Identification of an analgesic lipopeptide produced by the probiotic *Escherichia coli* strain Nissle 1917

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Administration of the probiotic *Escherichia coli* strain Nissle 1917 (EcN) decreases visceral pain associated with irritable bowel syndrome. Mutation of clbA, a gene involved in the biosynthesis of secondary metabolites, including colibactin, was previously shown to abrogate EcN probiotic activity. Here, we show that EcN, but not an isogenic clbA mutant, produces an analgesic lipopeptide. We characterize lipoamino acids and lipopeptides produced by EcN but not by the mutant by online liquid chromatography mass spectrometry. One of these lipopeptides, C12AsnGABA-OH, is able to cross the epithelial barrier and to inhibit calcium flux induced by nociceptor activation in sensory neurons via the GABA_b receptor. C12AsnGABA-OH inhibits visceral hypersensitivity induced by nociceptor activation in mice. Thus, EcN produces a visceral analgesic, which could be the basis for the development of new visceral pain therapies.

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Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by recurrent episodes of abdominal pain/discomfort and bowel habit changes (e.g., constipation, diarrhea). With a global prevalence of ~11%, IBS constitutes one of the most common conditions leading to gastrointestinal referral, and results in a considerable disease burden. While the pathophysiology of IBS is not fully understood, visceral hypersensitivity (VH; enhanced sensitivity of the intestinal wall to local stimuli) has been proposed as a key mechanism underlying abdominal pain, one of the most debilitating and most troublesome symptoms of this disorder. Current treatments for IBS are mainly symptoms orientated; however, the overall efficacy is low and there are no drugs specifically approved for abdominal pain. Thus, selective pharmacological tools targeting VH may be considered a suitable therapeutic approach for visceral pain treatment and development of novel IBS therapies.

Data from clinical research suggest that certain probiotic bacterial strains have the potential to modulate abdominal pain in IBS. Nonetheless, these data differ considerably among studies due to the probiotic bacterial strains used for the treatment and the heterogeneity of IBS groups included. Moreover, the mechanisms of action responsible for the claimed therapeutic effects differ from one strain to another.

Escherichia coli Nissle 1917 (EcN) is the active component of Mutass® (Ardeypharm GmbH, Herdecke, Germany), a probiotic drug licensed in several countries for the treatment of multiple intestinal disorders. Clinical trials have shown EcN to be effective for the treatment of abdominal pain in IBS patients, but very little is known about the specific mechanisms through which EcN exerts the ascribed analgesic effects. EcN is known to harbor a genomic island, named pks, which carries a cluster of genes that enables the synthesis of hybrid peptide polyketides and especially a genotoxin called colibactin (Fig. 1).

Colibactin is a structurally uncharacterized polyketide (PK) and in vivo. While an increased number of small molecules derived from the colibactin encoding hybrid PKS-NRPS biosynthetic gene clusters have been characterized, the mechanism of action responsible for the claimed therapeutic effects differ from one strain to another. Following activation by ClbA, the initiating NRPS ClbN uses Asn as a substrate to generate N-myristoyl-D-Asn (Fig. 1). The NRPS-PKS assembly line contains the synthesis of precolibactin compound(s) using malonyl-CoA and different amino acids as substrates. The precolibactin is then cleaved by peptidase ClbP to liberate colibactin and N-myristoyl-D-Asn (C14AsnOH; Fig. 1)

Surprisingly, although colibactin was shown to be a bona fide virulence factor and a putative carcinogenic agent, its genotoxicity is also produced by EcN. The probiotic activity of EcN can apparently not be dissociated from its genotoxic activity, since inactivation of clbA required for the activation of the NRPS and PKS enzymes leading to colibactin production also attenuates the probiotic activity of EcN in experimental colitis. A possible explanation for the dual role of colibactin in EcN may be that the pks island codes for additional bioactive compounds distinct from colibactin and involved in the probiotic activity. Hence, the identification and functional characterization of new molecules derived from the colibactin encoding hybrid PKS-NRPS biosynthetic gene clusters may help to decipher some of the mechanisms, supporting the capacity of EcN to modulate abdominal pain, and thereby allowing the design of novel analgesic agents devoid of genotoxic properties.

Here, we use liquid chromatography coupled to electrospray source (ESI) with tandem high-resolution mass spectrometry (LC-HRMS and LC-HRMS/MS) to identify a metabolite encoded by the pks island that shows anti-nociceptive properties in vitro and in vivo. While an increased number of small molecules derived from the colibactin encoding hybrid PKS-NRPS biosynthetic gene clusters have been described, this study characterizes a non-genotoxic bioactive metabolite. This visceral analgesic produced by probiotic bacteria may represent a promising therapeutic agent in visceral pain.

Results

Identification of lipoamino acid and lipopeptides produced by EcN. In order to characterize the lipids potentially implicated in probiotic properties of EcN, we performed a comparative lipodomic analysis by LC-HRMS of lipids extracted from the wild-type probiotic strain (EcNwt) and an isogenic mutant for clbA that has lost its probiotic activity (EcNΔclbA) in a model of colitis. The total ion chromatograms (TIC) obtained from the ESI were compared to characterize compounds with a relative higher concentration in EcNwt compared to EcNΔclbA. As the relative intensity of the peak eluted at 15.3 min constitutes more

![Fig. 1 Schematic representation of the pks genomic island. This cluster of genes encodes the enzymes and accessory proteins (arrows) necessary for synthesis of precolibactin in E. coli. Precolibactin is cleaved by a peptidase encoded by clbP (green arrow) into active colibactin and a cleavage product, mainly C14AsnOH, synthesized by the initiating NRPS encoded by clbN (blue arrow).](image-url)
than 80% of the relative intensity in EcNwt, and only 2.5% in EcNΔclbA, we focused our interest on this peak (Fig. 2a). With the accurate m/z 341.2448 ratio obtained we determined an elemental composition of the deprotonated molecule like [(C18H34N2O4)-H]-. Then, the natural isotopic profile of the molecular species in the experimental mass spectrum obtained at 15.33 min was compared to its simulation generated from the formula (calculated: m/z 341.2446). These two isotopic profiles were similar and the accuracy of the m/z measurements of monoisotopic peak was 0.6 ppm (Fig. 2b), as well as the m/z measurements of its first major natural isotopic [(13CnC18-1H34N2O4)-H]- peak supporting the proposed elemental composition. Finally, MS/MS analyses were performed under HR measurement conditions by using collisional activation (Fig. 2c).

The product ion spectrum (under normalized collision energy (NCE) = 35%) of the carboxylate anion [M- H]– (m/z 341) generated in electrospray from the LC peak eluted at 15.33 min (Supplementary Fig. 2b) reflects the consecutive CO2 and HCONH2 losses, the free functional groups of the C14-Asparagine lipoamino acid. Furthermore, the m/z 226.2176 fatty amide ion is generated by release of the fumaric acid amide (Supplementary Fig. 2c). On the other hand, the m/z 131.0455 ion is characteristic of the deprotonated asparagine resulting of the C14 alkyl ketene loss (Supplementary Fig. 2d). Thus, with the fragmentation pattern we identified as expected the C14-Asparagine lipoamino acid, which is the cleavage product of precolibactin18,21,22.

Consequently, the extracted ion chromatogram (EIC) of the diagnostic product ion at m/z 131.0455 obtained from dissociations of the various deprotonated molecular species of the mixture allowed us to identify the CnAsnOH molecules with Cn ≠ C14. Based on the mass resolution of the mass spectrometer allowing the accurate m/z measurements and the analysis of product ion spectra, we identified several lipopeptides with different hydrocarbon chain lengths as already published21,22. On the other hand, the characterization of other amino acids linked to C-terminus of the C12AsnOH and C14AsnOH chains through peptidic bond was then performed by the use of detection of the m/z 295.2021 and m/z 323.2335 product ions. They correspond to the formal [(CnAsnOH-H)-H2O]– fragment ions (n = 12 and 14), respectively. By this mean, the EICs of these product ions—as reported in Supplementary Figs. 1a, 3a, respectively—allow to determine each m/z values of the parent ions of m/z 295.2021 and m/z 323.2335 corresponding to m/z 426.2980 and m/z 454.3294, respectively. Thus, a common
neutral loss (i.e., 131.0959u) occurs from dissociation of these precursor ions to yield the formal [(CnAsnOH-H)-H2O]− fragment ions, with n = 12 or 14, at m/z 295.2021 and m/z 323.2335. This common neutral release could correspond to a leucine or an isoleucine (Supplementary Figs. 1, 3). The proposed interpretation of formation of these product ions is reported in Supplementary Note 1 and Supplementary Fig. 4a.

We also focused our interest on the ion at m/z 398.2663 eluted at 12.96 min (Fig. 3a). The EICs showed that this molecule is present in EcNwt but not in the mutant EcNΔclbA (Fig. 3a). m/z ratio accuracy of the monoisotopic peak of [C12AsnLeuOH-H]− at m/z 398.2663 displayed by the ESI mass spectrum, under LC-HRMS analysis conditions, allowed to identify its elemental composition, which was confirmed by the simulated natural
The series of $m/z$ 312.2276 product ion (Supplementary Figs. 4c and 9c, Supplementary Note 1) is detected only from the BABA structure;

(ii) The series of $m/z$ 155.0822, $m/z$ 138.0557, and $m/z$ 137.0716 product ions characterizes the presence of GABA (Fig. 3c) and AABA (Supplementary Fig. 9f); and

(iii) The product ions at $m/z$ 113.0352 (Supplementary Fig. 9d), $m/z$ 102.0553 (Supplementary Figs. 4a, 9f and Supplementary Note 1), $m/z$ 96.0090 (Fig. 3c, Supplementary Note 1 and Supplementary Fig. 10) are the largest peak at the low $m/z$ ratio range (<$m/z$ 200) for the BABA, AABA, and GABA substitutions, respectively.

The four synthesized isomers were also analyzed on low-resolution triple quadrupole mass spectrometer coupled online to a liquid chromatography. A 15 min separation method was developed for the separation of each isomer (Fig. 3d). The analysis of bacterial pellets showed the presence of only C12AsnGABA0H and C12AsnBABA0H (Fig. 3d).

$\text{C12AsnGABA0H and C12AsnBABA0H synthesis depends on the pks island.}$ $E.\text{coli} \ K-12 \ MG1655$ was transformed with the bacterial artificial chromosome (BAC) harboring the entire pks island (BAC pks+). Unlike the MG1655wt, the strain MG1655 +BAC pks+ was shown to produce C12AsnBABA0H and C12AsnGABA0H demonstrating the role of the pks island in the production of these two compounds (Supplementary Fig. 11). To further elucidate the role of the pks island in the synthesis of C12AsnGABA0H and C12AsnBABA0H, EcNΔclbA and additional isogenic mutants of the EcN strain were generated. These mutants ($\Delta\text{clbA}, \Delta\text{clbN}, \Delta\text{clbB}, \Delta\text{clbC}, \text{and} \Delta\text{clbP}$) were not capable of producing the genotoxin colibactin inducing double-strand breaks in eukaryotic cells, contrary to the parental EcN strain. The lipid metabolite profiles were then characterized in EcN and the mutants, as well as in their culture supernatants. The inactivation of clbA, coding for the PPTase, induced a drastic decrease of both C12AsnGABA0H and C12AsnBABA0H in bacteria and culture supernatants (Fig. 4a, b), confirming the relevance of this gene for the synthesis of these molecules. The clbN, clbB, clbC, and clbP genes code for enzymes involved in the first steps and last steps of the biosynthesis of colibactin, namely,
the elongation and the cleavage of the colibactin pro-drug scaffold. Deletion of \( \text{clbN} \) completely abrogated the production and secretion of \( \text{C12AsnGABA} \text{OH} \) and \( \text{C12AsnBABA} \text{OH} \) in bacteria and their culture supernatants, evidencing the essential role of this gene in the production of these molecules. In a similar manner, \( \text{clbB} \) inactivation abolished the synthesis and secretion of \( \text{C12AsnBABA} \text{OH} \), and reduced significantly the synthesis and secretion of \( \text{C12AsnGABA} \text{OH} \). On the contrary, mutation of \( \text{clbC} \) (a gene coding for a trans-acyl-transferase PKS that catalyzes an additional round of PK extension, after \( \text{ClbN} \) and \( \text{ClbB} \)) did not induce significant changes in the concentration of any of the two molecules in both bacteria and supernatants. Likewise, the \( \text{clbP} \)
deletion did not modify the synthesis and secretion pattern of any of these molecules, demonstrating that C12AsnGABA\textsubscript{OH} and C12AsnBABA\textsubscript{OH} are not cleavage products. In contrast, the concentration of the cleavage product (C14AsnOH) was drastically decreased by clb\textsubscript{P} deletion (Supplementary Fig. 12), as expected. Thus, C12AsnGABA\textsubscript{OH} and C12AsnBABA\textsubscript{OH} are two new molecules dependent on at least three genes of the clb\textsubscript{P} biosynthetic gene cluster, confirming the hypothesis that the pks island could mediate the formation of compounds with potential probiotic activity\textsuperscript{23}, in addition to molecules inducing DNA damage\textsuperscript{13,28}. As among the aminobutyric acid isomers, \(\gamma\)-aminobutyric (GABA) acid is the primary inhibitory neurotransmitter in the mammalian brain, we hypothesized that C12AsnGABA\textsubscript{OH} was responsible for the anti-nociceptive properties EcN.

**C12AsnGABA\textsubscript{OH} inhibits neuronal activation.** To determine whether C12AsnGABA\textsubscript{OH} or C12AsnBABA\textsubscript{OH} are capable of signaling to sensory nerves, calcium mobilization studies were performed on primary cultures of mouse dorsal root ganglia (DRG) neurons. None of the isomers (10 \(\mu\text{M}\)) induced calcium mobilization under basal (unstimulated) conditions (Supplementary Fig. 13). The same experiments were thereafter performed in neurons activated by either an agonist of the receptor calcium channel TRPV1 (capsaicin) or by a mix of agonists (histamine, serotonin, and bradykinin) for G-protein-coupled receptors (GPCR) implicated in VH. Exposure of neurons to either capsaicin (125 nM) or the mix of GPCR agonists (histamine, bradykinin, serotonin, 10 \(\mu\text{M}\) each) induced an increase in calcium flux as shown by the higher % of responding neurons and amplitude of the response (\(\Delta F/F\)) compared to the vehicle (Fig. 5a, b). The calcium flux increase induced by both nociceptive stimuli was prevented by C12AsnGABA\textsubscript{OH} pretreatment in a dose-dependent manner (Fig. 5a, b), whereas C12AsnBABA\textsubscript{OH} had no effect (Supplementary Fig. 14). Thus, C12AsnGABA\textsubscript{OH} does not induce calcium mobilization in sensory neurons but inhibits neuronal activation induced by pro-nociceptive stimuli in a concentration range similar to GABA alone (Supplementary Fig. 15). To investigate whether the inhibitory effect of C12AsnGABA\textsubscript{OH} was associated to the GABA residue, neurons were treated with saclofen (10, 50, and 100 \(\mu\text{M}\)), a competitive antagonist of the GABA\textsubscript{B} receptor. Treatment with saclofen abolished the inhibitory effect of C12AsnGABA\textsubscript{OH} against capsaicin and the mix of GPCR agonists in a dose-dependent manner (Fig. 5c, d). Taken together, these results demonstrate that C12AsnGABA\textsubscript{OH} is capable of inhibiting calcium signaling in primary afferents via the GABA\textsubscript{B} receptor.
crosses the epithelial barrier. The initial barrier for any drug absorption is the intestinal epithelial cell wall after penetration of the mucus layer. To evaluate whether C12AsnGABA is capable of crossing the epithelial barrier and then stimulate GABA receptors on neurons, human epithelial cells monolayers (fully differentiated Caco-2 cells) were treated at the apical side with C12AsnGABA. LC-MS/MS quantification of this compound was performed in cells and in apical and control cultures.
basolateral side of transwell chambers after an incubation period of 24 h. Approximately 50% of C12AsnGABA OH added in the apical chamber (800 ng) was found in the basolateral chamber (Fig. 6a), whereas epithelial cells contained low levels of C12AsnGABA OH after 24 h. The transport of GABA alone across the cell monolayer was also assessed. For this purpose, commercial GABA was added in the apical chamber (800 ng) and after 3, 6, and 16 h, the presence of this molecule was quantified by LC-HRMS in basal and apical chambers (Fig. 6b). GABA was not detected in the basal chamber following the incubation period, showing that this molecule did not cross the intestinal epithelial monolayer. Thus, the addition of the (C12AsnOH) by the bacteria to the GABA confers the capacity for this neuromediator to cross the intestinal epithelial barrier.

To assess the effect of C12AsnGABA OH on paracellular permeability, transport of dextran 4 kDa fluorescein isothiocyanate (FITC) across a Caco-2 cell monolayer was investigated. As shown in the Fig. 6c, the percentage of 4 kDa FITC traversing the cell monolayers after 24 h was not modified by C12AsnGABA OH (10 µM) treatment, evidencing that C12AsnGABA OH does not alter paracellular permeability. In parallel, the release of CXCL8 from Caco-2 cells into the medium, of both apical and basolateral sides of the transwell, was assessed by ELISA. None of the tested doses (1, 10, 100 µM) and neither the vehicle modified the secretion of CXCL8 by Caco-2 cells (Fig. 6d). Thus, the addition of (C12AsnOH) to GABA by the bacteria confers the capacity for this neuromediator to cross the intestinal epithelial barrier without altering paracellular permeability. We hypothesized that intralocronal administration of C12AsnGABA OH could mimic the luminal production of the angesis lipopeptide by EcN and its diffusion across the epithelial barrier.

C12AsnGABA OH inhibits VH. In a first set of experiments, we assessed the in vivo ability of C12AsnGABA OH and GABA alone to cross the epithelial barrier. Intracocular administration of C12AsnGABA OH in mouse increased the concentration of this compound in the colonic tissue and blood (Fig. 7a, b). In contrast, following its intralocronal administration, GABA concentration were not increased in the colonic wall or in the blood (Fig. 7c). Based on the inhibitory effect of C12AsnGABA OH on calcium mobilization in sensory neurons and on its capacity to cross the epithelial barrier in vivo, we evaluated the analgesic potency of this lipoprotein. The impact of C12AsnGABA OH on VH was assessed by measuring visceromotor responses (VMR) to colorectal distension (CRD). VMR recordings were initiated 15 min after intracocular administration of either capsaicin (100 µg per animal; 100 µL) or the vehicle (EtOH 40%). Capsaicin evoked an increase (p < 0.05) in VMR to CRD pressures of 15–60 mm Hg compared to vehicle (Fig. 7d, e). This increase was significantly prevented for all distension pressures in animals pretreated with the C12AsnGABA OH (250 µg/mL) treatment (250 µg/mL). Bacteria were then inoculated at OD600 = 0.1 in 10 mL of minimal medium A (K2HPO4, 10.5 g L−1; KH2PO4, 4.5 g L−1; (NH4)2SO4, 1 g L−1; sodium citrate 0.5 g L−1; MgSO4, 0.2 g L−1; and glycerol 0.2%) supplemented with kanamycin or chloramphenicol (25 µg mL−1) when required. After overnight incubation at 37 °C, single colonies for each strain were seeded in 4 mL of LB with antibiotics (when needed) and incubated overnight at 37 °C in agitation (250 rpm). Bacteria were then incubated at OD600 = 0.1 in 10 mL of minimal medium A (K2HPO4, 10.5 g L−1; KH2PO4, 4.5 g L−1; (NH4)2SO4, 1 g L−1; sodium citrate 0.5 g L−1; MgSO4, 0.2 g L−1; and glycerol 0.2%) supplemented with kanamycin or chloramphenicol (when required) and cultures were grown for 24 h at 37 °C under shaking conditions (250 rpm). The last step was repeated one more time.

Conclusion

Here, we identified a lipopeptide related to GABA—the main inhibitory transmitter of the central nervous system—exhibiting analgesic properties in visceral pain. Three enzymes implicated in the synthesis of this C12AsnGABA OH have been identified. Surprisingly, these enzymes were encoded by a genomic island, named pks, which carries the cluster of genes that enables the synthesis of hybrid peptide polyketides and especially the genotoxin colibactin, a bona fide virulence factor and a putative carcinogenic agent. Our results illustrate how the colibactin NRPS–PKS biosynthetic pathway represents a rich source of unusual assembly-line enzymology coding for additional bioactive compounds distinct from colibactin. The addition of the C12AsnOH moiety confers to GABA the capacity to diffuse across the epithelial barrier and subsequently to act on sensory neurons. Interestingly, C12AsnGABA OH does not modify the physiology of the intestinal epithelium or the intestinal motility, suggesting that it might have fewer side effects than prototypical analgesics such as morphine. Thus, C12AsnGABA OH may represent a promising therapeutic agent for the management of visceral pain.

Methods

Chemicals Lipoxin A4 deuterated (Lx4-a-d5); leukotriene B4 deuterated (LTB4-d4) and SS-hydroxy-eicosatetraenoic acid deuterated (5-HETE-d8) were purchased from Cayman Chemicals (Interchim, Montluçon, France). Methanol (MeOH), Hank’s balanced salt solution (HBSS), HEPES, Collagenase type I from Clostridium histolyticum, dipase II, papain, cytosine β-D arabinofuranoside (ARAc), 5-fluorouracil (5-FU), uridine, 4 kilodalton FITC-labeled dextran, capsacin (transient receptor potential vaniloid [TRPV1]-1 agonist), pluronic F-127 were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Saclofen (GABAB receptor antagonist) and GABA were obtained from Tocris Bioscience (R&D Systems Europe, Lille, France). The mix of GPCR agonists (histamine, bradykinin, serotonin) was provided by Sigma-Aldrich (Saint Quentin Fallavier, France), Fluo-4 acetoxyethyl (Fluo-4 AM, molecular probes) was from Life Technologies (Eugene, OR, USA), Leibovitz’s L-15 Medium was from Gibco (Invitrogen Life Technologies, Paisley, UK).

Animals

Male C57Bl6 mice (6–8 weeks, Janvier St Quentin-Fallavier, France) were used to produce primary cultures of DRG sensory neurons for calcium flux experiments, and to perform studies of colorectal distention and intestinal isometric contraction. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the European Council and were approved by the Animal Care and Ethics Committee of USO06/CREFE (CETHA-122).

Bacterial strains and culture conditions. E. coli MG1655, MG1655+BAC pks+, EcN 1917 (Mutator, DSM 6601, serotype 06:K5:H1), the isogenic mutants EcNΔcbA, EcNΔcbB, EcNΔcbC, and EcNΔcbP were used in this study (Supplementary Table 2). Gene inactivation was performed using the lambda red recombinase method and deletions were confirmed by using flanking primers. MG1655, MG1655+BAC pks+, EcNwt, EcNΔcbA, EcNΔcbB, EcNΔcbC, EcNΔcbB were grown on LB agar plates supplemented with kanamycin (50 µg mL−1) or chloramphenicol (25 µg mL−1) when required. After overnight incubation at 37 °C, single colonies for each strain were seeded in 4 mL of LB with antibiotics (when needed) and incubated overnight at 37 °C in agitation (250 rpm). Bacteria were then inoculated at OD600 = 0.1 in 10 mL of minimal medium A (K2HPO4, 10.5 g L−1; KH2PO4, 4.5 g L−1; (NH4)2SO4, 1 g L−1; sodium citrate 0.5 g L−1; MgSO4, 0.2 g L−1; and glycerol 0.2%) supplemented with kanamycin or chloramphenicol (when required) and cultures were grown for 24 h at 37 °C under shaking conditions (250 rpm). The last step was repeated one more time.

Extraction of lipoprotein acids and lipopeptides. Bacterial cultures were centrifuged at 755 g for 15 min and the recovered bacterial pellets were immediately crushed with a FastPrep-24 Instrument (MP Biomedical, Santa Ana, CA) in 200 µL HBSS and 5 µL internal standard (IS) mixture (Deuterium-labeled compounds) (400 ng mL−1). After two crush cycles (6.5 m s−1, 30 s), 10 µL of suspensions were withdrawn for protein quantification and 0.3 mL of cold methanol (MeOH) was added. In parallel, bacteria culture supernatants were filtered through a 0.22-µm pore size filter (Millipore) to remove residual bacterial cells, and 1 mL of supernatant was collected for lipid extraction after addition of 5 µL IS mixture and 0.5 mL of 20% methanol and 0.5 mL of hexane. Both bacterial pellets and supernatant samples were spotted at 1016 x 15 µm for 15 min (4°C) and the resulting supernatants were submitted to solid-phase extraction of lipids using HRX-50 mg 96-well plates (Merck Hayzel, Hayes...
Hoerd, France). Briefly, plates were conditioned with 2 mL MeOH and 2 mL H2O/MeOH (90:10, v/v). Samples were loaded at a flow rate of about one drop per 2 s and the spots were visualized by the addition of 4% vanillin–H2SO4. The columns were thereafter dried under aspiration and lipids were eluted with 2 mL MeOH. Solvent was evaporated under N2 and samples were resuspended in 10 μL MeOH for liquid chromatography/tandem mass spectrometry analysis14.

Characterization of C12-Asn-amino·butyric acid. The characterization of C12-Asn-agmatine was performed by liquid chromatography/tandem mass spectrometry (U3000, Thermo Fisher Scientific, Waltham, MA, USA) coupled on line to a Fourier Transform Mass Spectrometer (FT/MS) equipped with a Synapt G2-S from Waters equipped with an ESI source. High-resolution mass data were acquired at Laboratoire de Mesures Physiques (Université de Montpellier) on a Synapt G2-S from Waters equipped with an ESI source. The mass spectrometer was operated in the negative ion FTMS mode at a resolution of 30,000. The source temperature was 325 °C, source heating temperature was 300 °C, sheath gas flow rate was 30 a.u. (arbitrary unit), auxiliary gas flow rate was 10 a.u., and source voltage was −2.9 kV. Samples were injected on a ZorbAX SB 120 C18 column (2.1 μm × 100 mm, 2.7 μm) (Agilent Technologies) maintained at 40 °C. Solvent A was 0.1% formic acid in H2O and solvent B was 0.1% formic acid in acetonitrile at a flow rate of 350 μL min−1. The multi step gradient was as follows: 25% B at 0 min, 40% B at 10 min, 47.5% B at 10.1 min, 73.8% B at 21 min, 100% B at 21.2 min, 100% B at 23.2 min, 25% B at 25.4 min. The autosampler was set at 5 °C and the injection volume was 5 μL. The identification was performed using Xcalibur software (Thermo Fisher Scientific). The MS/MS experiments were performed by collisional activation within non-resonant excitation mode (HCD)15. The excitation energy was optimized in the range of the 20 and 35% NCE33 such as the precursor ion survived to the dissociation processes within a relative abundance between 30 and 150% of the most abundant product ion displayed in the HCD spectrum recorded with a resolution of 30,000.

Synthesis of C12:0-AsnAABA, C12:0AsnBBA, and C12:0AsnGABA. All reactions requiring anhydrous conditions were conducted in flame dried glassware with magnetic stirring under an atmosphere of nitrogen unless otherwise mentioned. Anhydrous CH2Cl2 was obtained from the Innovative Technology PS-Micro solvent purification system. Other solvents and reagents were obtained as supplied by the suppliers (Aldrich, Alfa Aesar, Acros) unless otherwise noted. Reactions were monitored by TLC using plates precoated with silica gel 60 (Merck). Characterization of C12-Asn-aminobutyric acid.

1H NMR spectra (Supplementary Figs.6a, 8a, 17a) were obtained at 300 or 500 MHz on a Bruker Avance 500 spectrometer using DMSO-d6 and CDCl3 as solvents. 13C NMR (75 MHz, DMSO; Supplementary Fig.10b) and 2D NMR analyses were performed in the negative ion FTMS mode at a resolution of 30,000. Mass spectrometry data were obtained in a scan range from 100 to 1000 u with 2 mL MeOH. Solvent was evaporated under N2 and samples were resuspended in 10 μL MeOH for liquid chromatography/tandem mass spectrometry analysis14.

MS (ESI+) [M+H]+: 738.17; [M+Na]+: 760.50; MS (ESI−) [M−H]+: 698.25; [M+Na]−: 720.50; MS (ESI−) [M−Na]−: 514.58.

Characterization of C12-Asn-aminobutyric acid. The characterization of C12-Asn-agmatine was performed by liquid chromatography/tandem mass spectrometry (U3000, Thermo Fisher Scientific, Waltham, MA, USA) coupled on line to a Fourier Transform Mass Spectrometer (FT/MS) equipped with a Synapt G2-S from Waters equipped with an ESI source. High-resolution mass data were acquired at Laboratoire de Mesures Physiques (Université de Montpellier) on a Synapt G2-S from Waters equipped with an 5 kV. High-resolution mass data were acquired at Laboratoire de Mesures Physiques (Université de Montpellier) on a Synapt G2-S from Waters equipped with an ESI source. The mass spectrometer was operated in the negative ion FTMS mode at a resolution of 30,000. The source temperature was 325 °C, source heating temperature was 300 °C, sheath gas flow rate was 30 a.u. (arbitrary unit), auxiliary gas flow rate was 10 a.u., and source voltage was −2.9 kV. Samples were injected on a ZorbAX SB 120 C18 column (2.1 μm × 100 mm, 2.7 μm) (Agilent Technologies) maintained at 40 °C. Solvent A was 0.1% formic acid in H2O and solvent B was 0.1% formic acid in acetonitrile at a flow rate of 350 μL min−1. The multi step gradient was as follows: 25% B at 0 min, 40% B at 10 min, 47.5% B at 10.1 min, 73.8% B at 21 min, 100% B at 21.2 min, 100% B at 23.2 min, 25% B at 25.4 min. The autosampler was set at 5 °C and the injection volume was 5 μL. The identification was performed using Xcalibur software (Thermo Fisher Scientific). The MS/MS experiments were performed by collisional activation within non-resonant excitation mode (HCD)15. The excitation energy was optimized in the range of the 20 and 35% NCE33 such as the precursor ion survived to the dissociation processes within a relative abundance between 30 and 150% of the most abundant product ion displayed in the HCD spectrum recorded with a resolution of 30,000.
C12:0-Asn-BABA-OH was synthesized following the procedure described above using (S)-α-aminobutyric acid as a starting material. At the last step, 280 mg of C12:0-Asn-BABA-OH was purified by HPLC to give C12:0-Asn-BABA-OH (2 mg, 13%). Analytical LC-MS Rt: 1.16 min; [u]25 = -60 (c 5, DMSO); [H] NMR (300 MHz, DMSO; Supplementary Fig. 11a) δ 7.88 (d, J = 7.9 Hz, 1H), 7.68 (d, J = 8.0 Hz, 1H), 7.20 (s, 1H), 6.81 (s, 1H), 4.42 (q, J = 7.2 Hz, 1H), 4.07–3.86 (m, 1H), 2.51–2.11 (m, 5H), 2.03 (t, J = 7.1 Hz, 2H), 1.41 (s, 6H), 1.19 (d, 6H), 1.01 (d, J = 6.4 Hz, 3H), 0.80 (t, J = 6.5 Hz, 3H), 3.72 (t, J = 7.0 Hz, 4H), 1.70 (d, J = 6.0 Hz, 2H), 1.50 (m, 4H), 1.14 (m, 4H), 0.80 (t, J = 6.7 Hz, 4H). HRMS [M+H]+calc: 400.2811 found: 400.2815.

Quantification of C12AsnGABAOH and C12AsnBABAOH. The quantification of C12AsnGABAOH and C12AsnBABAOH was performed on a high-performance liquid chromatography (Agilent 1290 Infinity) coupled to a triple quadrupole mass spectrometer (G6460A Agilent). Samples were injected on a Zorbax SB C18 120 C18 column (2.1 × 100 mm, 2.7 µm) and the injection was 5 µL. The solvent A was 0.1% formic acid in H2O and solvent B was 0.1% formic acid in 50% acetonitrile at a flow rate of 350 µL min⁻¹. The linear gradient was as follows: 30% A at 0 min, 85% B at 15 min, 100% B at 1 min, 100% B at 16.5 min, and 30% A at 17.6 min. The flow rate was set at 5 °C and the spray voltage was adjusted to −3.5 kV. Analyses were performed in Selected Reaction Monitoring mode using nitrogen as collision gas. The specific transition was (m/z 398 → m/z 295) corresponding to the [M−H]⁻/[(M-H)ABA]⁻ abundance ratio (product ions displayed in Fig. 2a). Fragmentor and collision energy were, respectively, 120 and 18 V. Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies). Finally, the quantification of C12AsnGABAOH and C12AsnBABAOH was performed using a calibration curve calculated by the IS method. Six biological replicates were performed for each strain.

GABA analysis by LC-HRMS. GABA was analyzed by liquid chromatography (Vanquish System, Dionex, Sunnyvale, CA, USA) coupled with a Orbitrap Q Exactive plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated ESI probe. MS analyses were performed in the positive FTMS mode at a resolution of 70,000 (at m/z 400) with the following source parameters: capillary temperature was 320 °C, source heater temperature was 250 °C, sheath gas flow rate was 40, auxiliary gas flow rate was 15, S-Lens RF level was 50%, and source voltage was 4 kV. Samples were injected on a Supelco HS F5 Discovery column (150 mm × 2.1 mm; 5 µm particle size) equipped with a Supelco HS F5 guard column (20 mm × 2.1 mm; 5 µm particle size). Solvent A was 0.1% formic acid in H2O and solvent B was 0.1% formic acid in acetonitrile at a flow rate of 400 µL min⁻¹. The linear gradient was as follows: 15% B at 0 min, 100% B at 5 min, 100% B at 6.5 min, and 15% B at 7 min. The flow rate was adjusted to −3.5 kV. Analyses were performed in Selected Reaction Monitoring mode using nitrogen as collision gas. The specific transition was (m/z 398 → m/z 295) corresponding to the [M−H]⁻/[(M-H)ABA]⁻ abundance ratio (product ions displayed in Fig. 2a). Fragmentor and collision energy were, respectively, 120 and 18 V. Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies). Finally, the quantification of C12AsnGABAOH and C12AsnBABAOH was performed using a calibration curve calculated by the IS method. Six biological replicates were performed for each strain.

Cell culture and absorption/permeability experiments. Caco-2 cells (Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures GmbH, catalog number: ACC 169) were grown in Glutamax DMEM (Gibco, Invitrogen Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen Life Technologies, Paisley, UK), 1% nonessential amino acids, and 1% antibiotics (100 U ml⁻¹ penicillin and 100 µL ml⁻¹ streptomycin; Gibco, Invitrogen Life Technologies, Grand Island, NY, USA) at 37 °C in a 5% CO2, water-saturated atmosphere. Cells were seeded on Transwell inserts (Costar, Sigma-Aldrich, Saint Quentin Fallavier, France) and absorption studies of C12AsnGABAOH and GABA were performed 16 days later. These compounds were detected in both apical and basolateral chambers as well as in cells. The effect of C12AsnGABAOH (100 µM) on paracellular permeability was also measured by mucosal-to-serosal flux of 4 kDa FITC-labeled dextran as previously described34. All experiments were performed in serum-free medium. CXXCL8 concentration in apical and basolateral chambers was determined 24 h after C12AsnGABAOH addition using a commercial enzyme-linked immunosorbent assay kit (BD Biosciences, Erembodegen, Belgium) following manufacturer's recommendations35.

Calcium imaging of sensory neurons. Mouse DRG were dissociated as previously described8. After 40–42 h of culture, cells were incubated with HBSS containing 20 mM HEPES, 1 mM fluo-4 acetoxyethyl (AM), and 20% pluronic F-127 for 30 min at 37 °C plus 30 min in the dark at RT. The plates were then washed with HBSS and 100 µL of HBSS were added to each well. Live cell imaging of calcium was carried out on an automated microscope (A potscope 2, Zeiss) with a x10 objective. Images were acquired using the Zen imaging software and a kinetic of 80 records (one per second) was performed. The first five images were used to determine the baseline and, from 60 to 90 s, neurons were exposed to either a mix of GPCR agonists (histamine, bradykinin, serotonin, 10 µM), capsaicin (125 nM), or vehicle (HBSS). After 60 s, neurons were treated with KCl (50 mM) in order to discriminate neurons from glial cells. The ImageJ software was used to perform the analysis of calcium flux. In a first set of experiments, neurons were pre-treated with either C12-Asn-GABA, C12-Asn-BABA, or C12-Asn-AABA (0.1, 1, and 10 µM), or vehicle (HBSS/DMSO 0.01%), then treated for 3 min with C12-Asn-GABA (10 µM) or vehicle and finally stimulated with either capsaicin or the mix of GPCR agonists and vehicle.

CRD and electromyography (EMG) recordings. Nickel–chrome electrodes were implanted in the abdominal external oblique musculature of anesthetized mice in order to detect EMG activity as previously described37. CRD was performed 3 days post-surgery by inserting a distension catheter (Fogarty catheter for arterial embolomcy, 4 F; Edwards LifeSciences, Nijmegen, the Netherlands) into the colon at 5 mm from the anus. The balloon was progressively inflated in a stepwise of 15 mm Hg (from 0 to 60 mm Hg) performing 10-s distension for each pressure (in triplicate) and with resting intervals of 5 min as previously described37. In a first set of experiments, four groups of mice (n = 7–8 per group) were pre-treated with a 100 µL intraluminal injection of either C12-Asn-GABA (10 µM) in EIOH 40% or vehicle, and 30 min later animals were administered either capsaicin (100 µg per animal) or vehicle (EtOH 40%) intracolonically. CRD was performed 15 min after capsaicin/vehicle administration, and VMR to different colorectal pressures were recorded.

Intestinal isotonic contractions. Duodenum segments obtained from euthanized mice were washed in Krebs–Ringer bicarbonate/glucose buffer (pH 7.4) in an atmosphere of 95% O2, 5% CO2. Duodenum segments were then incubated in oxygenated Krebs–Ringer solution and attached to an isotonic transducer as previously described38. Isotonic contractions were recorded by means of BDAS software (Hugo Sachs Elektronik) following the transducer displacement. Basal contractions were recorded after duodenum segment attachment for 10 min. Subsequently, 100 µL of either C12-Asn-GABA (10 µM) or Krebs–Ringer solution were added in survival medium, and contractions were recorded for 10 min. The amplitudes were also recorded for 10 min at 10-s intervals, and their average was compared to average basal contractions. Contractions amplitudes are presented as percentage relative to basal response, while contraction frequencies are expressed as a number of contractions per min.

Statistical analysis. Data are presented as means ± SEM. The software GraphPad Prism 6.0 (GraphPad, San Diego, CA) was used for statistical analysis. Multiple comparisons within groups were performed by Kruskal–Wallis test followed by Dunns post-test or by two-way Anova followed by Bonferroni post-test. Statistical significance was accepted at p < 0.05.

Data availability. The authors declare that the relevant data supporting the findings of the study are available in this article and its Supplementary Information, or from the corresponding author upon request.

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Author contributions

T.P.-B. designed and conducted experiments, performed data acquisition, analysis and interpretation, and wrote the manuscript. J.P. carried out experiments, performed data acquisition, analysis and interpretation. P.M. carried out experiments and contributed to data interpretation and drafting of the manuscript. L.F. and J.C.T. carried out experiments, performed data acquisition, analysis and interpretation, and participated in the manuscript writing. J.-M.G., A.G. and T.D. conducted experiments and contributed with the drafting of the manuscript. F.B., C.K., S.T. and M.H. conducted experiments and performed analysis of data. J.B.-M. contributed to data analysis and interpretation and edition of the manuscript. G.D. participated in the manuscript writing. E.O. designed experiments, performed interpretation of data, helped with manuscript drafting, and supervised the study. N.C. designed and conducted experiments, performed analysis and interpretation of data, wrote the manuscript, and directed the project.

Additional information

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