Published in final edited form as:

Mol Neurobiol. 2020 January ; 57(1): 461–468. doi:10.1007/s12035-019-01721-7.

Gut microbiota dysbiosis enhances migraine-like pain via TNF α upregulation

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Abstract

Migraine is one of the most disabling neurological diseases worldwide; however, the mechanisms underlying migraine headache are still not fully understood and current therapies for such pain are inadequate. It has been suggested that inflammation and neuro-immune modulation in the gastrointestinal tract could play an important role in the pathogenesis of migraine headache, but how gut microbiomes contribute to migraine headache is unclear. In the present study, we investigated the effect of gut microbiota dysbiosis on migraine-like pain using broad-spectrum antibiotics and germ-free (GF) mice. We observed that antibiotics treatment prolonged nitroglycerin (NTG)-induced acute migraine-like pain in wild-type (WT) mice and the pain prolongation was completely blocked by genetic deletion of tumor necrosis factor- α (TNF α) or intra-spinal trigeminal nucleus caudalis (Sp5C) injection of TNF α receptor antagonist. The antibiotics treatment extended NTG-induced TNF α upregulation in the Sp5C. Probiotics administration significantly inhibited the antibiotics-produced migraine-like pain prolongation. Furthermore, NTG-induced migraine-like pain in GF mice was markedly enhanced compared to that in WT mice and gut colonization with fecal microbiota from WT mice robustly reversed

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Compliance with Ethical Standards: All animal procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Texas A&M University College of Dentistry Institutional Animal Care and Use Committee.

Potential Conflicts of Interest: The authors have no conflicts of interest to declare.

microbiota deprivation-caused pain enhancement. Together, our results suggest that gut microbiota dysbiosis contributes to chronicity of migraine-like pain by upregulating TNF α level in the trigeminal nociceptive system.

Keywords

Gut microbiota; Migraine headache; Tumor necrosis factor-alpha; Spinal trigeminal nucleus caudalis

Introduction

Migraine is the third most prevalent disease in the world [1], and more than 37 million people in the United States suffer from migraine. Migraine headache is often associated with some gastrointestinal (GI) symptoms including nausea, vomiting, diarrhea, constipation, and dyspepsia [2]. Inflammation and neuro-immune modulation in the GI tract could play an important role in the pathogenesis of migraine headache [3,4]. The GI tract is colonized by trillions of microorganisms collectively termed gut microbiota [5]. Accumulating evidence suggests that alterations in gut microbiota not only regulate GI function, immune system maturation and brain development, but also influence migraine [2,5–7]. However, how gut microbiomes are involved in migraine headache is still unclear.

The composition of gut microbiota is essential for maintaining important functions of healthy hosts, including maintenance of intestinal homeostasis, peristalsis, intestinal mucosal integrity, protection against pathogens, and priming of immune responses [8,9]. Conversely, many GI disorders, such as irritable bowel syndrome, inflammatory bowel disease, celiac disease, and food allergies, are associated with an imbalance in gut microbial population [10–13]. Moreover, differences in diversity of gut microbiota and its composition have been linked to extra-intestinal diseases [14–17]. The brain and the GI tract are strongly connected via neural, endocrine, metabolic, and immune pathways [18–24]. The communications between gut and brain are bidirectional and termed as the gut-brain axis [25,15,26–28]. Previous studies have shown that perturbation of the gut-brain axis is involved in some neurological diseases [29–35], such as anxiety, depression, autism, Alzheimer disease, stroke, and Parkinson's disease.

GI disorders appear to be more frequent in migraineurs than in the general population [36,4], but it is still unknown whether and how alteration of gut microbiomes affects migraine headache. In the present study, we investigated the effect of gut microbiota dysbiosis on nitroglycerin (NTG)-induced migraine-like pain and examined the underlying mechanism. The administration of NTG in rodents causes sensory hypersensitivity associated with migraine and produces light-averse behaviors [37–39]. Previous studies showed that blood level of tumor necrosis factor-alpha (TNF α) was higher in migraineurs and markedly increased in NTG-induced migraine rodent model [40,41]. Thus, we will investigate the involvement of TNF α in the effect of gut dysbiosis on chronicity of migraine-like pain.

Materials and Methods

Mice

Male C57BL/6 wild-type (WT) mice (Jackson Lab, Stock # 000664), germ-free (GF) mice (The Core for Integrated Microbiota Research at Texas A&M University) and TNF α knockout (KO) mice (Jackson Lab, Stock # 005540) with C57BL/6 genetic background (8–10 weeks) were used in this study. Mice were housed under standard conditions with a 12 h light-dark cycle, with water and food pellets available *ad libitum*. GF mice were housed under sterile conditions with a sterile standard pellet diet and sterile water. GF mouse sterility was verified by Gram Stain, aerobic and anaerobic culture, and by qPCR. In all behavioral experiments, we acclimated the mice in our animal facility for one week and habituated them in the test room for 30–60 min before behavioral testing. All animal procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The experiments were carried out by researchers in a blinded manner.

Drug Administration

Mice received i.p. injection of NTG or vehicle solution at a 10 ml/kg volume. NTG (American Regent, Shirley, NY) in a stock solution (5 mg/ml) was freshly diluted with 0.9% saline to a dose of 10 mg/kg. We conducted microinjection of R-7050 (0.5 μ l, 0.1 mM in 0.9% saline; Santa Cruz Biotechnology, Inc.) into bilateral spinal trigeminal nucleus caudalis (Sp5C) using a Hamilton syringe at 30 min prior to NTG injection or 1 day after NTG. Intra-Sp5C injection of R-7050 or saline was performed at 0.25 μ l/min and the needle was remained in place for additional three minutes. At the end of experiments, the microinjection site was confirmed histologically.

Oral Gavage of Antibiotics and Probiotics

All oral gavage treatments were performed for 10 days before NTG injection. Mice orally received broad-spectrum antibiotics ABX (including 10 mg/kg streptomycin, 10 mg/kg neomycin, 5 mg/kg vancomycin, and 10 mg/kg metronidazole, dissolved in 0.9% saline, Sigma-Aldrich) every 12 hours. Additionally, ampicillin (1 g/l, Sigma-Aldrich) was added into drinking water. The mice in control group received oral gavage of saline every 12 hours and normal drinking water [42]. For oral gavage of probiotics, mice received probiotics mixture VSL#3 (reconstituted with 0.9% saline, Sigma-Tau Pharmaceuticals) every 24 hours at a dose of 4.5×10^9 bacteria in 0.2 ml. VSL#3 contains eight live, freeze-dried bacterial species: *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Streptococcus salivarius* subsp. *Thermophilus* [43,44].

Mouse Gut Colonization with Fecal Microbiota Transplant (FMT)

For gut colonization of GF mice, a fecal slurry was prepared under anaerobic condition by obtaining fecal pellets from specific pathogen-free WT mice and vortexing with reduced PBS (100 mg/ml). The GF mice were gavaged with fresh fecal slurry (200 μ l per mouse) and then housed in sterilized ventilating isolators. Control GF mice received 200 μ l sterile PBS

by oral gavage and were tested negative for microbial contaminants by Gram Stain, aerobic and anaerobic culture, and qPCR. After two weeks, microbiota profiling in the GF mouse fecal samples was examined with the same methods to confirm the gut colonization. The following universal primers were used in qPCR to amplify the genes encoding 16S rRNA from all bacteria as described previously [45]: Forward: 5'-GTGSTGCAYGGYTGTCGTCA-3'; Reverse: 5'-ACGTCRTCCMCACCTTCCTC-3'.

Orofacial Pain Test

The calibrated von Frey filaments were used to test orofacial mechanical hypersensitivity before and after NTG injection. Each mouse was placed into a 10-cm long restraining glass cylinder and allowed to poke out their heads and forepaws, but the restrainer prevented them from turning around [46]. After acclimation for 5 min, the filament was applied to the midline of the forehead at the level of the eyes (innervated by trigeminal nerve V1 branch). A positive response was defined as a sharp withdrawal of the head upon stimulation. Each filament was applied five times to the V1-innervated skin area for 1–2 s with a 10 s interval, starting from the lowest force of filament (0.08 g) continuing in ascending order. The head withdrawal threshold was calculated as the force at which the positive response occurred in three of five stimuli.

Western Blotting

Mice were sacrificed after different treatments under isoflurane anesthesia and the Sp5C tissues were harvested on ice. The affinity-purified antibody against TNF α (1:2000, Cat # PA5–19810, Life Technologies) was used to assess the expression level of TNF α . β -actin (1:200000, Cat # A5316, Sigma) served as a loading control in all Western blotting experiments. The specificity of TNF α antibody has been verified using the brain tissue from TNF α KO mice. The intensities of bands in the Western blotting were quantified with densitometry. The intensity values of TNF α bands were normalized to β -actin and expressed as a ratio of TNF α / β -actin.

Locomotor Function Test

A rotarod apparatus was used to measure locomotor function of mice. The rods in the apparatus were accelerated from 4 to 40 rpm over 5 min. The falling speed and the time to fall off the rod were recorded.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. All statistical analyses were performed by SigmaStat software. A Student's *t*-test was used for analyzing Western blotting data. Two-way ANOVA with the post-hoc Student-Newman-Keuls test was performed for behavioral testing data. *P* < 0.05 was considered statistically significant.

Results

Gut Microbiota Perturbation Significantly Prolongs NTG-Induced Migraine-like Pain

To explore if gut microbiota dysbiosis affects migraine-like pain, we administered broad-spectrum antibiotics ABX by oral gavage for 10 days before NTG injection and found that ABX treatment did not affect basal head withdrawal threshold, but significantly prolonged NTG-induced orofacial mechanical hypersensitivity indicated by decreased head withdrawal threshold in WT mice (Fig. 1a). We further observed that oral gavage of probiotics VSL#3 for 10 days reversed the antibiotics-produced pain prolongation in the mouse NTG model, though the probiotics alone had no effect on basal head withdrawal threshold and did not alter NTG-induced acute migraine-like pain (Fig. 1a).

Gut Colonization Robustly Reverses Microbiota Deprivation-Produced Prolongation of NTG-Induced Migraine-like Pain

To confirm the involvement of gut microbiota in the pathogenesis of migraine-like pain, we employed GF mice in this study. The basal head withdrawal thresholds in GF mice were at similar level to those in WT mice (Fig. 1b). However, gut microbiota deprivation in the GF mice dramatically prolonged NTG-induced migraine-like pain compared to that in WT mice. We observed that NTG-decreased head withdrawal thresholds only exist for 2 h in WT mice but the decreased thresholds at least last for 48 h in the GF mice (Fig. 1b). Interestingly, 2-week colonization of GF mice with fecal microbiota from WT mice prevented the prolonged migraine-like pain. We observed that NTG significantly decreased head withdrawal thresholds at 2 h post-NTG and the head withdrawal thresholds went back to baseline level from 8 h post-NTG in the GF mice with the gut colonization (Fig. 1b).

Using Gram Stain, aerobic and anaerobic culture and qPCR to test the presence of bacteria in mouse gut, we observed that all the tests showed negative in fecal samples from GF mice and all the tests showed positive in fecal samples from WT mice. To verify the gut colonization with FMT in this study, we tested fecal samples from GF mice after two weeks of FMT and we observed that all the fecal samples were positive.

Gut Microbiota Dysbiosis Prolongs NTG-Induced TNF α Upregulation in the Sp5C

We showed that although ABX treatment for 10 days did not change TNF α expression in the Sp5C (Fig. 2a, b), this treatment significantly increased Sp5C TNF α at both 2 h and 24 h post-NTG (Fig. 2c–f). However, the Sp5C TNF α upregulation in NTG alone group only occurred at 2 h post-NTG (Fig. 2c, d), but not at 24 h post-NTG (Fig. 2e, f).

Genetic Deletion of TNF α or Antagonism of TNF α Receptors Blocks Gut Microbiota Dysbiosis-Prolonged Migraine-like Pain

To verify the contribution of TNF α on gut microbiota dysbiosis-caused pain enhancement in the NTG-induced migraine-like pain model, we employed TNF α KO mice in our study. We showed that genetic deletion of TNF α in the KO mice not only significantly inhibited NTG-induced acute migraine-like pain, but also completely blocked ABX treatment-produced prolongation of the migraine-like pain (Fig. 3a). The basal head withdrawal thresholds in the TNF α KO mice were at the similar level to those in WT mice, but NTG-decreased head

withdrawal thresholds in the KO mice with/without ABX treatment were markedly elevated compared to WT mice (Fig. 3a).

To further determine if TNF α in the trigeminal nociceptive system controls the pain prolongation produced by antibiotics treatment in the NTG-induced migraine-like pain model, we injected R-7050, a specific TNF α receptor antagonist, into the Sp5C and examined the effect of antagonism of TNF α receptors on migraine-like pain in WT mice with/without ABX treatment. We found that bilateral intra-Sp5C injection of R-7050 at 30 min prior to NTG not only significantly inhibited NTG-induced acute migraine-like pain, but also completely blocked ABX treatment-produced prolongation of the migraine-like pain (Fig. 3b). As a control, we observed that intra-Sp5C injection of R-7050 alone had no effect on basal head withdrawal thresholds (Fig. 3b). We further observed that intra-Sp5C injection of R-7050 on Day 1 after NTG significantly inhibited ABX treatment-produced prolongation of the migraine-like pain (see Supplemental Figure 1).

Systemic NTG injection and oral gavage of antibiotics and/or probiotics have no toxic effects on locomotor function of mice

To exclude potential toxic effects produced by systemic NTG injection (10 mg/kg, i.p.) as well as oral gavage of ABX and/or VSL#3, we performed locomotor function testing using a rotarod apparatus. We recorded falling speed and time to fall off the rod before and after treatments. Our data showed that both NTG injection and oral gavage administration did not affect locomotor function of mice (Fig. 4). We observed that the falling speed (Fig. 4a) and the time to fall (Fig. 4b) in the rotarod test had no significant changes after these treatments.

Discussion

Previous studies have shown that NTG not only triggers migraine attacks in migraine-susceptible patients, but also causes headache in healthy people [47–50]. In a mouse model, we and other laboratories have demonstrated that systemic injection of NTG can induce an acute migraine-like pain [37,46,38,51,39]. In this study, we found that chronic use of antibiotics produces chronicity of NTG-induced acute migraine-like pain by disturbing gut microbiota and recovering microbiomes with probiotics prevents the chronicity of such pain. By using GF mice, we further found that gut microbiota deprivation in the GF mice prolongs NTG-induced migraine-like pain and the pain prolongation is completely reversed by gut colonization with fecal microbiota from WT mice. These results suggest that gut microbiota dysbiosis is critical for migraine-like pain chronicity and recovering disturbed gut microbiomes to a normal level could be an effective approach to treat such pain.

In the present study, we discovered that gut microbiota dysbiosis enhances migraine-like pain via TNF α upregulation in the Sp5C. TNF α is a proinflammatory cytokine and plays an important role in the development of chronic pain [52–54]. It has been reported that TNF α increases α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) GluA1 phosphorylation and trafficking in the spinal dorsal horn neurons and contributes to inflammatory pain [55,56]. Our recent study found that AMPA GluA1 Ser831 phosphorylation is critical for NTG-induced migraine-like pain [39]. Moreover, AMPA phosphorylation can alter synaptic AMPA subunit composition and then lead to AMPA

switch from Ca^{2+} -impermeable to Ca^{2+} -permeable receptors [57,58]. A reduction in Ca^{2+} -permeable AMPARs in the spinal cord dorsal horn causes a loss of nociceptive plasticity, whereas an increase in the spinal Ca^{2+} -permeable AMPARs promotes nociceptive plasticity and inflammatory pain chronification [59,60]. Therefore, activation of the $\text{TNF}\alpha$ -AMPA pathway in the trigeminal nociceptive system is likely one of the underlying mechanisms for gut microbiota dysbiosis-enhanced migraine-like pain. It has been demonstrated that gut microbiota can regulate microglia maturation and function by producing short-chain fatty acids [61]. Thus, gut microbiota dysbiosis may activate microglia and secrete more $\text{TNF}\alpha$ in the Sp5C to enhance migraine-like pain.

In conclusion, our results indicate that gut microbiota dysbiosis contributes to chronicity of migraine-like pain and recovering disturbed gut microbiomes could be developed as a new therapy for chronic migraine headache. Moreover, $\text{TNF}\alpha$ in the Sp5C may mediate gut microbiota dysbiosis-produced prolongation of migraine-like pain, and $\text{TNF}\alpha$ in the trigeminal nociceptive system could be a potential therapeutic target for migraine chronicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was supported by National Institutes of Health Grants R01 DE022880 (F.T.) and K02 DE023551 (F.T.).

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Fig. 1. Gut microbiota dysbiosis or deprivation prolongs NTG-induced migraine-like pain.

a Orofacial pain tests for baseline-1 and baseline-2 measurements were carried out before and after 10-day oral gavage of saline, ABX and/or VSL#3, respectively. NTG was injected (10 mg/kg, i.p.) into WT mice with different treatments after baseline-2 measurement ($n = 6-9$ per group). The treatment of ABX (broad-spectrum antibiotics) significantly prolonged NTG-decreased head withdrawal thresholds for at least 48 h post-NTG compared to the saline-treated control group ($*P < 0.05$ vs. the saline control group by two-way ANOVA with the post-hoc Student-Newman-Keuls test). Simultaneous oral gavage of probiotics VSL#3 and ABX did not show prolongation of NTG-decreased head withdrawal thresholds ($\#P < 0.05$ vs. the ABX-treated group by two-way ANOVA with the post-hoc Student-Newman-Keuls test), though the VSL#3 alone had no effect on NTG-induced acute migraine-like pain. **b** Orofacial pain baseline was measured twice for WT and GF mice (baseline-1 and baseline-2). For the GF mouse colonization group (GFC), baseline-1 and baseline-2 were measured before and after 2-week fecal colonization, respectively. NTG was injected (10 mg/kg, i.p.) into these mice after baseline-2 measurement ($n = 6$ per group). Gut microbiota deprivation in the GF mice significantly prolonged NTG-decreased head withdrawal thresholds for at least 48 h post-NTG compared to those in WT mice ($@P < 0.05$ vs. WT mice group by two-way ANOVA with the post-hoc Student-Newman-Keuls test). Gut colonization with fecal microbiota from WT mice in the GFC group completely prevented the gut microbiota deprivation-produced prolongation of NTG-induced migraine-like pain ($&P < 0.05$ vs. the GF mice group by two-way ANOVA with the post-hoc Student-Newman-Keuls test), though GF mice with the colonization still showed NTG-induced acute migraine-like pain. Data are shown as mean $\pm$ SEM.

Fig. 2. Gut microbiota dysbiosis prolongs NTG-induced TNFα upregulation in the Sp5C. **a** and **b** ABX treatment had no effect on the expression of TNFα in the Sp5C (**a**) and statistical analysis showed no significant difference of the ratio of TNFα/β-actin between control and ABX-treated groups (**b**). **c** and **d** At 2 h after NTG injection, TNFα expression in the Sp5C was upregulated in both NTG alone and “ABX+NTG” groups (**c**) and statistical analysis showed that the ratios of TNFα/β-actin in the two groups significantly increased compared to that in the control group (**d**). * $P < 0.05$ vs. the control group by Student’s *t*-test. **e** and **f** At 24 h after NTG injection, TNFα upregulation in the Sp5C still existed in the “ABX+NTG” group, but not in NTG alone group (**e**) and statistical analysis showed that the ratio of TNFα/β-actin in the “ABX+NTG” group significantly increased compared to those in the control and NTG alone groups (**f**). * $P < 0.05$ vs. the control group and # $P < 0.05$ vs. the NTG alone group by Student’s *t*-test. Data are shown as mean $\pm$ SEM. $n = 3$ for each group.

Fig. 3. Genetic deletion of TNF α or antagonism of TNF α receptors blocks the gut microbiota dysbiosis-prolonged migraine-like pain.

a Orofacial pain tests for baseline-1 and baseline-2 measurements were carried out before and after 10-day oral gavage of saline or ABX, respectively. NTG was injected (10 mg/kg, i.p.) into WT and TNF α KO mice with different treatments after baseline-2 measurement ($n = 6-9$ per group). Genetic deletion of TNF α in the KO mice not only significantly inhibited NTG-induced acute migraine-like pain, but also completely blocked ABX treatment-produced prolongation of such pain ($*P < 0.05$ vs. the ABX-treated WT mice group and $^{\#}P < 0.05$ vs. the saline-treated WT mice group by two-way ANOVA with the post-hoc Student-Newman-Keuls test). **b** Orofacial pain tests for baseline-1 and baseline-2 measurements were carried out before and after 10-day oral gavage of saline or ABX, respectively. And baseline-3 was measured following intra-Sp5C injection of vehicle or R-7050 (a specific TNF α receptor antagonist). NTG was injected (10 mg/kg, i.p.) into WT mice with different treatments after baseline-3 measurement ($n = 5$ per group). Same as the effects of genetic deletion of TNF α , bilateral intra-Sp5C injection of R-7050 not only significantly inhibited NTG-induced acute migraine-like pain, but also completely blocked ABX treatment-produced prolongation of such pain ($@P < 0.05$ vs. the “ABX+Vehicle” group and $\&P < 0.05$ vs. the “Saline+Vehicle” group by two-way ANOVA with the post-hoc Student-Newman-Keuls test). Data are shown as mean $\pm$ SEM.

Fig. 4. Systemic NTG injection and oral gavage of antibiotics and/or probiotics have no toxic effects on locomotor function of mice.

a and b A rotarod test was performed to assess locomotor function of mice. We recorded falling speed and time to fall off the rod before and after different treatments (NTG injection and oral gavage of saline, ABX, VSL#3 or “ABX+VSL#3”). These treatments did not affect falling speed (**a**) and the time to fall (**b**) in the rotarod test. Data are shown as mean $\pm$ SEM. $n = 6-9$ for each group.