α-tubulin expression and are presented as mean (95% CI) from three independent biological replicates ($n = 3$). Each biological replicate represents an independent experiment performed on separate cell cultures. Individual data points represent biological replicates. Statistical analysis was performed on biological replicates using the unpaired two-tailed Student's t-test. (**C**) RT-qPCR quantification of HMGB1 mRNA expression in SH-SY5Y cells with and without transfection for 24 h. Gene expression level was normalized to GAPDH. Data were presented as mean (95% CI) from three independent biological replicates ($n = 3$). Each biological replicate represents an independent experiment performed on separate cell cultures. Individual data points represent biological replicates. Statistical analysis was performed on biological replicates. *$p < 0.05$; **$p < 0.01$

expression following siHMGB1 transfection. Quantitative analysis of HMGB1 protein levels (Fig. 2B) confirmed a significant reduction compared with control cells. Consistently, RT-qPCR analysis (Fig. 2C) showed a marked decrease by approximately 77%, in HMGB1 mRNA expression following siHMGB1 transfection.

To further determine whether HMGB1 contributes to CGRP production under inflammatory and hypoxic conditions, SH-SY5Y cells were treated with LPS + $CoCl_2$ in the presence or absence of HMGB1 knockdown. Representative Western blot images, shown in Fig. 3A, illustrate changes in NF-κB, p-NF-κB, HMGB1, and CGRP expression across experimental conditions. Compared with the LPS + $CoCl_2$ group, HMGB1 knockdown significantly reduced CGRP expression and decreased the p-NF-κB/NF-κB ratio (Fig. 3B). Similarly, HMGB1

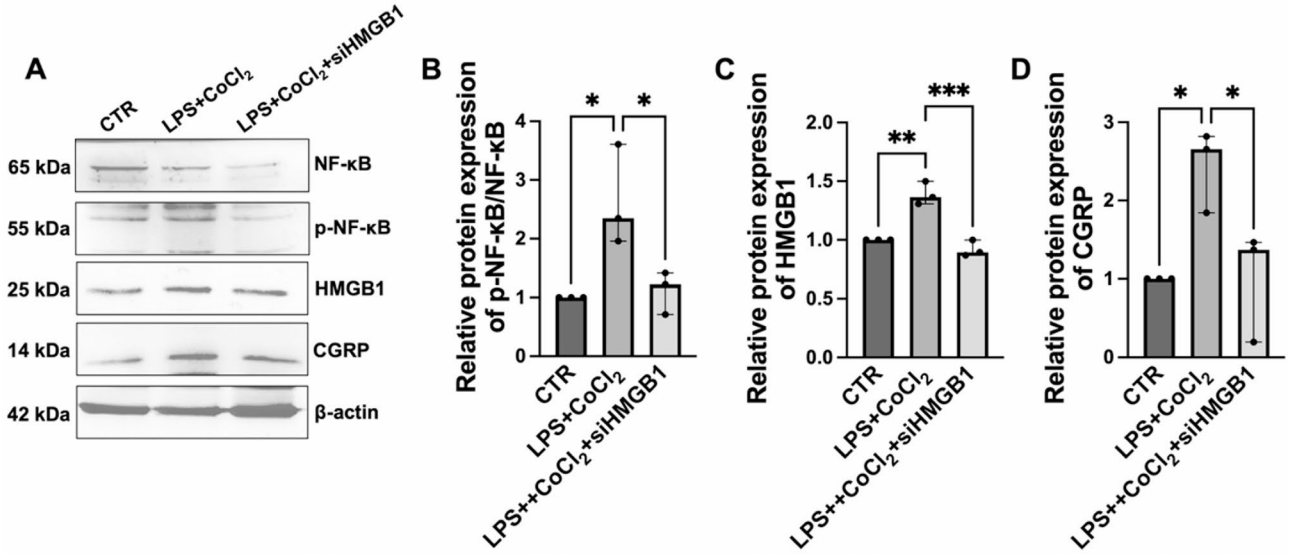


Fig. 3 HMGB1 promotes CGRP production through the NF-κB signaling pathway in SH-SY5Y cells. (**A**) Representative Western Blot bands of NF-κB, p-NF-κB, HMGB1 and CGRP in SH-SY5Y cells treated with LPS+$CoCl_2$ or LPS+$CoCl_2$+siHMGB1 for 48 h compared with untreated control cells. (**B**-**D**) Quantitative analysis of p-NF-κB/NF-κB (**B**), HMGB1 (**C**) and CGRP (**D**) protein expression in SH-SY5Y cells. Data normalized to α-tubulin or β-actin expression were presented as mean (95% CI) from three independent biological replicates ($n = 3$). Each biological replicate represents an independent experiment performed on separate cell cultures. Individual data points represent biological replicates. Statistical analysis was performed on biological replicates. $^{**}p < 0.01$, $^{***}p < 0.001$

protein levels (Fig. 3C) and CGRP expression (Fig. 3D) were markedly decreased in the LPS + $CoCl_2$ + siHMGB1 group. These findings suggest that HMGB1 contributes to CGRP upregulation under inflammatory-hypoxic conditions, likely through NF-κB–related signaling.

These findings confirm that inflammatory-hypoxic stimulation induces HMGB1 expression, providing the basis for subsequent experiments investigating the role of HMGB1 in CGRP regulation.

Given that SH-SY5Y cells are widely used as a neuronal model to study trigeminal-related neuroinflammation [44], these results further suggest that HMGB1-NF-κB-CGRP signaling may reflect mechanisms contributing to TVS activation in migraine.

CGRP secreted by SH-SY5Y cells promotes HMGB1 production through the NF-κB signaling pathway in HUVECs

HUVECs have been widely used to establish an in vitro model of vascular endothelial cells relevant to migraine research [45]. Accordingly, HUVECs were used in this study to model migraine-related vascular endothelial components of the TVS. To investigate whether neuron-derived signals influence endothelial inflammatory responses, HUVECs were treated with conditioned medium derived from SH-SY5Y cells exposed to LPS + $CoCl_2$ stimulation. To assess the role of CGRP in regulating HMGB1 production, the CGRP receptor antagonist olcegepant (BIBN4096) was used to block CGRP signaling in HUVECs. Representative Western blot images are shown in Fig. 4A, illustrating the expression of NF-κB pathway components, HMGB1, and CGRP in HUVECs under different treatment conditions.

Quantification of CGRP levels in conditioned medium (Fig. 4B) confirmed increased CGRP release from stimulated SH-SY5Y cells. The absolute CGRP concentration increased from approximately 30.96 pg/mL in control samples to 102.10 pg/mL in the LPS + $CoCl_2$ group. Quantitative analyses of p-NF-κB/NF-κB, HMGB1, and CGRP expression in HUVECs are presented in Fig. 4C–E. These results show that conditioned medium derived from stimulated neuronal cells activates NF-κB signaling and increases HMGB1 expression in endothelial cells, effects that are attenuated by CGRP receptor blockade.Taken together with the findings from SH-SY5Y cells, these data suggest the existence of a positive feedback loop in which neuronal HMGB1 enhances CGRP production, and CGRP in turn stimulates HMGB1 expression in endothelial cells. This reciprocal interaction may amplify neurovascular inflammation within the TVS and potentially contribute to migraine pathophysiology.

HMGB1 promotes SH-SY5Y cell migration under inflammatory and hypoxic conditions

To evaluate whether HMGB1 influences cellular activation dynamics under inflammatory-hypoxic conditions, migration behavior of SH-SY5Y cells by tracking individual cell trajectories. Representative migration trajectories are shown in Fig. 5A–C, corresponding to control cells, LPS + $CoCl_2$–treated cells, and LPS + $CoCl_2$ + siHMGB1

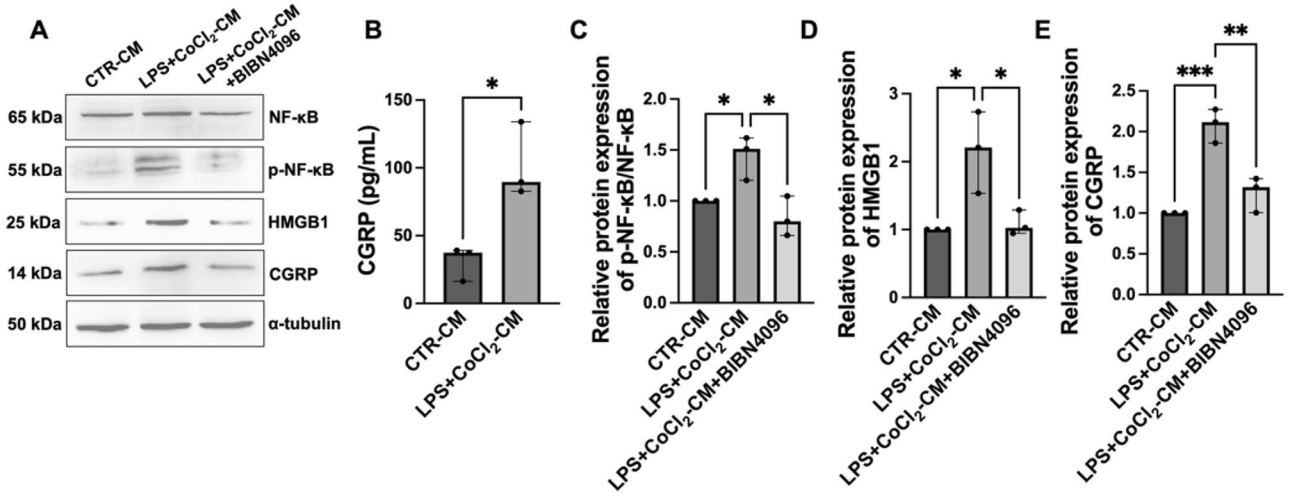


Fig. 4 CGRP secreted by SH-SY5Y cells promotes HMGB1 production through the NF-κB signaling pathway in HUVECs. (**A**) Representative Western Blot bands of NF-κB, p-NF-κB, HMGB1 and CGRP in HUVECs treated with CM collected from SH-SY5Y cells for 24 h. The CTR-CM group received CM from untreated SH-SY5Y cells, the LPS+$CoCl_2$-CM group received CM from SH-SY5Y cells stimulated with LPS+$CoCl_2$, and the antagonist group received CM from LPS+$CoCl_2$-treated SH-SY5Y cells together with 100 nM BIBN4096. (**B**) Quantitative analysis of CGRP in CM collected from SH-SY5Y cells. Data were presented as mean (95% CI) from three independent biological replicates ($n = 3$). Statistical significance was analyzed using the unpaired two-tailed Student's t-test. (**C**-**E**) Quantitative analysis of p-NF-κB/NF-κB (C), HMGB1 (**D**) and CGRP (**E**) protein expression in HUVECs treated with CM collected from SH-SY5Y cells. Data, normalized to α-tubulin expression, were presented as mean (95% CI) from three independent biological replicates ($n = 3$). Individual data points represent biological replicates. Statistical analysis was performed on biological replicates using Student's t-test or one-way ANOVA followed by Tukey's post hoc test, as appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

cells, respectively. Quantitative analyses of migration velocity and displacement presented in Fig. 5D–E, showed a significant increase in, both migration velocity and displacement of SH-SY5Y cells following inflammatory-hypoxic stimulation. In contrast, HMGB1 knockdown markedly attenuated the promigratory effects induced by inflammatory and hypoxic stimuli.

To further elucidate the potential mechanisms underlying HMGB1-mediated regulation of cell migration, C-X-C motif chemokine receptor 4 (CXCR4) expression in SH-SY5Y cells was assessed using immunofluorescence staining (Fig. 5F). The results showed that CXCR4 expression was markedly upregulated under inflammatory and hypoxic conditions. Conversely, CXCR4 expression was significantly reduced following HMGB1 downregulation (Fig. 5G). These findings suggest that HMGB1 enhances the migratory capacity of SH-SY5Y cells under inflammatory and hypoxic conditions through the regulation of CXCR4 expression.

HMGB1 contributes to oxidative stress in SH-SY5Y cells under inflammatory and hypoxic conditions

To investigate whether HMGB1 contributes to oxidative stress responses under inflammatory and hypoxic conditions, intracellular ROS levels were assessed in SH-SY5Y cells. Representative confocal images of ROS fluorescence shown in Fig. 6A, demonstrated an increased intracellular ROS accumulation following combined inflammatory and hypoxic stimulation. Quantitative analysis of ROS fluorescence intensity (Fig. 6B) confirmed a significant elevation in intracellular ROS levels in stimulated cells compared with controls. In contrast, HMGB1 knockdown significantly attenuated ROS accumulation. These results indicate that HMGB1 contributes to oxidative stress response in SH-SY5Y cells under inflammatory and hypoxic conditions.

Discussion

This study investigated the role of HMGB1 within the TVS under migraine-like conditions, with a particular focus on neuron-vascular endothelial interactions under inflammatory and hypoxic conditions. Our primary findings indicate that HMGB1 promotes CGRP expression in neuron-like cells; while CGRP, in turn, induces HMGB1 expression in vascular endothelial cells, forming a positive feedback amplification loop within the TVS. Furthermore, HMGB1 enhanced the migration of neuron-like cells and induced oxidative stress responses (Fig. 7). Together, these findings suggest that HMGB1 may play an important role in the activation and maintenance of peripheral TVS in migraine.

In recent years, the role of inflammation and hypoxia in migraine pathophysiology has attracted increasing attention. Both MOH animal models and clinical patients exhibit elevated levels of pro-inflammatory mediators including HMGB1, CGRP, IL-6, LPS, and LPS-binding protein, as well as increased expression of HIF-1α [26, 27, 46–48]. Animal studies have shown that during

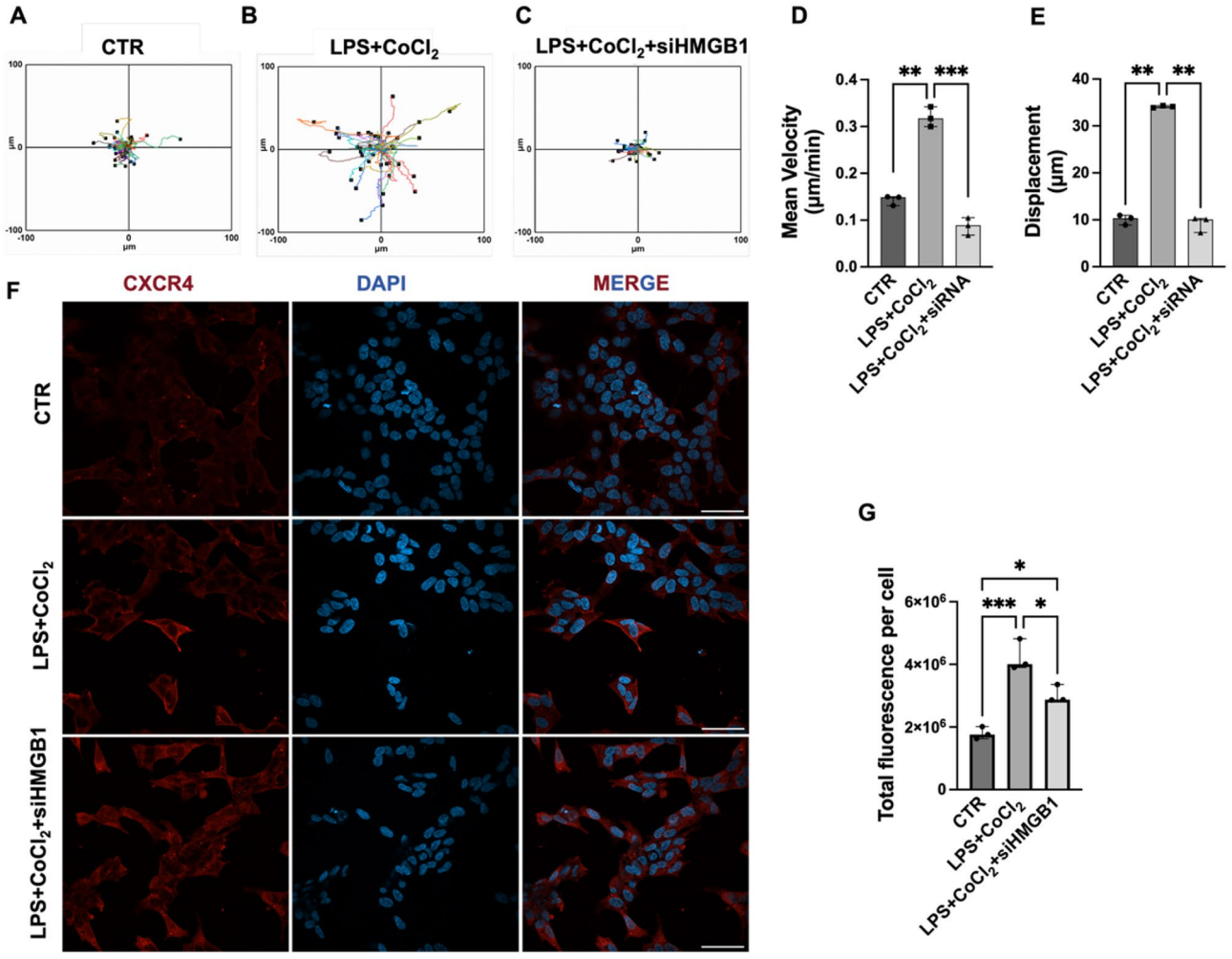


Fig. 5 HMGB1 promotes SH-SY5Y cell migration under inflammatory and hypoxic conditions. (**A**-**C**) Representative migration trajectories of SH-SY5Y cells tracked over 3 h are shown. Each line represents the trajectory of a single cell with all trajectories originating from a common starting point. Approximately 30–40 cells per condition were analyzed within each independent experiment. The migration trajectories of untreated control SH-SY5Y cells (A) are compared with cells treated with LPS+$CoCl_2$ (**B**) or LPS+$CoCl_2$+siHMGB1 (**C**) for 48 h compared with. Colors indicate different cells. (**D**) and (**E**) Quantitative analysis of migration velocity (**D**) and displacement (**E**) of SH-SY5Y cells. Experiments were repeated on separate, independently prepared cell cultures ($n = 3$ biological replicates). Data were presented as mean (95% CI). Statistical analysis was performed using a linear mixed-effects model, followed by Holm–Šídák multiple-comparisons testing. (F) Representative confocal images of CXCR4 in SH-SY5Y cells treated with LPS+$CoCl_2$ or LPS+$CoCl_2$+siHMGB1 for 48 h compared with untreated control cells. (**G**) Quantitative analysis of CXCR4 fluorescence intensity in SH-SY5Y cells. Data were presented as mean ± S.E.M. from three independent biological replicates ($n = 3$). Statistical analysis was performed on biological replicates using the Kruskal–Wallis test followed by Dunn's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

migraine aura-related CSD, cerebral blood flow and tissue oxygen tension are reduced while the cerebral metabolic rate of oxygen remains elevated, resulting in a state of relative cerebral hypoxia [49, 50]. These observations indicate that hypoxia may be involved in the pathophysiology of migraine. LPS is commonly used to establish in vitro inflammatory models by activating the NF-κB signaling pathway and inducing the release of inflammatory mediators such as HMGB1 [51]. $CoCl_2$ is a classical chemical hypoxia mimetic that stabilizes HIF-1α, thereby activating the NF-κB signaling pathway and promoting HMGB1 expression [52, 53]. Based on these mechanisms, the present study employed combined LPS and $CoCl_2$ stimulation of SH-SY5Y cells to mimic the inflammatory and hypoxic microenvironment associated with migraine. In this model, the expression levels of CGRP, HMGB1, HIF-1α, and the p-NF-κB/NF-κB ratio were significantly increased. These results are consistent with previous migraine-related studies [46, 54–56], suggesting that the model successfully mimics the molecular characteristics associated with migraine attack. Although viability assays were not reported in this study, no overt morphological signs of cell damage were observed with the selected concentrations and exposure times of LPS and $CoCl_2$, and normalization of protein expression to housekeeping controls remained consistent across

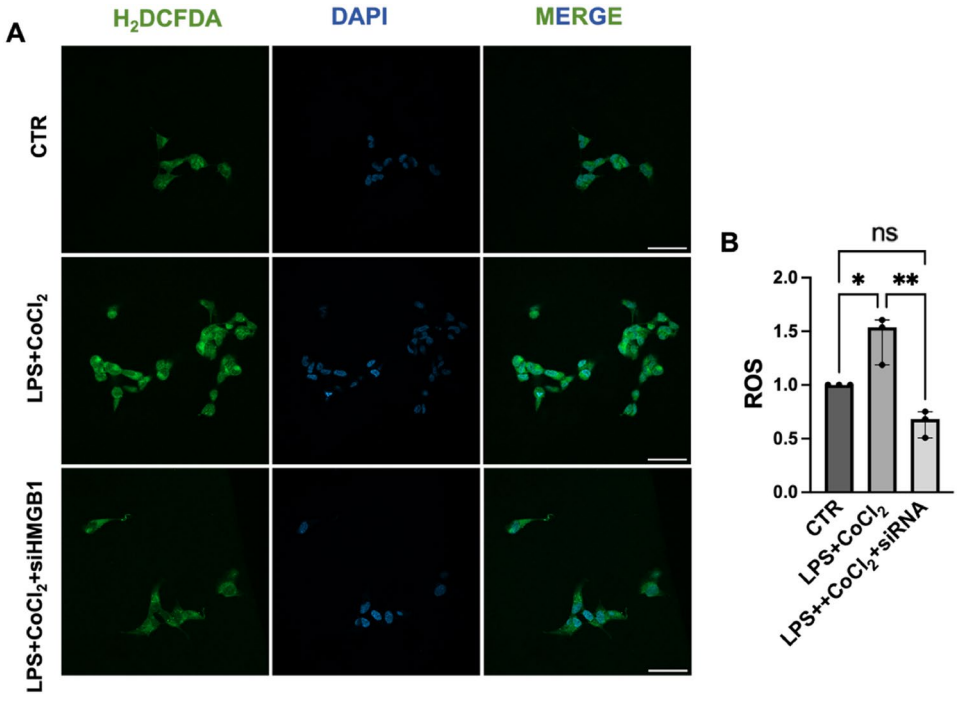


Fig. 6 HMGB1 contributes to oxidative stress in SH-SY5Y cells under inflammatory and hypoxic conditions. (**A**) Representative confocal images of intracellular ROS in SH-SY5Y cells treated with LPS+$CoCl_2$ or LPS+$CoCl_2$+siHMGB1 for 48 h compared with untreated control cells. (**B**) Quantitative analysis of intracellular ROS fluorescence intensity in SH-SY5Y cells. For fluorescence quantification, multiple cells (~30 per condition) were analyzed per replicate and averaged to obtain one value per biological replicate. Data were presented as mean (95% CI) from three independent biological replicates ($n=3$). Statistical significance was analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. ns = not significant, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

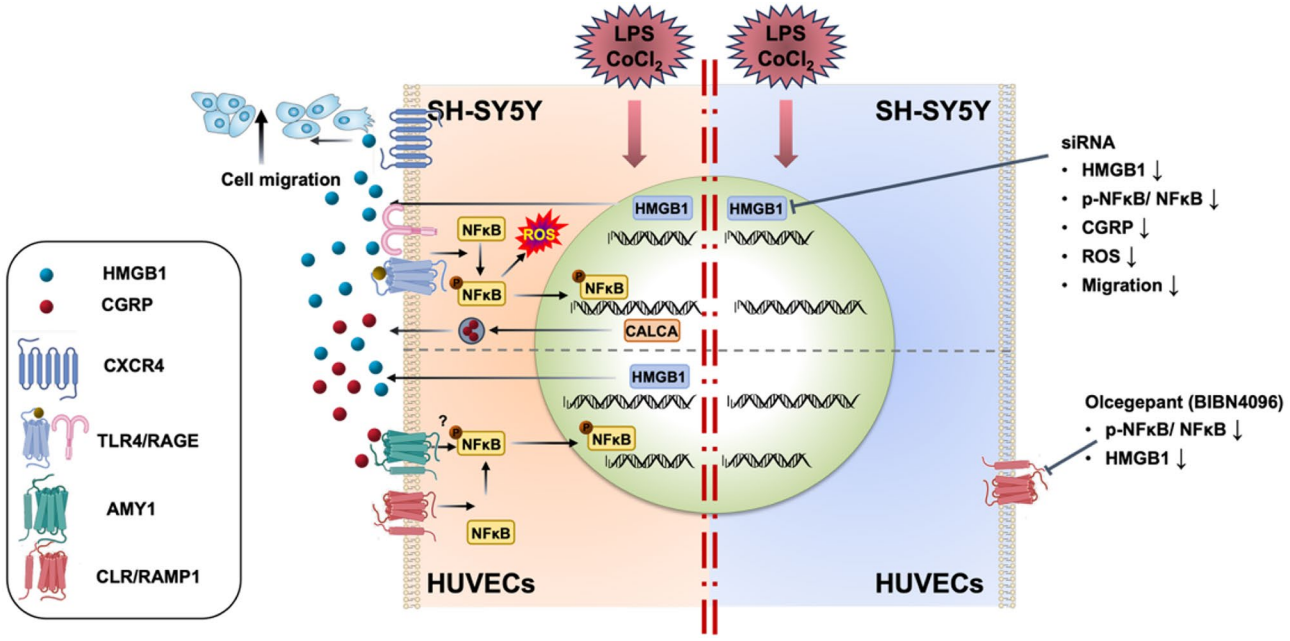


Fig. 7 Proposed role of HMGB1 in the TVS under inflammatory and hypoxic migraine-like conditions. HMGB1 promotes CGRP production through activation of the NF-κB signaling pathway in SH-SY5Y cells exposed to inflammatory and hypoxic stimuli. CGRP released from SH-SY5Y cells can act on endothelial cells through CGRP-responsive receptor complexes, including the canonical CGRP receptor (CLR/RAMP1) and the AMY1 receptor, thereby activating NF-κB signaling and promoting HMGB1 expression in HUVECs. This interaction forms a positive neuron–endothelial feedback loop within the trigeminovascular system. In addition, HMGB1 enhances neuronal migration and promotes oxidative stress responses under inflammatory and hypoxic conditions

groups. Nevertheless, future studies incorporating dedicated viability assays (e.g., MTT or LDH release) would further strengthen the interpretation that observed CGRP modulation is pathway-specific rather than secondary to cellular stress.

Peripheral mechanisms of migraine indicate that CGRP release is a key trigger for initiating and sustaining TVS activation [57]. However, the upstream molecular mechanisms responsible for abnormal CGRP release remain poorly understood. HMGB1 is regarded as a prototypical DAMP that translocates from the nucleus to the cytoplasm and is subsequently released into the extracellular space *via* vesicular pathways upon neuronal stimulation [58]. Extracellular HMGB1 binds to TLR4 or RAGE receptors expressed on neurons and glial cells, activating the NF-κB signaling pathway and inducing the expression of inflammatory mediators, thereby contributing to neuroinflammatory responses [59–61]. Although the present findings support involvement of NF-κB signaling, additional experiments using pharmacological NF-κB inhibitors or nuclear translocation assays would further strengthen the causal relationship between HMGB1 activation and NF-κB signaling in this model. Previous studies have emphasized the role of HMGB1 in promoting migraine-related inflammation via NF-κB activation [55, 62]. However, its involvement in regulating neuropeptide release, especially CGRP expression, has not been previously reported. Herein, siRNA-mediated downregulation of HMGB1 expression in SH-SY5Y cells significantly suppressed NF-κB signaling, consistent with previous research [63]. Interestingly, CGRP expression was also markedly reduced following downregulation of HMGB1. These results suggest that HMGB1 not only participates in regulating the inflammatory response in migraine but also acts as an upstream regulator of CGRP expression in trigeminal sensory neurons through NF-κB activation. This finding provides new insights into the upstream control of CGRP release and highlights HMGB1 as a key molecular link between inflammation and neuropeptide signaling in the peripheral migraine mechanisms.

A significant characteristic of TVS is the interaction between neurons and vascular endothelial cells. CGRP is a key neuropeptide released by trigeminal sensory neurons, performing a central role in the development of migraine [10]. Traditionally, CGRP has been considered to contribute to migraine mainly by inducing vasodilation via actions on cerebral vascular smooth muscle cells [64]. However, recent studies indicate that human cerebral vascular endothelial cells also express functional CGRP receptors and respond to CGRP stimulation by increasing intracellular cAMP levels [64–66]. Based on these findings, CM derived from SH-SY5Y cells, enriched in CGRP, was used to stimulate HUVECs to investigate neuron-derived CGRP effects on endothelial cells. The results suggested that CGRP significantly promoted activation of the NF-κB signaling pathway in HUVECs and upregulated HMGB1 expression, whereas these effects were markedly attenuated by CGRP receptor antagonists. While earlier reports emphasized the role of CGRP in glia-migraine-related inflammatory responses [67–69], the present study identifies vascular endothelial cells as active participants in CGRP-mediated neurovascular inflammation. These findings suggest that endothelial cells are not merely passive CGRP targets but may actively amplify neurovascular inflammatory signaling via HMGB1 release. Taken together, the present findings support the existence of a neuron-vascular endothelial positive feedback amplification loop within the TVS in which HMGB1 promotes neuronal CGRP production, and CGRP, in turn, induces HMGB1 expression in endothelial cells. This reciprocal interaction, mediated by activation of the NF-κB signaling pathway, may amplify local inflammatory responses and neural sensitization, thereby contributing to the persistence and propagation of migraine-related inflammation.While HMGB1 knockdown efficiency was confirmed by both RT-qPCR and Western blot analysis, the inclusion of a scrambled siRNA control would further strengthen methodological specificity. Future studies incorporating additional control sequences will help further validate the target specificity of HMGB1 silencing.

In addition to its role in neurovascular signaling, HMGB1 was found to significantly promote SH-SY5Y cell migration and induce oxidative stress. Neuronal migration is essential for cerebral cortex development and is implicated in multiple neurological disorders, including epilepsy, lissencephaly, schizophrenia, and autism [70–72]. Moreover, excessive migration under pathological conditions is also considered as a hallmark of cellular hyperactivation. Previous studies have primarily focused on the migratory responses of microglia and astrocytes to inflammatory or mechanical stimuli [73, 74]. Notably, studies of cancer pain have shown that increased Schwann cell migration represents a hyperactivated cellular state that contributes directly to pain amplification [75]. In the present study, both migration velocity and displacement of SH-SY5Y cells were significantly increased under combined inflammatory and hypoxic stimulation, suggesting that neurons within the TVS may adopt a hyperactivated phenotype under migraine-like conditions. This effect was significantly inhibited by downregulating HMGB1 expression, indicating that HMGB1 plays a crucial regulatory role in neuronal migration. However, whether increased migration directly reflects altered neuronal excitability or represents a stress-induced motile response requires further investigation. Although neuronal migration in the adult nervous system is limited, changes in migratory behavior

in vitro reflect cytoskeletal remodeling and cellular activation in response to inflammatory stress. The observed HMGB1-dependent upregulation of CXCR4 suggests engagement of the HMGB1/CXCL12/CXCR4 axis, a pathway implicated in cellular activation and sensitization processes [76–78]. HMGB1-dependent increases in migration may reflect enhanced responsiveness of trigeminal-like neurons under inflammatory conditions relevant to migraine. Notably, we observed increased expression of CXCR4 following inflammatory-hypoxic stimulation, which was attenuated after HMGB1 knockdown. The CXCL12/CXCR4 signaling axis has been implicated in inflammatory cell recruitment, neuronal activation, and pain sensitization processes. Therefore, the increased migratory dynamics observed in this model may reflect activation of chemokine-mediated signaling pathways that contribute to neuroinflammatory responses within the trigeminovascular system.

Oxidative stress is also recognized as a critical pathological mechanism in neuronal dysfunction and central sensitization [79]. It arises from an imbalance between ROS production and antioxidant defenses [80], leading to damage of DNA, lipids, and proteins and activation of inflammation-related signaling pathways [81]. Clinical studies have reported that migraine patients exhibit a pronounced imbalance between serum oxidant and antioxidant levels, characterized by reduced activity of antioxidant enzymes, such as superoxide dismutase and catalase, alongside elevated levels of oxidative stress markers, including nitric oxide and malondialdehyde [82]. Consistently, animal studies have demonstrated significantly increased ROS levels in the trigeminal ganglion and trigeminal nucleus caudalis in migraine models, promoting neuronal sensitization and sustaining the pain state [28, 31, 83]. Consistent with these findings, the present study showed that inflammatory and hypoxic stimulation significantly increased ROS levels in SH-SY5Y cells. Importantly, HMGB1 knockdown markedly reduced ROS accumulation, suggesting that HMGB1 is a key mediator of oxidative stress under these conditions, in agreement with previous reports [84]. These findings suggest that inflammation and hypoxia may drive excessive ROS production through HMGB1, thereby inducing oxidative stress and contributing to migraine-related neuronal damage and the persistence of pain.

Conclusion

Although the in vitro model used in this study cannot fully replicate the intricate anatomical structure of the TVS in vivo, it provides a well-controlled system for investigating neuron-endothelial molecular interactions relevant to the TVS. Cerebral microvascular endothelial cells would provide higher anatomical specificity, and future studies should validate these findings in more specialized models. Further validation in animal models and clinical samples will be necessary to confirm the relevance of the HMGB1-CGRP feedback loop in migraine.

In conclusion, using an in vitro neurovascular model exposed to inflammatory and hypoxic stress, we identify HMGB1 as a potential upstream modulator of CGRP-associated signaling and oxidative stress responses. The data support the existence of a bidirectional neuron–endothelial amplification loop that may contribute to trigeminovascular activation. While further validation in primary and in vivo systems is required, these findings expand the mechanistic framework linking inflammation to CGRP dysregulation in migraine. .

Abbreviations

TVS	Trigeminovascular system
CGRP	Calcitonin gene-related peptide
HMGB1	High-mobility group box 1
LPS	Lipopolysaccharide
$CoCl_2$	Cobalt chloride
HUVECs	Human umbilical vein endothelial cells
DAMP	Damage-associated molecular pattern
TLRs	Toll-like receptors
RAGE	Receptor for advanced glycation end products
CSD	Cortical spreading depression
FHM1	Familial hemiplegic migraine type 1
MOH	Medication-overuse headache
CM	Conditioned medium
siRNA	Small interfering RNA
siHMGB1	HMGB1-targeting siRNA
RT-qPCR	Real-time quantitative polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
ROS	Reactive oxygen species
p-NF-κB	Phosphorylated NF-κB

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10194-026-02357-4.

Supplementary Material 1

Acknowledgements

We thank Roberta Grillo for useful technical support. We also thank Giovanna Loffredo and Manuela Marchetti for practical administrative support.

Author contributions

M.S. designed and performed the experiments, the outcome parameters and assessments. M.S. performed Western Blot and ELISA assays. B.C. performed confocal imaging; ; C.D.L., G.G., G.F, M.S. and B.C. analysed and interpreted the data. M.S. drafted the manuscript writing. All authors contributed to the revision of the manuscript. G.C. and B.C. are the project supervisors and are responsible for supervision and resources. All authors discussed the results and contributed to the final manuscript.

Funding

We gratefully acknowledge the support from the China Scholarship Council program (202408830004). The research leading to these results has received funding from AIRC under IG 2021 - ID. 26328 project –"P.I. Cortese Barbara". The authors are also grateful to the "Tecnopolo per la medicina di precisione" (TecnoMed Puglia) - Regione Puglia: DGR n.2117 del 21/11/2018, CUP: B84I18000540002 and "Tecnopolo di Nanotecnologia e Fotonica per la medicina di precisione" (TECNOMED) - FISR/MIUR-CNR: delibera CIPE n.3449 del 7-08-2017, CUP: B83B17000010001.

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

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
G. Coppola declares to be Associate Editor of The Journal of Headache and Pain, Cephalalgia, Cephalalgia Reports, Frontiers in Neurology (Neurotechnology section), BMC Neurology (Pain section), Frontiers in Human Neuroscience (Brain Imaging and Stimulation section), Headache and Pain Research, Confinia Cephalalgica. He has received honoraria as a moderator from AbbVie, Pfizer, and Eli-Lilly, has received consulting fees from AbbVie, Eli-Lilly, and Pfizer, and has been the PI in trials sponsored by Pfizer and AbbVie. He has received research grants from AbbVie. G. Sebastianelli reports receiving personal fees from AbbVie and serves as a member of the Editorial Board of Neurology Residents and Fellows Sections and The Junior Editorial Board of The Journal of Headache and Pain. The other authors declare that they have no competing interests in regard to this research.

Author details
[1]Department of Neurology, Headache Center, The First Hospital of Shanxi Medical University, Taiyuan, China
[2]Department of Molecular Medicine, Sapienza University, Viale Regina Elena, 324, 00161 Rome, Italy
[3]Institute of Nanotechnology, National Research Council (CNR-NANOTEC), 73100 Lecce, Italy
[4]Department of Experimental Medicine, University of Salento, 73100 Lecce, Italy
[5]Tecnomed Puglia - Tecnopolo per la Medicina di Precisione (Biotech Lecce Hub), c/o Campus Ecotekne, Lecce, Italy
[6]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome Polo Pontino ICOT, Latina, Italy
[7]IRCCS Neuromed, Pozzilli, Italy
[8]National Research Council-Institute of Nanotechnology (CNR Nanotec), University La Sapienza, c/o Edificio Fermi, Pz.le Aldo Moro 5, 00185 Rome, Italy

Received: 10 February 2026 / Accepted: 1 April 2026
Published online: 11 April 2026

References

1. Raggi A et al (2024) Hallmarks of primary headache: part 1 - migraine. J Headache Pain 25(1):189
2. Global (2024) regional, and national burden of disorders affecting the nervous system, 1990–2021: a systematic analysis for the Global Burden of Disease Study 2021. Lancet Neurol 23(4):344–381
3. Moskowitz MA et al (1979) Neurotransmitters and the fifth cranial nerve: is there a relation to the headache phase of migraine? Lancet 2(8148):883–885
4. Noseda R, Burstein R (2013) Migraine pathophysiology: anatomy of the trigeminovascular pathway and associated neurological symptoms, cortical spreading depression, sensitization, and modulation of pain. Pain 154(Suppl 1):S44–53
5. Goadsby PJ et al (2017) Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiol Rev 97(2):553–622
6. Akerman S, Holland PR, Goadsby PJ (2011) Diencephalic and brainstem mechanisms in migraine. Nat Rev Neurosci 12(10):570–584
7. Goadsby PJ, Edvinsson L (1993) The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 33(1):48–56
8. Goadsby PJ, Edvinsson L, Ekman R (1988) Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. Ann Neurol 23(2):193–196
9. Ashina M et al (2019) Migraine and the trigeminovascular system-40 years and counting. Lancet Neurol 18(8):795–804
10. Chen ST, Wu JW (2020) A new era for migraine: The role of calcitonin gene-related peptide in the trigeminovascular system. Prog Brain Res 255:123–142
11. Huang Q et al (2025) Circulating CGRP as a diagnostic biomarker in female migraine patients: a single-center case-control study. J Headache Pain 26(1):222
12. van Dongen RM et al (2017) Migraine biomarkers in cerebrospinal fluid: A systematic review and meta-analysis. Cephalalgia 37(1):49–63
13. Alpuente A et al (2022) Salivary CGRP can monitor the different migraine phases: CGRP (in)dependent attacks. Cephalalgia 42(3):186–196
14. Edvinsson L et al (2018) CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat Rev Neurol 14(6):338–350
15. Haghdoost F et al (2023) Evaluating the efficacy of CGRP mAbs and gepants for the preventive treatment of migraine: A systematic review and network meta-analysis of phase 3 randomised controlled trials. Cephalalgia 43(4):3331024231159366
16. Chen PY et al (2023) Neuronal NLRP3 inflammasome mediates spreading depolarization-evoked trigeminovascular activation. Brain 146(7):2989–3002
17. Kursun O et al (2021) Migraine and neuroinflammation: the inflammasome perspective. J Headache Pain 22(1):55
18. Seong SY, Matzinger P (2004) Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol 4(6):469–478
19. Zeh HJ, Lotze MT (2005) Addicted to death: invasive cancer and the immune response to unscheduled cell death. J Immunother 28(1):1–9
20. Fan H et al (2020) Inhibiting HMGB1-RAGE axis prevents pro-inflammatory macrophages/microglia polarization and affords neuroprotection after spinal cord injury. J Neuroinflammation 17(1):295
21. Feng Y et al (2025) HMGB1 Mediates Microglia-Astrocyte/Neuron Crosstalk and Pyroptosis by the TLR4/NF-κB Pathway in Multiple Sclerosis. Front Biosci (Landmark Ed) 30(5):37838
22. Yang Y et al (2025) Microglial macrophage-derived ds-HMGB1 in DRG orchestrates neuropathic pain through immune-neural signaling. Cell Rep 44(12):116671
23. Karatas H et al (2013) Spreading depression triggers headache by activating neuronal Panx1 channels. Science 339(6123):1092–1095
24. Dehghani A et al (2023) Optogenetic cortical spreading depolarization induces headache-related behaviour and neuroinflammatory responses some prolonged in familial hemiplegic migraine type 1 mice. J Headache Pain 24(1):96
25. Vuralli D et al (2024) Leaky gut and inflammatory biomarkers in a medication overuse headache model in male rats. Turk J Med Sci 54(1):33–41
26. Vuralli D et al (2024) Lipopolysaccharide, VE-cadherin, HMGB1, and HIF-1α levels are elevated in the systemic circulation in chronic migraine patients with medication overuse headache: evidence of leaky gut and inflammation. J Headache Pain 25(1):23
27. Vuralli D et al (2024) Microbiota alterations are related to migraine food triggers and inflammatory markers in chronic migraine patients with medication overuse headache. J Headache Pain 25(1):192
28. Liu Y et al (2024) Unveiling the therapeutic potential of DI-3-n-butylphthalide in NTG-induced migraine mouse: activating the Nrf2 pathway to alleviate oxidative stress and neuroinflammation. J Headache Pain 25(1):50
29. Bernecker C et al (2011) Oxidative stress is associated with migraine and migraine-related metabolic risk in females. Eur J Neurol 18(10):1233–1239
30. Chen Y et al (2024) SIRT1 activation by Ligustrazine ameliorates migraine via the paracrine interaction of microglia and neurons. Phytomedicine 135:156069
31. Guan XY et al (2025) Mitochondrial dysfunction in trigeminal ganglion contributes to nociceptive behavior in a nitroglycerin-induced migraine mouse model. Mol Pain 21:17448069251332100
32. Yang YS et al (2018) Synaptic transmission and excitability during hypoxia with inflammation and reoxygenation in hippocampal CA1 neurons. Neuropharmacology 138:20–31
33. Frank F et al (2022) Short Report of Longitudinal CGRP-Measurements in Migraineurs During a Hypoxic Challenge. Front Neurol 13:925748
34. Zhang Q et al (2021) HAGLR aggravates neuropathic pain and promotes inflammatory response and apoptosis of lipopolysaccharide-treated SH-SY5Y cells by sequestering miR-182-5p from ATAT1 and activating NLRP3 inflammasome. Neurochem Int 145:105001
35. Park CH, Park JY, Cho WG (2024) Chemical hypoxia induces pyroptosis in neuronal cells by caspase-dependent gasdermin activation. Int J Mol Sci, 25(4)

36. Basilico B et al (2025) YAP/TAZ cytoskeletal remodelling is driven by mechanotactic and electrotactic cues. Mater Adv 6(1):248–262
37. Fratini N et al (2026) The extracellular matrix HA promotes the YAP/hypoxia axis of glioblastoma cells on 3D agar/HA scaffolds. Biomater Adv 181:214664
38. Moutaoukil ME et al (2025) Doxorubicin and NFL-TBS.40–63 peptide loaded gold nanoparticles as a multimodal therapy of glioblastoma. Discov Nano 20(1):72
39. Chia JSM et al (2020) Zerumbone modulates α(2A)-adrenergic, TRPV1, and NMDA NR2B receptors plasticity in CCI-induced neuropathic pain In vivo and LPS-induced SH-SY5Y neuroblastoma In vitro models. Front Pharmacol 11:92
40. Kourti M et al (2022) Enhanced Ca(2+) Entry sustains the activation of Akt in glucose deprived SH-SY5Y cells. Int J Mol Sci, 23(3)
41. Gómez-Arnaiz S, Tate RJ, Grant MH (2020) Cytotoxicity of cobalt chloride in brain cell lines - a comparison between astrocytoma and neuroblastoma cells. Toxicol Vitro 68:104958
42. Merlo S et al (2020) SIRT1 mediates melatonin's effects on microglial activation in hypoxia: in vitro and in vivo evidence. Biomolecules, 10(3)
43. Shademan B et al (2025) LPS-induced neuroinflammation disrupts brain-derived neurotrophic factor and kinase pathways in Alzheimer's disease cell models. Cell Mol Neurobiol 45(1):102
44. Zupin L, Crovella S (2022) Blue laser light counteracts HSV-1 in the SH-SY5Y neuronal cell model of infection. Life (Basel), 12(1)
45. Ouyang J et al (2019) Curcumin protects human umbilical vein endothelial cells against H(2)O(2)-induced cell injury. Pain Res Manag 2019:3173149
46. Topa EGA et al (2025) Altered expressions of CGRP, SULT1A1, HMGB1, and HIF-1α in the trigeminal ganglion in medication overuse headache in female rats. J Headache Pain 26(1):221
47. Dağıdır HG et al (2023) Medication overuse headache is associated with elevated lipopolysaccharide binding protein and pro-inflammatory molecules in the bloodstream. J Headache Pain 24(1):150
48. Liang J et al (2025) Association between hypoxia-inducible factor-1α and neurological diseases: a bidirectional two-sample mendelian randomization analysis. Brain Behav 15(3):e70398
49. Piilgaard H, Lauritzen M (2009) Persistent increase in oxygen consumption and impaired neurovascular coupling after spreading depression in rat neocortex. J Cereb Blood Flow Metab 29(9):1517–1527
50. Takano T et al (2007) Cortical spreading depression causes and coincides with tissue hypoxia. Nat Neurosci 10(6):754–762
51. Zhenzhen Z et al (2021) Targeting HMGB1-TLR4 signaling by miR-216a-5p elevation alleviates the inflammatory behavioral hypersensitivity. Neurosci Lett 759:136043
52. Cervellati F et al (2014) Hypoxia induces cell damage via oxidative stress in retinal epithelial cells. Free Radic Res 48(3):303–312
53. Jin G et al (2014) Development of a novel neuroprotective strategy: combined treatment with hypothermia and valproic acid improves survival in hypoxic hippocampal cells. Surgery 156(2):221–228
54. Sun S et al (2024) Microglia TREM1-mediated neuroinflammation contributes to central sensitization via the NF-κB pathway in a chronic migraine model. J Headache Pain 25(1):3
55. Uzay B et al (2024) The effect of P2X7 antagonism on subcortical spread of optogenetically-triggered cortical spreading depression and neuroinflammation. J Headache Pain 25(1):120
56. Chen H et al (2022) IL-17 crosses the blood-brain barrier to trigger neuroinflammation: a novel mechanism in nitroglycerin-induced chronic migraine. J Headache Pain 23(1):1
57. Iyengar S et al (2019) CGRP and the Trigeminal System in Migraine. Headache 59(5):659–681
58. Kaya Z et al (2023) Vesicular HMGB1 release from neurons stressed with spreading depolarization enables confined inflammatory signaling to astrocytes. J Neuroinflammation 20(1):295
59. Yang H, Andersson U, Brines M (2021) Neurons Are Prim Driv Inflamm via Release HMGB1 Cells, 10(10)
60. Kaya Z et al (2025) Spreading depolarization triggers pro- and anti-inflammatory signalling: a potential link to headache. Brain 148(7):2522–2536
61. Kim SW et al (2011) Extracellular HMGB1 released by NMDA treatment confers neuronal apoptosis via RAGE-p38 MAPK/ERK signaling pathway. Neurotox Res 20(2):159–169
62. Dehghani A et al (2021) Widespread brain parenchymal HMGB1 and NF-κB neuroinflammatory responses upon cortical spreading depolarization in familial hemiplegic migraine type 1 mice. Neurobiol Dis 156:105424
63. Moraes TR et al (2024) Spinal HMGB1 participates in the early stages of paclitaxel-induced neuropathic pain via microglial TLR4 and RAGE activation. Front Immunol 15:1303937
64. Jansen-Olesen I, Mortensen A, Edvinsson L (1996) Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. Cephalalgia 16(5):310–316
65. Oliver KR et al (2002) Immunohistochemical localization of calcitonin receptor-like receptor and receptor activity-modifying proteins in the human cerebral vasculature. J Cereb Blood Flow Metab 22(5):620–629
66. Jansen-Olesen I et al (2003) In-depth characterization of CGRP receptors in human intracranial arteries. Eur J Pharmacol 481(2–3):207–216
67. Thalakoti S et al (2007) Neuron-glia signaling in trigeminal ganglion: implications for migraine pathology. Headache 47(7):1008–1023 discussion 24 – 5
68. Zhou Y et al (2025) Caveolin-1 negatively regulates the calcitonin receptor-like receptor and neuroinflammation in a female mouse model of migraine. J Neuroinflammation 22(1):134
69. Yao G et al (2020) NO up-regulates migraine-related CGRP via activation of an Akt/GSK-3β/NF-κB signaling cascade in trigeminal ganglion neurons. Aging 12(7):6370–6384
70. Zhang Z et al (2023) CXCR5 regulates neuronal polarity development and migration in the embryonic stage via F-Actin homeostasis and results in epilepsy-related behavior. Neurosci Bull 39(11):1605–1622
71. Wu Q et al (2014) The dynamics of neuronal migration. Adv Exp Med Biol 800:25–36
72. Barth PG (1987) Disorders of neuronal migration. Can J Neurol Sci 14(1):1–16
73. Goshi N et al (2020) A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation. J Neuroinflammation 17(1):155
74. Mohamed T et al (2023) Protein kinase C epsilon activation regulates proliferation, migration, and epithelial to mesenchymal-like transition in rat Schwann cells. Front Cell Neurosci 17:1237479
75. Salvo E et al (2021) TNFα promotes oral cancer growth, pain, and Schwann cell activation. Sci Rep 11(1):1840
76. Pirani E et al (2024) Tumor cells express and maintain HMGB1 in the reduced isoform to enhance CXCR4-mediated migration. Front Immunol 15:1358800
77. Ding M et al (2023) HMGB1 can activate cartilage progenitor cells in response to cartilage injury through the CXCL12/CXCR4 pathway. Endokrynol Pol 74(1):74–82
78. Schiraldi M et al (2012) HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med 209(3):551–563
79. Shim HS et al (2019) Peripheral and central oxidative stress in chemotherapy-induced neuropathic pain. Mol Pain 15:1744806919840098
80. Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp Physiol 82(2):291–295
81. Valko M et al (2004) Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 266(1–2):37–56
82. Gross EC et al (2021) Mitochondrial function and oxidative stress markers in higher-frequency episodic migraine. Sci Rep 11(1):4543
83. Zhang X et al (2024) SIRT1-regulated ROS generation activates NMDAR2B phosphorylation to promote central sensitization and allodynia in a male chronic migraine rat model. Front Mol Neurosci 17:1387481
84. Massey N et al (2019) HMGB1-RAGE signaling plays a role in organic dust-induced microglial activation and neuroinflammation. Toxicol Sci 169(2):579–592

Publisher's note