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NON-MIGRAINE RELATED PAIN BEHAVIOURS IN A TRANSGENIC "MIGRAINE MOUSE" WITH CIRCADIAN DISRUPTION

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Research Submission

No Gastrointestinal Dysmotility in Transgenic Mouse Models of Migraine

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Objective.—To determine whether transgenic mouse models of migraine exhibit upper gastrointestinal dysmotility comparable to those observed in migraine patients.

Background.—There is considerable evidence supporting the comorbidity of gastrointestinal dysmotility and migraine. Gastrointestinal motility, however, has never been investigated in transgenic mouse models of migraine.

Methods.—Three transgenic mouse strains that express pathogenic gene mutations linked to monogenic migraine-relevant phenotypes were studied: CADASIL (*Notch3*-Tg88), FASP (*CSNK1D*-T44A), and FHM1 (*CACNA1A*-S218L). Upper gastrointestinal motility was quantified by measuring gastric emptying and small intestinal transit in mutant and control animals. Gastrointestinal motility was measured at baseline and after pretreatment with 10 mg/kg nitroglycerin (NTG).

Results.—No significant differences were observed for gastric emptying or small intestinal transit at baseline for any of the 3 transgenic strains when compared to appropriate controls or after pretreatment with NTG when compared to vehicle.

Conclusions.—We detected no evidence of upper gastrointestinal dysmotility in mice that express mutations in genes linked to monogenic migraine-relevant phenotypes. Future studies seeking to understand why humans with migraine experience delayed gastric emptying may benefit from pursuing other modifiers of gastrointestinal motility, such as epigenetic or microbiome-related factors.

Key words: migraine, headache, gastroparesis, gastrointestinal motility

Abbreviations: CADASIL cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, FASP familial advanced sleep phase, FHM familial hemiplegic migraine, IBS irritable bowel syndrome, IG idiopathic gastroparesis, NTG nitroglycerin

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INTRODUCTION

Migraine is a prevalent episodic brain disorder characterized by severe headaches as well as sensory, cognitive, and autonomic disturbances.[1] Symptoms vary between attacks and between individuals. About 22% of patients with migraine reports gastrointestinal symptoms during the premonitory phase of migraine,[2] and up to 96% report symptoms with migraine attacks

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that may reflect the dysfunction of the gastrointestinal system, including nausea, vomiting, abdominal discomfort, and diarrhea.[3]

Mothers with migraine are more likely to have a baby with colic.[4] In children and adolescents, abdominal migraine and cyclic vomiting syndrome may occur in the absence of abnormal gastrointestinal examinations (or of headache), but are associated with the development of migraine later in life.[5,6] Functional gastrointestinal disorders, such as irritable bowel syndrome (IBS) and idiopathic gastroparesis (IG), are more prevalent in adults with migraine and *vice versa*. Patients with IBS are ~2.7 times more likely to have migraine[7] and those with migraine are ~2.0 times more likely to have IBS.[8] IG is defined as "a chronic motility disorder of the stomach that involves delayed emptying of solids and liquids, without evidence of mechanical obstruction."[9] Both migraine and IG have a higher prevalence in women than men (3-fold higher and 4-fold higher, respectively).[10,11] Symptoms typically reported with migraine are often the symptoms of IG, including nausea (92% of IG subjects), vomiting (84%), and early satiety (60%).[12] In a survey of 243 patients with IG from gastroenterology specialty clinics, 41% also reported having migraine, whereas, 59% of those patients with severe IG symptoms reported migraine vs 32% with mild IG symptoms.[13]

Kaufman and Levine[14] were the first to report an association between transient IG and migraine attacks. They reported "a tremendously dilated stomach" and a failure to empty orally administered contrast agent as shown by abdominal X-rays in a patient during a migraine attack that was accompanied by nausea and vomiting, whereas no such radiological abnormality was observed after the attack. Patients with migraine retain orally administered medication in their stomach, limiting passage into the small intestine, and its subsequent detection in serum, consistent with IG. Oral salicylate administered during migraine attacks resulted in lower serum drug levels, compared to administration either between attacks[15] or during attacks pretreated with prokinetic agents (eg, metoclopramide), though this study was limited by failure to use subjects as their own controls.[15,16] However, similar findings have been reported with the administration of paracetamol and tolfenamic acid.[17-19] Slower rates of gastric emptying have also been correlated with greater headache severity in migraine.[20] Migraine-associated gastroparesis has been confirmed with both liquid and solid gastric scintigraphy.[21-23]

The etiology of gastroparesis in patients with migraine is unclear. Calcitonin gene-related peptide (CGRP) has effects on gastrointestinal motility and has been implicated in migraine pathophysiology.[24] In some[25,26] but not all[27] human studies, CGRP is elevated in jugular venous blood. CGRP receptors are located throughout the gastrointestinal system including the stomach, and small and large intestines.[28] In mice, CGRP has been shown to induce diarrhea, which can be prevented with concurrent anti-CGRP receptor antibodies, but the mechanisms that cause this effect are not known.[29] Constipation has been reported with anti-CGRP receptor antibodies in humans.[30,31] An animal model of migraine-related gastroparesis would be useful to further elucidate the mechanisms underlying this finding.

Mutations in several genes have been linked to rare monogenic migraine-relevant phenotypes. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a heritable cause of migraine, stroke, and dementia in humans. CADASIL is a consequence of specific missense mutations in the *NOTCH3* gene that encodes a transmembrane receptor protein primarily expressed in arterial smooth muscle.[32] Familial advanced sleep phase (FASP) is syndromic with migraine with or without aura in members of 2 identified families.[33] The FASP-migraine syndrome in these families is associated with missense mutations in the *CSNK1D* gene encoding casein kinase 1δ, a serine-threonine kinase that phosphorylates circadian clock protein Per2, among other substrates. Familial hemiplegic migraine (FHM) is a variant of migraine with aura

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[*]Adam S. Sprouse Blum and Brigitte Lavoie contributed equally to the work.

Conflict of Interest: None

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characterized by transient motor weakness during attacks. Several mutations have been linked to FHM, including the *CACNA1A* gene (FHM1) that encodes the pore-forming α_{1A} subunit of neuronal voltage-dependent $Ca_V2.1$ (P/Q-type) calcium channels.[34] Migraine attacks in patients with mutations in the CADASIL (*NOTCH3*), FASP (*CSNK1D*) and FHM (*CACNA1A, ATP1A2, SCN1A*) genes exhibit most of the clinical features also seen in those with the common forms of migraine,[33,35,36] but no study has evaluated the presence of IG in these patients.

For pathogenic mutations identified in the genes *NOTCH3, CSNK1D, and CACNA1A*, transgenic knock-in or conventional overexpressor mouse strains have been generated that consistently reproduce features of the respective migraine-associated phenotypes (such as increased susceptibility to cortical spreading depolarization and increased pain response when exposed to nitroglycerin [NTG]).[33,37-40] Gastrointestinal motility, however, has not been investigated in any of these well-established mouse models of migraine. We investigated whether these transgenic mutant mice exhibit delays in gastrointestinal motility reminiscent of what has been observed in patients with migraine.

METHODS

Mice.—Three transgenic mouse models carrying monogenic migraine gene mutations were studied: (1) CADASIL: male and female mice that overexpressed the rat *Notch3 cDNA*, driven by the SM22 alpha-smooth muscle cell gene promoter that contained the R169C missense mutation that causes CADASIL (Tg*Notch3*^{R169C}; line 88 mice).[41] (2) FASP: male and female mice that overexpressed the human *CSNK1D* gene that contained the T44A missense mutation that causes FASP (*CSNK1D*-T44A; line 827).[33] (3) FHM1: female knock-in mice that expressed the S218L missense mutation (in the endogenous *Cacna1a* gene) that causes FHM1 (*CACNA1A*-S218L).[42] For the FHM1 strain, only females were studied as they have been shown to express a more severe phenotype than male mutant mice; only mice homozygous for the S218L mutation were studied due to higher genetic load.[10,42]

For the genotypic comparison, age- and sex-matched mice of the respective strains were used. For CADASIL and FASP strains, because the mutant animals overexpressed the mutant gene, overexpressors of the wild-type gene (Tg*Notch3*^{WT}; line 129[41] and *CSNK1D*-WT; line 433, respectively) were used as controls. FHM1 (*CACNA1A*-S218L) mutants were compared to wild-type littermates.

Mouse ages ranged from 13 to 19 weeks. Animals were group-housed with a 12-hour-light/12-hour-dark cycle and water and food *ad libitum*. The University of Vermont Institutional Animal Care and Use Committee approved all care and experimental procedures.

Gastrointestinal Motility Measures and Experimental Groups.—Animals were divided into 3 groups to test different conditions: (1) baseline (no pretreatment); (2) pretreatment with nitroglycerin (NTG); and (3) pretreatment with vehicle.

Animals that underwent pretreatment were administered 10 mg/kg NTG, which came diluted in 5% dextrose (100 mg/250 mL) (Baxter Healthcare Corp., Deerfield, IL), or corresponding vehicle (5% dextrose) subcutaneously at the nape of the neck by tenting the skin. CADASIL and FASP animals received serial NTG injections on Days 0, 2, 4, 6, and 8 (the day of the gastrointestinal motility procedure), similar to prior reports in mice[43] and rats,[44,45] with the intent to maximize the effects of pretreatment with NTG. FHM1 animals were injected only once (to limit the handling of the animals) on the day of the procedure given the higher risk for mortality in homozygous *CACNA1A*-S218L mice.[46]

The night prior to the gastrointestinal motility procedure, food and cage bedding were removed. In animals that underwent pretreatment, animals were weighed and then administered NTG or vehicle subcutaneously 4 hours prior to dissection. In all animals, water was removed 3 hours prior to dissection. Fifteen minutes prior to euthanasia, 100 μL of a non-absorbable fluorescent solution containing 2.5 mg/mL of Rhodamine B Dextran (MW: 70KDa: Invitrogen Corp. Carlsbad, CA) and 2% methylcellulose (Sigma-Aldrich, St. Louis, MO) in tap water was administered via gastric gavage. Two minutes prior to euthanasia, animals were placed in an induction chamber where they underwent an overdose of isoflurane anesthesia followed by euthanasia by decapitation. Next, the abdomen was opened along the midline and hemostats

were placed at the gastroesophageal and gastroduodenal junctions. The stomach was removed and placed into a conical tube containing 4 mL of 0.9% saline. A third hemostat was placed at the ileocecal valve. The small intestine was removed and divided into 10 segments of equal length (~35-45 mm), and subsequently placed into numbered conical tubes, also containing 4 mL of 0.9% saline. The tissues were then homogenized and centrifuged (15 minutes, 500 g, 4°C) and subsequently, 250 μL of the fluorescence containing supernatant was pipetted into black bottom 96-well plates. Fluorescence was quantified at 450 nm absorbance using a BioTek Synergy H4 Hybrid Microplate Reader (BioTek, Winooski, VT).

The primary endpoints were: (1) gastric emptying: the percentage of tracer bolus that progressed from the stomach into the small intestine at 15 minutes and (2) geometric center: the distance that the fluorescent tracer bolus traveled through the small intestine calculated as the $\sum$(Percentage of total fluorescent signal in each segment × The segment number) / Total intestinal fluorescence. Geometric center has been validated as a measurement of intestinal transit in rat.[47] Both endpoints have been used by our laboratory and others in mice.[48,49]

Statistics.—For each group of data, a Kruskal-Wallis one-way ANOVA with Dunn post hoc test was used for *P* value calculations with $P \leq .05$ considered significant. GraphPad Prism (version 7.0, GraphPad Software, La Jolla, CA) was used for statistical analysis.

RESULTS

In experiments with CADASIL (Tg*Notch3*^{R169C} vs Tg*Notch3*^{WT}) mice, no genotypic differences were observed for either the gastric emptying or geometric center endpoints, neither at baseline nor after serial NTG induction in either sex (Fig. 1). Also for experiments in FASP (*CSNK1D*-T44A vs *CSNK1D*-WT) mice, no genotypic differences were observed for any of the endpoints whether at baseline or after serial NTG induction in either sex (Fig. 2). Finally, also for experiments in FHM1 (*CACNA1A*-S218L vs WT littermates) mice, no differences between genotypes were observed for gastric emptying or geometric center endpoints after a single NTG injection in female mutant animals compared to (1) vehicle-pretreated female mice of the same genotype or (2) NTG-pretreated female WT control animals (Fig. 3).

DISCUSSION

Gastroparesis is a well-recognized feature of migraine attacks in humans, but the etiology of this comorbidity is poorly understood. The current study was designed to investigate whether 3 monogenic mouse models of migraine (ie, CADASIL, FASP, or FHM1) also exhibit a gastroparesis phenotype. We recognized that findings in transgenic animals may not be generalizable to common (polygenic) forms of migraine but elected to study transgenic animals as a first attempt to increase the chance of observing the signs of gastrointestinal dysmotility. If the gene mutations (which confer increased susceptibility to migraine) also increased susceptibility to gastroparesis, this finding would have added weight to the concept that gastroparesis is a primary feature of migraine, rather than an associated trait. While neither gastroparesis nor delayed small intestinal motility were observed in any of the mutant mouse strains at baseline or after induction with NTG, the failure to identify gastroparesis in this study does not negate prior evidence linking gastroparesis to migraine, nor does it suggest further studies of gastroparesis in animal models of migraine should not be pursued.

There are multiple explanations for why we did not detect gastrointestinal dysmotility in mutant mice. First, patients with CADASIL, FASP, or FHM1 may not experience gastroparesis, in which case the mouse models of these phenotypes would not be expected to do so either. While many patients with CADASIL,[50] FASP (unpublished observations), and FHM1[51] experience nausea and/or vomiting with attacks, such symptoms may not arise from or be associated with gastroparesis. Second, it is possible that gastrointestinal dysmotility is present in humans with monogenic migraine phenotypes but not captured in mouse models with the gene mutations as some gastrointestinal motility behaviors are not observed in rodents (eg, vomiting)[52] and autonomic dysfunction may not be prevalent in these genetic models. Third, other modifiers of gastrointestinal motility may be critical in the expression of its pathophysiology, such as epigenetic factors[53] or altered microbiome[54] that may require a specific type of exposure to which the mutant mice have not been exposed. Fourth, the protocol we employed for NTG administration via repeated subcutaneous injections may have limited our ability to detect gastrointestinal

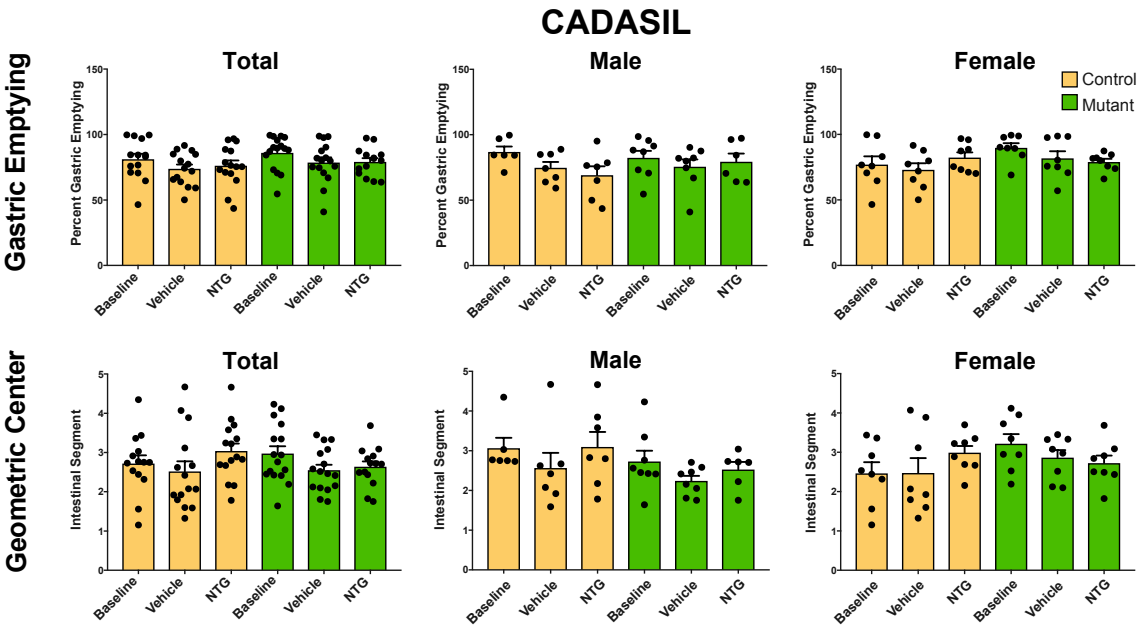

Fig. 1.—Gastric emptying and geometric center measurements in male and female CADASIL (Tg*Notch*^{R169C}) overexpressor mutant mice and their respective controls (Tg*Notch3*^{WT}; line 129). Measurements were performed at baseline and after serial induction with NTG or vehicle. Data are represented as mean ± SEM and analyzed by Kruskal-Wallis one-way ANOVA *P* values: Gastric Emptying: Total, *P* = .17; Male, *P* = .15; Female, *P* = .61. Geometric Center: Total, *P* = .64; Male, *P* = .54; Female, *P* = .66. [Color figure can be viewed at wileyonlinelibrary.com]

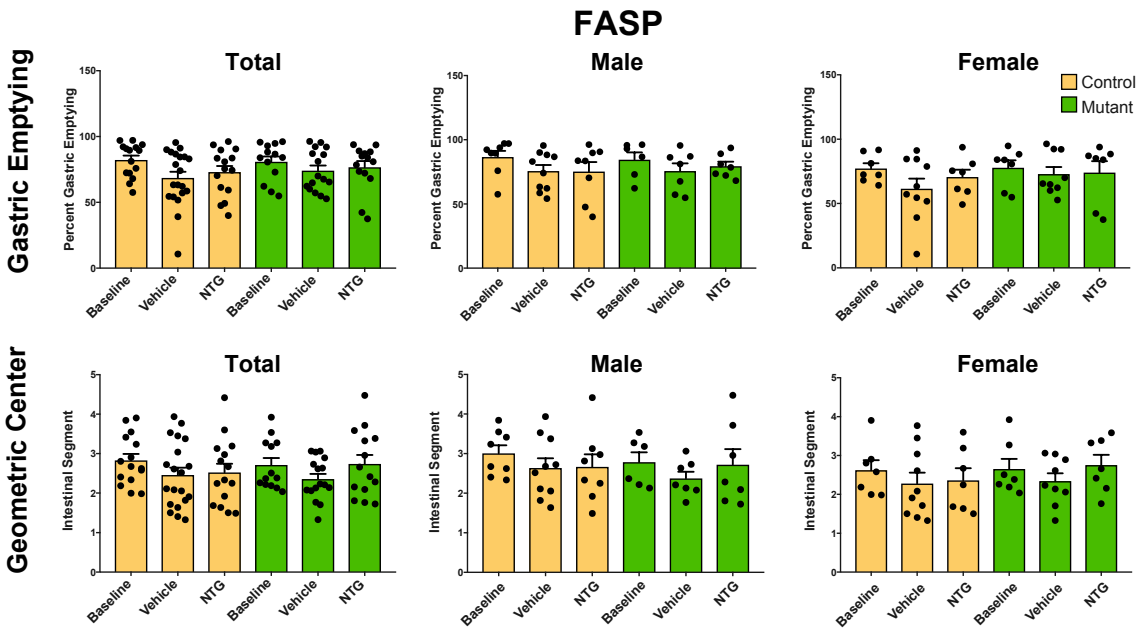

Fig. 2.—Gastric emptying and geometric center measurements in male and female FASP (*CSNK1D*-T44A) overexpressor mutant mice and their respective controls (*CSNK1D*-WT; line 433). Measurements were taken at baseline and after serial induction with NTG or vehicle. Data are represented as mean ± SEM and analyzed by Kruskal-Wallis one-way ANOVA *P* values: Gastric Emptying: Total, *P* = .29; Male, *P* = .36; Female, *P* = .25. Geometric Center: Total, *P* = .14; Male, *P* = .09; Female, *P* = .31. [Color figure can be viewed at wileyonlinelibrary.com]

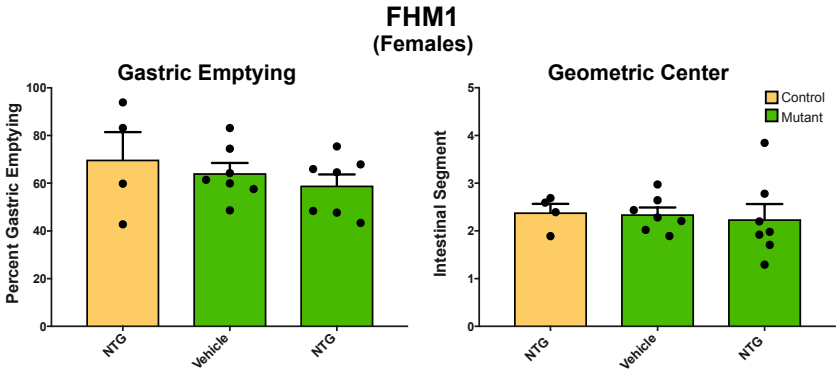

Fig. 3.—Gastric emptying and geometric center measurements in female FHM1 (*CACNA1A*-S218L) mutant mice and wild-type control littermates. Measurements were taken after single induction with NTG or vehicle. Data are represented as mean ± SEM and analyzed by Kruskal-Wallis one-way ANOVA *P* values: Gastric Emptying: Female, *P* = .82. Geometric Center: Female, *P* = .67. [Color figure can be viewed at wileyonlinelibrary.com]

dysmotility. Preliminary studies in our laboratory in which intraperitoneal injections were used showed that direct (external) application of NTG to the small intestine altered motility in WT mice (data not shown). In support of this finding, nitric oxide has previously been shown to have a direct inhibitory effect on gastrointestinal smooth muscle.[55] Therefore, we used subcutaneous injection of NTG in this study. We did not independently confirm the subcutaneous NTG induction protocol leads to similar findings as observed in mice with intraperitoneal NTG injections (eg, mechanical and thermal hyperalgesia or cFos-activation in the trigeminal nucleus caudalis).[56] Previous studies in rats demonstrated an increase in spontaneous trigeminal neuronal firing, trigeminal neuronal hypersensitivity,[57] and cFos and calmodulin-dependent protein kinase II (CamKII) activation 4 hours after subcutaneous NTG administration.[58-61] Finally, the ability of nitric oxide to induce migraine attacks in humans with FHM1 is an unsettled question.[10,62] Due to the unavailability of mice, our studies did not include a comparison of gastrointestinal motility between vehicle and NTG in female wild-type littermates of FHM1 S218L mice. However, the female mice homozygous for the FHM1 S218L mutation did not demonstrate any gastrointestinal dysmotility following NTG injections.

CONCLUSIONS

We found no evidence of gastroparesis or delayed small intestinal motility at baseline or following repeated subcutaneous NTG administration in any of the 3 investigated monogenic mouse models of migraine, that is, in CADASIL (*Notch3*-Tg88), FASP (*CSNK1D*-T44A), and FHM1 (*CACNA1A*-S218L) mice. Future studies seeking to understand why humans with migraine experience gastrointestinal dysmotility may benefit from studying other animal migraine models[63] or investigating potential modifiers of gastrointestinal motility, such as epigenetic or microbiome-related factors.

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