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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Enteric Glia Mediate Neuron Death in Colitis Through Purinergic Pathways That Require Connexin-43 and Nitric Oxide

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SUMMARY

Death of enteric neurons contributes to motility dysfunction in gastrointestinal disorders. Our work provides the first evidence of glial activation as a driver of enteric neurodegeneration.

BACKGROUND & AIMS: The concept of enteric glia as regulators of intestinal homeostasis is slowly gaining acceptance as a central concept in neurogastroenterology. Yet how glia contribute to intestinal disease is still poorly understood. Purines generated during inflammation drive enteric neuron death by activating neuronal P2X7 purine receptors (P2X7R); triggering adenosine triphosphate (ATP) release via neuronal pannexin-1 channels that subsequently recruits intracellular calcium ($[Ca^{2+}]_i$) in surrounding enteric glia. We tested the hypothesis that the activation of enteric glia contributes to neuron death during inflammation.

METHODS: We studied neuroinflammation in vivo using the 2,4-dinitrobenzene sulfonic acid model of colitis and in situ using whole-mount preparations of human and mouse intestine. Transgenic mice with a targeted deletion of glial connexin-43 (Cx43) [$GFAP::Cre^{ERT2+/-}/Cx43^{f/f}$] were used to specifically disrupt glial signaling pathways. Mice deficient in inducible nitric oxide (NO) synthase ($iNOS^{-/-}$) were used to study NO production. Protein expression and oxidative stress were measured using immunohistochemistry and in situ Ca^{2+} and NO imaging were used to monitor glial $[Ca^{2+}]_i$ and $[NO]_i$.

RESULTS: Purinergic activation of enteric glia drove $[Ca^{2+}]_i$ responses and enteric neuron death through a Cx43-dependent mechanism. Neurotoxic Cx43 activity, driven by NO production from glial iNOS, was required for neuron death. Glial Cx43 opening liberated ATP and Cx43-dependent ATP release was potentiated by NO.

CONCLUSIONS: Our results show that the activation of glial cells in the context of neuroinflammation kills enteric neurons. Mediators of inflammation that include ATP and NO activate neurotoxic pathways that converge on glial Cx43 hemichannels. The glial response to inflammatory mediators might contribute to the development of motility disorders. *(Cell Mol Gastroenterol Hepatol 2016;2:77–91; http://dx.doi.org/10.1016/j.jcmgh.2015.08.007)*

Keywords: Enteric Nervous System; Hemichannels; Oxidative Stress; Purines.

Reflex behaviors of the intestine, such as peristalsis, are orchestrated by the enteric nervous system (ENS); a complex network of neurons and glia embedded in the gut wall. The basic neural circuitry of the ENS is now well defined and it is generally accepted that the breakdown of ENS control is a major contributing factor in the development of functional bowel disorders.[1] However, it is only recently that we are beginning to appreciate the potential roles of enteric glial cells in the physiology and pathophysiology of the ENS.[2] Despite intense interest in enteric glia as regulators of enteric neurons, the precise functions of enteric glia remain poorly defined.

Enteric glia are a unique population of peripheral astroglial cells that surround enteric neurons and are thought to sustain neural signaling and survival. In support, enteric glia secrete neuroprotective factors[3] and the selective ablation of glial signaling alters the neural control of motility.[4] Likewise, in vivo models of glial ablation cause enteric neuron death.[5,6] Thus, the loss of glial supportive functions is postulated as a potential mechanism contributing to enteric neuropathy.[2]

Abbreviations used in this paper: **A-740003, *N*-[1-[(*E*)-[(cyanoamino)-(quinolin-5-ylamino)methylidene]amino]-2,2-dimethylpropyl]-2-(3,4-dimethoxyphenyl)acetamide; ADP, adenosine 5′-diphosphate monopotassium salt dihydrate; ADPβS, adenosine 5′-[β-thio]diphosphate trilithium salt; ATP, adenosine triphosphate; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt; CNS, central nervous system; Cx43, connexin-43; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle medium; DNBS, dinitrobenzene sulfonic acid; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; GW274150, (2*S*)-2-amino-4-[2-(1-aminoethylideneamino)ethylsulfanyl] butanoic acid; iNOS, inducible nitric oxide synthase; KO, knock out; LMMP, longitudinal muscle myenteric plexus; L-NAME, $N_{\omega fs}$-nitro-L-arginine methyl ester; MRS2365, trisodium;[[(1*R*,2*R*,3*S*,5*S*)-4-(6-amino-2-methylsulfanylpurin-9-yl)-2,3-dihydroxy-1-bicyclo[3.1.0]hexanyl] methoxy-oxidophosphoryl] phosphate; NAC, *N*-acetyl cysteine; panx1, pannexin-1; PAPA NONOate, propylamine propylamine NONOate; PBS, phosphate-buffered saline; P2X7R, P2X7 receptor; P2Y1R, P2Y1 receptor; NO, nitric oxide; 1400W, *N*-([3-(aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride.**

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However, new data show that chronic astroglial activation, rather than glial cell loss, is responsible for driving neurodegeneration during neuroinflammation in the brain.[7] Indeed, the conversion of astroglia to reactive astrocytes can promote the secretion of factors that promote neuron death.[8]

We recently discovered that enteric glia are activated by purines released from enteric neurons before neuronal death during colitis.[9] Specifically, the activation of neuronal P2X7 purine receptors (P2X7Rs) triggers the release of adenosine triphosphate (ATP) from neurons through pannexin-1 (panx1) channels as a signal to enteric glia. In the brain, neuronal ATP release through panx1 is considered a danger signal that glial cells interpret as a "search and destroy" message, causing glia to execute otherwise healthy neurons. Given that stimulation of P2Y1Rs is a potent stimulus for reactive astrogliosis in the central nervous system,[10] we hypothesized that the activation of glial purine receptors contributes to neuropathy in the ENS.

We tested our hypothesis using a combination of in vivo models of colitis with inducible and conditional transgenic mice and ex vivo intestinal preparations to address specific mechanisms. Our data show that glial activation is sufficient to cause enteric neuron death via a mechanism that depends on the activation of connexin-43 (Cx43) hemichannels and subsequent ATP release. Surprisingly, our data show that glial-driven neuron death requires the gating of glial Cx43 hemichannels by nitric oxide (NO). In all, our results suggest that the activation of enteric glial cells is a central mechanism in the development of enteric neuropathy.

Materials and Methods

Animals

All work involving animals was approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. Male mice (8–10 weeks of age) were maintained on a 12-hour light/dark cycle with access to food and water ad libitum. C57Bl/6 mice were purchased from Charles River Laboratories (Hollister, CA) and the inducible nitric oxide synthase (iNOS) null mice (B6.129S2-*Nos2*^{tm1Mrl}N12; Taconic Labs; RRID: MGI_4837857; hereafter referred to as *iNOS*$^{-/-}$) from Taconic Farms (Germantown, NY).[11] Transgenic mice with an inducible and conditional deletion of Cx43 in glial fibrillary acidic protein (GFAP)-expressing glia (*GFAP::Cre*$^{ERT2+/-}$/*Cx43*$^{f/f}$; hereafter referred to as Cx43i-cKO) and their Cre-negative littermate controls (*GFAP::Cre*$^{ERT2+/+}$/*Cx43*$^{f/f}$) were generated in-house as previously described[4] by crossing *GFAP::Cre*$^{ERT2+/-}$ mice [(GFAP-cre/ERT2)505Fmv/J; Jackson Laboratory (Bar Harbor, ME); RRID: IMSR_JAX:012849] with *Cx43*$^{f/f}$ mice (B6.129S7-Gja1tm1Dlg/J; Jackson Laboratory; RRID: IMSR_JAX:008039). Cre recombinase activity was induced by feeding animals tamoxifen citrate in chow (400 mg/kg) for 2 weeks. Animals were returned to normal chow for 1 week to clear tamoxifen before beginning experiments.

Human Tissue

Work involving human tissue was approved by the institutional review board of Michigan State University (IRB 13-945M). Samples of live, full-thickness human jejunum were collected from a 57-year-old woman with hypertension and type 2 diabetes who underwent elective laparoscopic bariatric surgery for weight loss. The samples were placed in chilled Dulbecco's modified Eagle medium (DMEM)/F-12 medium during transfer to the laboratory. Live longitudinal muscle myenteric plexus (LMMP) whole-mount preparations were prepared by microdissection for calcium (Ca^{2+}) imaging.

Table 1. Primary Antibodies Used

Antibody	Source	Dilution	Catalog No.
Rabbit anti-iNOS	Abcam, Cambridge, MA	1:200	ab15323
Biotinylated mouse anti-human HuC/D	Molecular Probes, Grand Island, NY	1:200	A-21272
Chicken anti-GFAP	Abcam	1:1000	ab4674
Rabbit anti-nitrotyrosine	Millipore, Billerica, MA	1:100	06-284
Rabbit anti-P2Y1R	Alomone Labs, Jerusalem, Israel	1:200	APR-021

GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide synthase; P2Y1R, P2Y1 receptor.

Whole-Mount Immunohistochemistry

Whole-mount preparations of mouse colonic LMMP were prepared by microdissection from tissue preserved in Zamboni's fixative. Processing of LMMPs via immunohistochemistry was conducted as described elsewhere[4] with the primary and secondary antibodies listed in Tables 1 and 2, respectively. Briefly, LMMP preparations underwent three 10-minute washes in 0.1% Triton X-100 in phosphate-buffered saline (PBS) followed by a 45-minute incubation in blocking solution containing 4% normal goat serum, 0.4% Triton X-100 and 1% bovine serum albumin. Preparations were incubated in primary antibodies (listed in Table 1) for

Table 2. Secondary Antibodies Used

Antibody	Source	Dilution	Catalog No.
Alexa Fluor 488 Goat anti-rabbit	Invitrogen, Carlsbad, CA	1:200	A-11034
Alexa Fluor 488 Goat anti-chicken	Invitrogen	1:200	A-11039
Alexa Fluor 568 Goat anti-chicken	Invitrogen	1:200	A-11041
Alexa Fluor 594-conjugated streptavidin	Jackson Immuno Research, West Grove, PA	1:200	016-580-084