(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 January 2022 (13.01.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/010352 A1

(51) International Patent Classification:
A61K 31/404 (2006.01) *A61P 1/10* (2006.01)

(21) International Application Number:
PCT/NL2021/050434

(22) International Filing Date:
09 July 2021 (09.07.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/050,176 10 July 2020 (10.07.2020) US

(71) Applicant: **RIJKSUNIVERSITEIT GRONINGEN** [NL/NL]; Broerstraat 5, 9712 CP Groningen (NL).

(72) Inventors: **EL AIDY, Sahar**; c/o Nijenborgh 7, 9747 AG Groningen (NL). **WACLAWIKOVA, Barbora**; c/o Nijenborgh 7, 9747 AG Groningen (NL).

(74) Agent: **WITMANS, H.A.**; V.O., P.O. Box 87930, 2508 DH Den Haag (NL).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2022/010352 A1

(54) Title: 5-HYDROXYINDOLE AND ANALOGS THEREOF AS STIMULANTS OF GUT MOTILITY

(57) Abstract: The invention relates to 5-hydroxyindole (5-HI) and analogs thereof, and to their medical use, such as the treatment of conditions associated with reduced gut motility. Provided is a method for the treatment of a condition associated with reduced gut motility, comprising administering to a subject in need thereof a pharmaceutically effective amount of 5-HI or an analog thereof having the capability to increase gut motility.

Title: 5-HYDROXYINDOLE AND ANALOGS THEREOF AS STIMULANTS OF GUT MOTILITY

The invention relates to the field of pharmacology, in particular gastrointestinal pharmacology. In general, the invention relates to compounds that affect gut motility. The invention also relates to the treatment of conditions associated with reduced gut motility, such as constipation. More in particular, the invention relates to 5-hydroxyindole and analogs of 5-hydroxyindole as stimulants of gut motility.

The gastrointestinal (GI) tract is home to trillions of microbes. The gut microbiota produces a wide range of small bioactive molecules derived from various substrates, including dietary precursors and medications (Van Kessel *et al.*, *Nat. Commun.* 2019, 31, 1-31; Donia *et al.*, *Science* 2015, 349, 80). Such microbial conversion represents a significant regulatory mechanism by which gut microbes can alter intestinal host physiology, including gastrointestinal motility (Yano *et al.*, Cell 2015, 161, 264-276; Reigstad *et al.*, *FASEB J.* 2015, 29, 1395-1403). For example, tryptamine produced by bacterial decarboxylation of dietary tryptophan accelerates gastrointestinal transit by activating epithelial G-protein coupled receptors, serotonin receptor 4, and increasing anion-dependent fluid secretion in the proximal colon of mice (Bhattarai *et al.*, *Cell Host Microbe* 2018, 23, 775-785). Therefore, gut microbiota-derived molecules appear to functionally link the microbiota activity to the host gastrointestinal motility.

Gastrointestinal motility is a tightly controlled system involving two main neurotransmitters, acetylcholine (ACh) and serotonin (5-HT), and their receptors, which are respectively located on smooth muscle cells and neuronal structures of the enteric nervous system (ENS) (Hansen, *Physiol.*

Res. 2003, 52, 1-30). Following mucosal stimulation, 5-HT is released from a subset of enteroendocrine cells, enterochromaffin cells (EC cells), to act on intrinsic primary afferent neurons (IPANs), followed by the release of several molecules, including acetylcholine. Initiation of this signaling pathway results ultimately in the release of acetylcholine in the smooth muscle cells expressing muscarinic acetylcholine receptors (Hansen (2003)). Stimulation of the muscarinic receptors, in turn, induces contractions that depend on voltage-dependent and voltage-independent Ca^{2+} entry and intracellular Ca^{2+} release (Unno *et al.*, *Br. J. Pharmacol*. 2005, 146, 98-108) (cholinergic neurotransmission is extensively discussed in: Hansen (2003) and Sanders *et al.*, *Nat. Rev. Gastroenterol. Hepato*l. 2012, 9, 633-645). Within this tightly controlled system, any disturbance in these regulatory mechanisms will result in gastrointestinal motility disorders.

Constipation is a common, debilitating motility disorder affecting up to 27 % of the population (Sanchez *et al.*, *Can. J. Gastroenterol*. 2011, 25, 11-15). Constipation is also often associated with colorectal cancer, Parkinson's disease, childhood attention-deficit/hyperactivity disorder and autism spectrum disorder, as well as, mood disorders. Recently, the administration of 5-hydroxytryptophan (5-HTP) to a mouse model of depression resulted in a normalized total gastrointestinal transit time and increased colonic motility (Israelyan *et al.*, *Gastroenterology* 2019, 157, 507-521).

5-HTP (also known as oxitriptan) is a naturally occurring amino acid, as well as a chemical precursor and intermediate metabolite of the essential amino acid L-tryptophan in the biosynthesis of serotonin (Bertrand *et al.*, *Auton. Neurosci*. 2010, 153, 47-57). Several studies focus on using 5-HTP as a food supplement, or drug with or without other medications for the treatment of a wide variety of conditions, including depression, fibromyalgia, binge eating associated with obesity, chronic headaches, and insomnia (Birdsall, *Altern. Med. Rev*. 1998, 3, 271-280; Jacobsen *et al.*,

Neuropsychopharmacology 2019, 44, 2082-2090; Caruso *et al.*, *J. Int. Med. Res.* 1990, 18, 201-209; Nardini *et al.*, *Int. J. Clin. Pharmacol. Res.* 1983, 3, 239-250; Perez *et al.*, *Front. Pharmacol.* 2019, 10, 1-15).

Other studies focus on 5-HT receptors and 5-HT antagonists. For example, Gershon *et al.*, *Experientia* 1985, 41, 863-868 describes multiple molecules, including 5-hydroxyindole, that can displace serotonin from its neuronal binding sites identified in intestinal membranes isolated from rabbits, guinea pigs, and mice.

5-HTP is a structural homologue of L-tryptophan, which is metabolized by gastrointestinal bacterial tryptophanase (TnaA) enzyme to produce indole (Snell, *Adv. Enzymol. Relat. Areas Mol. Biol.* 1975, 42, 287-329). However, it remains unknown whether gut bacteria can also metabolize 5-HTP and whether the produced products can modulate gastrointestinal motility.

The present inventors recognized the need in the art for a stimulant of gut motility having low toxicity and high effectivity. In particular, they aimed at providing novel means and methods for increasing contractility of the gut. Another objective is to provide a stimulant that accelerates the total gut transit time.

The inventors surprisingly found that one or more of these objectives can, at least in part, be met by using 1*H*-indoles, particularly 5-hydroxyindole (5-HI) and analogs thereof 5-HI.

In a general sense, the invention is based on the judicious insight that 5-hydroxyindole and analogs thereof are able to stimulate motility of the mammalian gut. By (orally) administering such indole derivatives, which preferably have an oxygen-containing substituent at selected positions on benzene moiety of the indole core, reduced gut motility can be treated.

Accordingly, in a first aspect, the invention is directed to a method for the treatment of a condition associated with reduced gut

motility, comprising administering to a subject in need thereof a pharmaceutically effective amount of 5-hydroxyindole (5-HI) or an analog thereof having the capability to increase gut motility.

Gut motility or gastrointestinal (GI) motility as used herein refers to the movements of the digestive system, and the transit of the contents within it. When nerves or muscles in any portion of the digestive tract do not function with their normal strength and coordination, a subject can develop symptoms related to motility problems. There are a variety of motility disorders that can affect the GI tract from the very top (esophagus) to the very bottom (colon and rectum). Each part of the GI tract – esophagus, stomach, small intestine, and large intestine – has a unique function to perform in digestion, and each has a distinct type of motility and sensation. When motility or sensations are not appropriate for performing this function, symptoms occur.

The term "analog" as used in this disclosure includes compounds that are similar to 5-HI. Preferably, the analog is a structural analog of 5-HI. Useful structural analogs of 5-HI are described in this disclosure. The similarity between the compounds that are similar to 5-HI and 5-HI itself can be a result of a chemical reaction, thereby yielding a compound having a chemical structure which is similar to that of 5-HI.

The analog of 5-HI can have one or more substituents, preferably one substituent. The substituent can be any atom or molecule, other than hydrogen. The presence of an oxygen atom in the substituent was found to be surprisingly advantageous to reach a potent stimulatory effect on gut motility. Hence, the analog of 5-HI may comprise an oxygen-containing substituent that is directly attached at the benzene moiety of the indole ring system. Preferably, the analog has a hydroxyl group or a methoxy group. The one or more substituent(s) can be at the benzene ring of the analog. The one or more substituent(s) can be at the 4-, 5-, 6-, and/or 7-position of the phenyl. It may be that the presence of one or more substituents at the

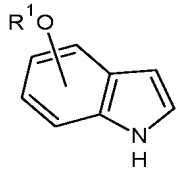
phenyl group of the 5-HI analog, especially those that have an oxygen atom, tend to increase the stimulating effect the compounds have on gut motility when used (as a medicament) in the treatment of a condition associated with reduced gut motility. In particular, the substituent is at the 4-, 5-, or 6-position. The substituent is preferably at the 5- or 6-position, and more preferably at the 5-position.

The term "alkoxy" includes any hydrocarbon group that is bonded through an oxygen atom. The hydrocarbon group may comprise one or more heteroatoms, *i.e.*, other than hydrogen, carbon, and oxygen. In particular, alkyls that are bonded through an oxygen atom are meant. The alkoxy group may be connected with its oxygen atom directly to the compound.

The alkoxy group can be linear, such as ethoxy, or branched, such as isopropoxy. In particular, the alkoxy group is linear.

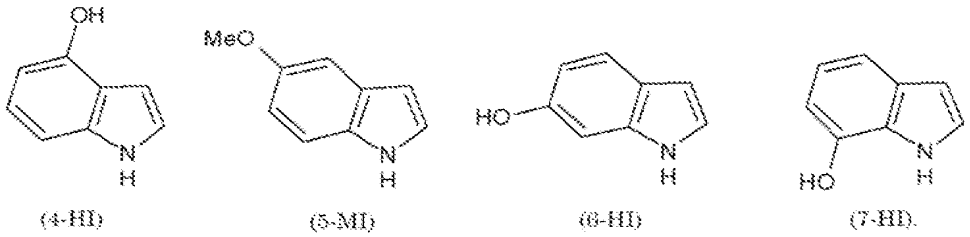
The alkoxy group can have 1-3 carbon atoms, such 1 or 2 carbon atoms. In particular, the alkoxy group is selected from the group consisting of methoxy or ethoxy. Preferably, the alkoxy group is a methoxy.

The 5-HI analog can be a compound of formula (I)



(I).

wherein R^1 is hydrogen, methyl or ethyl, preferably hydrogen or methyl. The OR^1 group can be at the 4-, 5-, 6-, or 7-position. The group can be at the 4-, 6-, or 7-position, and R^1 may be hydrogen. Preferably, the group is at the 6- or 7-position, and R^1 is hydrogen. The group can be at the 4-, 5-, 6- or 7-position, and R^1 may be methyl. Preferably, the group is at the 5-position, and R^1 is methyl. In particular, the analog is selected from



(4-HI) (5-MI) (6-HI) (7-HI)

Preferably, the analog is 5-MI, 6-HI or 7-HI. More preferably, the analog is 5-MI or 6-HI. Even more preferably, the analog is 5-MI.

According to the invention, 5-HI or an analog thereof can be administered in any conventional manner by any route where it is active or becomes active. Administration can be systemic or oral. Administration is preferably oral. The modus of administration can depend on the condition to be treated. The selection of the specific route of administration can be selected or adjusted by a clinician according to methods known to the clinician to obtain the desired clinical response.

5-HI or the analog thereof is preferably formulated in accordance with routine procedures as a pharmaceutical adapted for administration to humans. For oral administration, 5-HI or the analog thereof can be formulated by combining 5-HI or the analog with a pharmaceutically acceptable carrier, diluent or vehicle. The invention also provides a pharmaceutical composition comprising a 5-HI analog as herein disclosed, and with a pharmaceutically acceptable carrier, diluent or vehicle. Preferably, the composition is formulated for oral administration.

The phrase "pharmaceutically acceptable" refers to, for example, compounds, materials, compositions, and dosage forms which are, within the scope of the practitioner's medical judgment, suitable for use in contact with tissues of animals, in particular humans. The term may also include the approval by a (governmental) regulatory agency for use in animals, and more in particular for use in humans.

Such pharmaceutically acceptable carriers enable 5-HI or the analog thereof to be formulated as, for example, solutions; sustained-release

formulation; suppository; tablets; pills; drageés; capsules; emulsions; liquids; gels; syrups; caches; pellets; powders; granules; slurries; lozenges; aqueous; or oily suspensions for oral ingestion by the subject or patient to be treated. Pharmaceutical preparations for oral use can be obtained by, *e.g.*, adding a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or drageés. Suitable excipients include, but are not limited to, fillers, such as sugars, including, but not limited to, lactose, sucrose, mannitol, and sorbitol; cellulose preparations, such as, but not limited to, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and polyvinyl pyrrolidone. If desired, disintegrating agents can be added, such as, but not limited to, the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate.

A composition formulated for the oral administration of 5-HI or an analog thereof can contain one or more optional agents, *e.g.*, sweetening agents, such as fructose, aspartame, or saccharin; flavoring agents, such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions may be coated to delay disintegration and absorption in the gastrointestinal tract, particularly in the gut, thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for the oral administration. Oral compositions can include standard vehicles, such as mannitol; lactose; starch; magnesium stearate; sodium saccharine; cellulose; magnesium carbonate; etc. Such vehicles are suitably of pharmaceutical grade.

Drageés can be provided with suitable coatings. For this purpose, concentrated sugar solutions can be used, which can optionally contain Arabic gum; talc; polyvinyl pyrrolidone; carbopol gel; polyethylene glycol;

and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or drageé coatings for identification or to characterize different combinations of active compound doses. Pharmaceutical preparations which can be used orally include, but are not limited to, push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler, such as lactose or binders, such as starches, and/or lubricants, such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers can be added.

5-HI or an analog thereof as herein disclosed may be administered either alone or in combination (concurrently or serially) with other compounds, such as pharmaceutically acceptable compounds and/or prodrugs. For example, 5-HI or the analog thereof can be administered in combination with an antibiotic and/or other known drug compound, for example, for the treatment of a condition associated with reduced gut motility, such as one or more further contractile agent(s).

According to the invention, 5-HI or an analog thereof is preferably used in an amount or dosage which is pharmaceutically effective. The phrase "pharmaceutically effective amount" refers to the amount of active compound, or pharmaceutical, that elicits the biological or medicinal response that is being sought in a tissue, system or subject by a researcher, veterinarian, medical doctor or other clinician (practitioner). The pharmaceutic effect is dependent on the condition being treated, the biological effect desired, and/or the clinical result desired. As such, the pharmaceutic effect can be a decrease in the severity of symptoms associated with the condition and/or inhibition (partial or complete; at least in part) of the progression of the condition, or improved treatment, healing,

prevention or elimination of a condition, or side-effects. The amount needed to elicit the therapeutic response can be determined, for example, based on the species, age, health, size, gender, and/or sex of the subject. Optimal amounts can also be determined based on monitoring of the subject's response to treatment.

The dosage to be administered may depend on the characteristics of the subject being treated, *e.g.*, the particular animal or human treated, age, weight, health, types of concurrent treatment, if any, and frequency of treatments, and can be easily determined by, *e.g.*, a clinician. The dosing for 5-HI or analog thereof can be used and adjusted depending on, *e.g.*, the factors above. The selection of the specific dose regimen can be selected, adjusted, or titrated by a clinician according to methods known in the art to obtain the desired clinical response.

The dosage amount of 5-HI or analog thereof that is pharmaceutically effective in, *e.g.*, the treatment of a particular condition will depend on the nature and extent of the condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may be employed to help identify optimal dosage ranges. The precise dose to be employed also typically depends on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and the patient's condition(s). Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems (animal models). 5-HI or analog thereof may be used for a time sufficient to treat the condition.

In one embodiment, 5-HI or analog thereof is administered in a dosage range of 0.1 mg per kg of body weight or more. The dosage range can be 1000 mg/kg body weight or less. The dosage range can be 5-900 mg/kg body weight, such as 20-800 mg/kg body weight, 30-700 mg/kg body weight, or 40-600 mg/kg body weight. In particular, the dosage range is 0.1-100 mg/kg body weight, such as 20-90 mg/kg body weight, 30-80 mg/kg

body weight, or 40-70 mg/kg body weight. Preferably, the dosage range is 1-50 mg/kg body weight, such as 10-40 mg/kg body weight, 15-35 mg/kg body weight, or 20-30 mg/kg body weight.

5-HI or analog thereof or (pharmaceutical) compositions comprising 5-HI or analog thereof can be prepared in unit dosage form. In such form, the composition can be divided into unit doses containing appropriate quantities of 5-HI or analog thereof. The unit dosage form can be a packaged preparation, where the package contains discrete quantities of the preparations, *e.g.*, packeted tablets, capsules, and powders in vials or ampules. The unit dosage form can be a capsule, cachet, or tablet itself, or it can be the appropriate number of any of these packaged forms.

5-HI or an analog thereof can be administered orally to the subject in need thereof. The phrase "in need thereof" includes that a subject has been identified as having a need for the particular treatment. The identification of such a need can be by any means of diagnostics. In particular, in any of the methods and treatments described herein, the subject is in need thereof.

The terms "treatment" and "treating" are not meant to be limited to curing. Treating also includes alleviating at least one symptom of a condition, removing at least one symptom of a condition, lessen at least one symptom of a condition, and/or delaying the course of a condition. The term "treatment" includes methods of therapy and optionally diagnosis. Also, the terms include therapeutic treatment and prophylactic or preventative measures wherein the object is to prevent or slow down (lessen) an undesired physiological condition, or obtain beneficial or desired clinical results. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of condition, stabilized (*i.e.*, not worsening) state of condition, delay in onset or slowing of condition progression, amelioration of the condition state or remission (whether partial or total), whether detectable or undetectable, an amelioration of at

least one measurable physical parameter, not necessarily discernible by the patient; or enhancement or improvement of condition. Treatment includes eliciting a clinically significant response without excessive levels of side effects. Treatment may also include prolonging survival as compared to expected survival if not receiving treatment. Thus, treatment of a condition associated with reduced gut motility preferably means an activity that prevents, alleviates and/or ameliorates reduced gut motility caused by the condition.

The subject can be any animal, including vertebrates, *e.g.*, wild, domestic, and farm animals. Preferably, the subject is a human subject. The subject may suffer from abnormal motility patterns in the small intestine can lead to symptoms of intestinal obstruction. Symptoms of bloating, pain, nausea, and vomiting can result either from weak contractions or from disorganized (unsynchronized) contractions that result from intestinal muscle (visceral myopathy) or nerve (visceral neuropathy) problems. The symptoms of constipation are infrequent bowel movements [usually less than 3 per week], passage of hard stools, and sometimes difficulty in passing stools. One motility problem that can lead to constipation is a decrease in the number of high amplitude propagating contractions (slow transit) in the large intestine. The test used to detect this is a transit time (Sitzmark) study.

According to the invention, 5-HI or analog thereof is advantageously used for treating a condition associated with reduced gut motility. The condition can be, for example, constipation. The constipation can be associated with, for example, colorectal cancer or Parkinson's disease.

As mentioned in this disclosure, 5-HI or analog thereof can stimulate release of 5-HT from EC cells and/or L-VDCCs on colonic smooth muscle cells.

5-HI and analogs thereof, particularly those described in this disclosure, can be used in medical applications, such as in medicine. 5-HI

and analogs thereof, particularly those described in this disclosure, thereof can be used in methods for therapy and/or (*in vivo*) diagnostics. The method for therapy and/or (*in vivo*) diagnostics can be a method for detecting, preventing, and/or treating, preferably treating, a condition associated with reduced gut motility, particularly constipation. 5-HI and analogs thereof, particularly those described in this disclosure, can be used in the preparation or manufacture of a medicament for detecting, preventing, and/or treating, preferably treating, one or more conditions associated with reduced gut motility, particularly constipation. 5-HI and analogs thereof, particularly those described in this disclosure, can be used for the manufacture of a medicament for the prevention or treatment, preferably treatment, of a condition associated with reduced gut motility, such as constipation. The constipation may be associated with, for example, colorectal cancer or Parkinson's disease.

In another aspect, there is provided 5-hydroxyindole (5-HI) or an analog thereof for use in the treatment of a condition associated with reduced gut motility, wherein a pharmaceutically effective amount of 5-HI or analog thereof having the capability to increase gut motility is administered to a subject in need thereof.

The analog of 5-HI is preferably as described in this disclosure, such as a compound of formula (I). The analog can be selected from 4-HI, 5-MI, 6-HI, and 7-HI. In particular, the analog is selected from 5-MI, 6-HI and 7-HI. Preferably, the analog is 5-MI or 6-HI.

5-HI or the analog thereof is preferably administered as described in this disclosure, especially orally, to a subject in need thereof as described in this disclosure. The condition associated with reduced gut motility is preferably as described in this disclosure, in particular constipation.

5-HI or the analog thereof may be administered in a dosage range of 0.1 mg per kg of body weight or more. The dosage range can be 1000 mg/kg body weight or less. The dosage range can be 5-900 mg/kg body

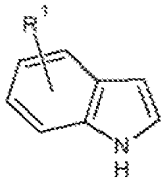
weight, such as 20-800 mg/kg body weight, 30-700 mg/kg body weight, or 40-600 mg/kg body weight. In particular, the dosage range is 0.1-100 mg/kg body weight, such as 20-90 mg/kg body weight, 30-80 mg/kg body weight, or 40-70 mg/kg body weight. Preferably, the dosage range is 1-50 mg/kg body weight, such as 10-40 mg/kg body weight, 15-35 mg/kg body weight, or 20-30 mg/kg body weight.

The treatment of conditions associated with reduced gut motility can be targeted to stimulate the release of 5-HT from EC cells. The treatment may instead, or in addition to, activate L-VDCCs. Hence, 5-HI or the analog thereof can stimulate the release of 5-HT from EC cells and/or activate L-VDCCs on colonic smooth muscle cells.

Any other known medicament, compound or composition used for the detection, prevention, and/or treatment of a condition associated with reduced gut motility can be used in co-therapy, co-administration, or co-formulation with the 5-HI or the analog thereof.

In yet another aspect, there is provided 5-HI or the analog thereof for use as an activator of gut motility or as a contractile agent. The analog may be as described in this disclosure, such as a compound of formula (I) or (II). The analog may be 4-hydroxyindole, 6-hydroxyindole, 7-hydroxyindole, or 5-methoxyindole. Preferably, the analog is 6-hydroxyindole, 5-methoxyindole or 7-hydroxyindole. More preferably, the analog is 5-methoxyindole or 6-hydroxyindole. Even more preferably, the analog is 5-methoxyindole.

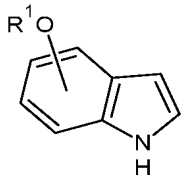
Also provided is a 5-HI analog for use as a medicament. The analog can be a compound of formula (II), wherein the formula is



(II).

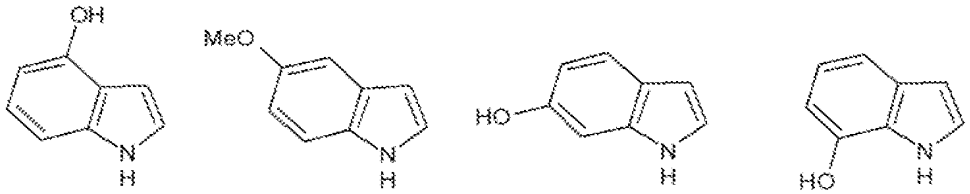
R^1 can be amino, hydroxyl, methoxy, or ethoxy. R^1 can be at the 4-, 5-, 6-, or 7-position. R^1 may be hydroxyl and can be at the 4-, 6-, or 7-position, preferably at the 4- or 6-position. R^1 may be methoxy and can be at the 4-, 5-, 6-, or 7-position, preferably at the 5-position. R^1 can be amino and can be at the 5-position.

In yet another aspect, there is provided a 5-HI analog for use as a medicament. The analog is a compound of formula (I), wherein the formula is



(I).

R^1 can be hydrogen or methyl. The OR^1 group can be at the 4-, 5-, 6- or 7-position. The group can be at the 4-, 6-, or 7-position, and R^1 may be hydrogen. The group can be at the 4-, 5-, or 6-position, and R^1 may be methyl. In particular, the analog is selected from



(4-HI) (5-MI) (6-HI) (7-HI).

Preferably, the analog is 5-MI, 6-HI or 7-HI. More preferably, the analog is 5-MI or 6-HI. Even more preferably, the analog is 5-MI.

There is also provided a pharmaceutical kit comprising one or more containers filled with 5-HI or an analog thereof as described in this disclosure. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration for treating a condition as described in this disclosure,

preferably a condition associated with reduced gut motility, such as constipation. The kit may contain one or more analogs of 5-HI, such as 4-hydroxyindole, 6-hydroxyindole, 7-hydroxyindole and/or 5-methoxyindole, preferably 6-hydroxyindole and/or 5-methoxyindole.

Also provided are the individual compounds as described and/or depicted in this disclosure, such as the individual analogs of 5-HI. Mixtures (compositions), including pharmaceutical compositions, comprising one or more of these compounds, are also provided. These compounds may be used as medicaments, for example, as pharmaceuticals, and may be used in medical applications, such as in medicine. These compounds may be used for treating (medical) conditions, especially conditions associated with reduced gut motility, such as constipation. These compounds may be used in a method of therapy, in particular to detect and/or treat, preferably to treat, a condition associated with reduced gut motility, especially in the treatment of constipation which can be associated with, *e.g.*, colorectal cancer or Parkinson's disease.

In yet another aspect, there is provided a pharmaceutical composition comprising a 5-HI analog as defined in this disclosure. In particular, the 5-HI analog is a compound of formula (I). Preferably, the 5-HI analog is selected from 4-hydroxyindole, 5-methoxyindole, 6-hydroxyindole, and 7-hydroxyindole. More preferably, the 5-HI analog is selected from 5-methoxyindole, 6-hydroxyindole and 7-hydroxyindole. The pharmaceutical composition may be suitable for oral administration. Hence, the pharmaceutical composition may be in a form for oral administration as described in this disclosure. For example, the pharmaceutical composition may comprise a pharmaceutically acceptable carrier and/or one or more optional agents, as described in this disclosure.

In yet another aspect, the invention is directed to a method for identifying an activator of gut motility. The method comprises contacting *in vitro* a 5-HI analog to be tested for activating gut motility with colonic

tissue. The method further comprises determining whether the compound induces muscle cell contractility. Either or both steps may be performed as described in this disclosure.

In yet another aspect, the invention is directed to a method for providing a compound having the capability to increase gut motility. The method comprises providing a library of 5-HI analogs. The method further comprises testing *in vitro* one or more members of the library, *i.e.*, one or more of the 5-HI analogs, on their capacity to activate L-VDCCs. The testing step may be performed as described in this disclosure.

The invention has been described by reference to various embodiments, and methods. The skilled person understands that features of various embodiments and methods can be combined with each other.

All references cited herein are hereby completely incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising", "having", "including" and "containing" are to be construed as open-ended terms (*i.e.*, meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as

indicating any non-claimed element as essential to the practice of the invention. For the purpose of the description and of the appended claims, except where otherwise indicated, all numbers expressing amounts, quantities, percentages, and so forth, are to be understood as being modified in all instances by the term "about". Also, all ranges include any combination of the maximum and minimum points disclosed and include any intermediate ranges therein, which may or may not be specifically enumerated herein.

When referring to a noun (*e.g.*, a compound, a pharmaceutically acceptable salt, a prodrug, *etc.*) in the singular, the plural is meant to be included, or it follows from the context that it should refer to the singular only.

Preferred embodiments of this invention are described herein. Variation of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject-matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context. The claims are to be construed to include alternative embodiments to the extent permitted by the prior art.

For the purpose of clarity and a concise description, features are described herein as part of the same or separate embodiments, however, it will be appreciated that the scope of the invention may include embodiments having combinations of all or some of the features described.

Hereinafter, the invention will be illustrated in more detail by means of specific examples. However, the invention may be embodied in

many different forms and should not be construed as being limited to the embodiments set forth herein. Rather, these example embodiments are provided so that this description will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

LEGEND TO THE FIGURES

Figure 1: Bacteria in fecal samples from healthy volunteers convert 5-HTP to 5-HI *ex vivo*.

A) From left to right: Chromatograms showing bacterial conversion of 100 μM 5-HTP to 5-HI after 6 and 24 h of incubation of human fecal samples (upper panel). Lower panel: quantification of 5-HTP consumption (substrate; black circles), and 5-HI production (product; white circles) observed in High (n = 8), Intermediate (n = 4), and Non-Converters (n = 6).

B) Heatmap of the levels of 5-HI on Day 1 (first ingestion of 5-HTP tablet), Day 3 and Day 8 (one day after last 5-HTP tablet was ingested), normalized to Day 0, detected in fecal samples of volunteers who administered 5-HTP tablets for 1 week. The numbers in the squares indicate fold change in 5-HI levels normalized to Day 0.

Figure 2: Bacteria in fecal samples from healthy volunteers convert 5-HTP to 5-HI.

A) Formation of 5-HI was identified and confirmed by LC-MS. Chromatograms show mass $[M+H]^+$ m/z = 134.0602, which corresponds to exact molecular weight of 5-HI in positive mode.

B) No basal levels of 5-HTP were detected in the control samples in the fecal samples from healthy individuals at time point 0 h.

C) Chromatogram shows that tryptophan was not converted to indole levels in the Non-Converters.

Figure 3: Gut bacteria containing tryptophanase are responsible for conversion of 5-HTP to 5-HI.

A) β-elimination reaction for tryptophan and 5-HTP.

B) Overnight culture of *E. coli* $BW25113^{WT}$ and *E. coli* $BW25113^{\Delta tnaA}$ incubated at 37 °C with agitation with 100 µM 5-HTP for 24 h. Curves represent one example of three biological replicates.

C,D) Michaelis-Menten kinetic curves for C) 5-HTP and D) tryptophan as substrates for 200 nM or 20 nM $TnaA_{BW25113}$, respectively. Reactions were performed in biological triplicate using 5-HTP concentrations ranging from 0 to 12 mM and tryptophan concentrations ranging from 0 to 14.58 mM. Enzyme kinetics were calculated using a nonlinear Michaelis-Menten regression model for 5-HTP and a non-linear substrate inhibition kinetic model for tryptophan. Error bars represent the SEM.

E) Phylogenetic tree created using iTOL online tool showing gut bacterial strains harboring the enzyme tryptophanase.

F,G) Overnight cultures of *F. nucleatum* subsp. *animalis* and *F. simiae* incubated anaerobically at 37 °C with 50 µM 5-hydroxytryptophan (5-HTP). Curves are a representative example of three biological replicates.

Figure 4: Gut bacteria harboring tryptophanase are responsible for conversion of 5-HTP to 5-HI.

A) Overnight culture of *E. coli* $BW25113^{WT}$ and *E. coli* $BW25113^{\Delta tnaA}$. Curves represent one example of three biological replicates.

B) High levels of tryptophan (Trp) do not prevent the conversion of 5-HTP and production of 5-HI in fecal samples after 24 h of anaerobic incubation.

Figure 5: Bacterial production of 5-HI is dependent on the microbiota composition and pH levels.

A) Microbial profiling of human fecal samples from High, Intermediate and Non-Converters shows decreasing relative abundance of *tnaA*-encoding gut

bacterial genera and increasing relative abundance of *Bifidobacteria* genera, respectively. Chromatograms on the left show variation in conversion of 5-HTP to 5-HI after 24 hours of anaerobic incubation with 5-HTP. B-D) Overnight cultures of *F. nucleatum* subsp. *animalis* incubated anaerobically at 37 °C with 100 µM 5-HTP B) in the absence or in the presence of either C) 10 % *Bifidobacterium breve* or D) 50 % *B. breve*.

Figure 6: Bacterial production of 5-HI is dependent on the microbiota composition.

Pie charts represent the difference in relative percentage of TnaA-proteins in the various bacterial phyla represented in the intestinal tract (exploded parts) among High, Intermediate and Non-Converters. Parts annotated in light blue (not exploded) show different relative percentage of *Bifidobacterium* genera among High, Intermediate and Non-Converters. Parts annotated in shades of grey represent the rest of bacterial genera detected in the samples.

Figure 7: 5-HI stimulates GI motility *in vivo*.

A) Graph shows effect of 5-HI on total gut transit in WTG rats. First, baseline measurement (before treatment) of the total gut transit time (TGTT) in all WTG rats was performed. After treatment, significantly decreased TGTT in WTG rats treated with 30 mg/kg of 5-HI (circles; n = 10) was observed. Vehicle group's (10 % sucrose; squares, n = 6) TGTT did not change after the treatment with 10 % sucrose. Data were analyzed using the RM 2-way ANOVA followed by Fisher's LSD test (*$p < 0.05$) (before and after treatment) and two-tailed unpaired t-test (*$p < 0.05$) was used for analysis of data from 5-HI-treated group and vehicle group after treatment. Error bars represent SEM.

B) Increased defecation frequency per 24 h per rat in rats treated with 30 mg/kg of 5-HI compared to the vehicle group (10 % sucrose). Data were analyzed using the two-tailed unpaired t-test (*$p < 0.05$).

C) Bar graph showing no difference between body weight increase in WTG rats treated with 30 mg/kg of 5-HI compared to the vehicle group. Data were analyzed using the two-tailed unpaired t-test (n.s. > 0.05). Error bars represent SEM.

Figure 8: 5-HI is a potent stimulator of gut contractility.

A) Bar graph represent a significantly increased 5-HT release, which is Ca^{2+} influx dependent, from RIN14B cells after stimulation with 100 µM of 5-HI. Data were analyzed using the two-tailed unpaired t-test (****$p < 0.0001$). Error bars represent SEM.

B) Bar graph and illustrative recording in the rat colon representing the enhanced response of the 5-HI on the gut contractility.

C) The dose response curve of 5-HI in the rat colonic tissue.

D-G) Bar graphs and illustrative recordings in the rat colon representing the enhanced response of the 5-HT-induced contractility by 100 µM 5-HI (D), which was not inhibited after adding 1 µM ondansetron (E), 1 µM SB-207266 (F), or 1 µM tetrodotoxin (TTX) (G).

H-K) Bar graphs and illustrative recordings in the rat colon representing enhanced response of the ACh-induced contractility by 100 µM 5-HI (H), which was not inhibited after the addition of 1 µM AQ-RA 741 (I), but was significantly increased after adding 100 nM DAU 5884 hydrochloride (J), and significantly inhibited with 1 µM nifedipine (K).

L) Bar graph and illustrative recording in the rat colon representing enhanced response by 100 µM 5-HI after colonic tissue contractile apparatus is altered by nifedipine (nifedipine is washed away from the tissue in the organ bath). Data represent 3-9 biological replicates. Data were analyzed using the Wilcoxon matched-pairs (before/after) signed rank test (*$p < 0.05$;

**p < 0.01). Error bars represent SEM. Quantitative analysis of the organ bath data is described in *Materials and methods to Examples 1-5 (Section A).*

Figure 9: 5-HI is a potent stimulator of gut contractility.

A) Bar graph represents remaining percentage of 5-HI after incubation for 90 min and 24 h in rat colonic tissue extracts (n = 3). Error bars demonstrate SEM.

B-E) Bar graphs and illustrative recordings representing the inhibitory effect of either 1 µM ondansetron (B), 1 µM SB-207266 (C), 1 µM tetrodotoxin (TTX) (D) or 1 µM hexamethonium (E) on the 5-HT-induced response.

F) Bar graph represents no inhibitory effect of 1 µM hexamethonium on 5-HI-induced response.

G-I) Bar graphs represent an inhibitory effect on ACh-induced response by the addition of either 1 µM AQ-RA 741, (G), 100 nM DAU 5884 hydrochloride (H) or 1 µM ML 204 and SAR 7334 (I).

J) Bar graph and its representative recording of colonic contractions showing no inhibitory effect on 5-HI-induced response when 1 µM ML 204 together with 1 µM SAR 7334 were added.

K) Bar graph represents contractile agent (ATP) acting independently of L-type Ca^{2+} channels. Data represent 3-5 biological replicates. Data were analyzed using the Wilcoxon matched-pairs (before/after) signed rank test (*p < 0.05; ***p < 0.001). Error bars represent SEM. Quantitative analysis of the organ bath data is described in *Materials and methods to Examples 1-5 (Section A).*

Figure 10: Proposed model for the mechanisms by which 5-HI or analog thereof accelerates intestinal motility.

Following bacterial conversion of 5-HTP to 5-HI, 5-HT is released from EC cells to IPANs. Stimulation of IPANs initiate a signaling pathway ending up with release of neurotransmitters to excitatory cholinergic motor neurons in the ENS releasing ACh to the smooth muscle cells causing stimulation of muscarinic receptors 2 and 3 (M2/3R), which induce contractions that depend on voltage-dependent and -independent Ca^{2+} entry followed by muscle contraction. In parallel, 5-HI translocates through the gut tissue, where it reaches the smooth muscle cells and accelerates gastrointestinal motility directly via activation of L-VDCCs. Abbreviations: NSSCs; non-selective Ca^{2+} channels.

Figure 11: Dose response curves and EC_{50} values of 5-hydroxyindole and analogs. Compounds were evaluated in an ex vivo rat colonic tissue model. 5-HI, 5-hydroxyindole; 6-HI, 6-hydroxyindole; 4-HI, 4-hydroxyindole; 5-MI, 5-methoxyindole; 5-AI, 5-aminoindole; 7-HI, 7-hydroxyindole.

Figure 12. Overlay of dose response curves and EC50 values of 5-HI and analogs thereof with hydroxy group at different position. Panel A: 5-HI vs. 6-HI; Panel B: 5-HI vs. 4-HI; Panel C: 5-HI vs. 7-HI; Pane; D: 6-HI vs. 4-HI; Panel E: 7-HI vs. 4-HI; Panel F: 6-HI vs. 7-HI.

Figure 13. Overlay of dose response curves and comparison of EC_{50} values of 5-HI and analogs thereof having a different substituent at position 5. Panel A: 5-HI vs. 5-AI (5-aminoindole); Panel B: 5-HI vs. 5-MI (5-methoxyindole); Panel C: 5-AI vs. 5-MI.

Figure 14. 5-HI and analogs and their effect on rat colonic contractility. Panel A: 5-HI. Panel B: 6-HI (6-hydroxyindole); Panel C: 4-MI (4-methoxyindole); Panel D: 5-AI (5-aminoindole); Panel E: 4-HI (4-hydroxyindole); Panel F: 7-HI (7-hydroxyindole); Panel G: 5-EI (5-

ethoxyindole). All compounds were tested at 100 μM. Data were analyzed using the Wilcoxon matched-pairs (before/after) signed rank test (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$). Error bars represent SEM.

Figure 15. 5-HI and analogs and their effect on rat colonic contractility compared to the maximum KCl contractility (n = 6). Data were analyzed using the Wilcoxon matched-pairs (before/after) signed rank test (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM.

EXPERIMENTAL SECTION

Section A

Example 1 - Gut bacteria convert 5-HTP to 5-HI

5-HTP is absorbed throughout the entire GI tract (Jacobsen, *et al.*, *Neuropsychopharmacology* 2016, 41, 2324-2334; Gijsman, *et al.*, *J. Clin. Psychopharmacol.* 2002, 22, 183-189) when taken as a food supplement or as an antidepressant, where it is partially converted in the gut into serotonin before it reaches the brain (Bertrand *(*2010)). To determine whether gut bacteria have the ability to metabolize 5-HTP before it is taken up by the intestinal tissue, human fecal samples from healthy volunteers (n = 18) were incubated anaerobically *ex vivo* with 100 μM 5-HTP and analyzed by High-Performance Liquid Chromatography coupled with Electrochemical detection and UV detection (HPLC-ED/UV). Chromatograms in Figure 1A revealed the formation of an unknown peak, which was further identified by Liquid Chromatography-Mass Spectrometry (LC-MS) to be 5-HI (Figure 2A). Interestingly, there was a variation among the tested fecal samples in their ability to convert 5-HTP into 5-HI, ranging from samples

that either completely or partially metabolized 5-HTP to 5-HI (High and Intermediate Converters; n = 8, and 4; respectively), to samples where 5-HTP was not metabolized to 5-HI at all (Non-Converters; n = 6) (Figure 1A; upper panel). Quantification of 5-HI production within the High and Intermediate Converters showed approximately 50 % and 10 % of 5-HTP metabolized after 6 h, respectively. After 24 h, 5-HTP was fully metabolized in the High Converters and partially metabolized (about 30 %) in the Intermediate Converters (Figure 1A; lower panel). No other metabolites were detected in the 5-HTP treated samples, suggesting that the gut microbiota metabolize 5-HTP only for production of 5-HI. Notably, no basal levels of 5-HTP were detected by HPLC-ED/UV in the control samples (Figure 2B; 0 h time-point). Similarly, no indole was detected in the Non-Converters samples (Figure 2C).

To further support the possibility that 5-HTP is metabolized in the gut lumen before it is absorbed in the gut tissue and is converted into serotonin, five healthy volunteers were randomly selected out of the 18 subjects, who participated in the fecal samples' donation. Subjects were orally administered 5-HTP tablets (Swanson Health Products, Fargo, ND, USA) (50 mg 5-HTP per tablet) daily for 1 week, as recommended by the manufacturer. Fecal samples were collected for targeted metabolomic analysis on Day 0 (control, no 5-HTP ingested), Day 1 (first ingestion of 5-HTP tablet), Day 3, and Day 8 (one day after last 5-HTP tablet was ingested), respectively. Targeted metabolomic analysis revealed that the levels of 5-HI in the fecal samples gradually increased during the period of 5-HTP intake (Figure 1B). Interestingly, there was a similar variation among the tested subjects in their ability to convert 5-HTP into 5-HI as observed in the *ex vivo* screening (Figure 1A). These results indicate a cumulative increase in the levels of produced 5-HI upon daily administration of 5-HTP over a period of 1 week with inter-individual variation. Taken together, the results show that gut bacteria can metabolize

5-HTP with interindividual differences in their ability to produce 5-HI.

Example 2 - Bacterial tryptophanase responsible for converting 5-HTP

Since 5-HI is a structural analog of indole, which is produced by bacterial degradation of L-tryptophan via TnaA enzyme, it was hypothesized that 5-HTP is also a substrate for the TnaA enzyme (Snell (1975)) (Figure 3A). To verify that hypothesis, *E. coli* BW25113$^{\Delta tnaA}$ mutant was incubated with 5-HTP, and was compared to the wild type *E. coli* BW25113^{WT} strain. Only, *E. coli* BW25113$^{\Delta tnaA}$ mutant was tested to confirm 5-HTP as a substrate for TnaA, based on previous reports proving the same function of TnaA enzyme (degradation of L-tryptophan to indole) in other bacterial species (Sasaki-Imamura, *et al.*, *Appl. Environ. Microbiol.* 2010, 76, 4260-4268; Lee, *et al.*, *FEMS Microbiol. Rev.* 2010, 34, 426-444). Overnight incubation of *E. coli* BW25113$^{\Delta tnaA}$ and *E. coli* BW25113^{WT} bacterial cells with 5-HTP showed that the production of 5-HI was completely abolished in the mutant strain as analyzed by HPLC-ED/UV (Figure 3B), indicating that 5-HTP is degraded to 5-HI by TnaA enzyme. Similarly, the production of indole from tryptophan was eliminated in the mutant strain, further confirming the involvement of TnaA enzyme in both conversions (Figure 4A). To further characterize the substrate specificity and kinetic parameters of the TnaA enzyme, *tnaA* gene from *E. coli* BW25113 was expressed in *E. coli* BL21 (DE3) and then purified. As expected, Michaelis-Menten kinetics indicated TnaA had a lower affinity (K_M) and catalytic efficiency (k_{cat}/K_M) for 5-HTP than its natural substrate tryptophan (Figures 3C and 3D; Table 1), illustrated by the K_M and k_{cat}/K_M values for 5-HTP (3.7 ± 0.2 mM and 21.9 min^{-1} mM^{-1}, respectively) compared to those for tryptophan (0.19 ± 0.009 mM and 2823.7 min^{-1} mM^{-1}, respectively).

Table 1. Enzyme kinetics determined by non-linear Michaelis-Menten regression model for 5-HTP and non-linear substrate inhibition kinetic model for tryptophan.

Michaelis-Menten kinetic parameters	
5-hydroxytryptophan (pH 7.4; Tryptophanase*E.coli* BW25113)	**L-Tryptophan (pH 7.4; Tryptophanase*E.coli* BW25113)**
E [nM] = 200	E [nM] = 20
K_M [mM] = 3.7 ± 0.2	K_M [mM] = 0.19 ± 0.009
V_{max} [mM/min] = 0.016 ± 0.0003	V_{max} [mM/min] = 0.011 ± 0.0001
K_i [mM] = n.a.	K_i [mM] = 69.79 ± 7.4

Table 1. Continued.

k_{cat} [min^{-1}] = 80.9 ± 1.6	k_{cat} [min^{-1}] = 536.5 ± 6.3
k_{cat}/K_M [min^{-1} mM^{-1}] = 21.9	k_{cat}/K_M [min^{-1} mM^{-1}] = 2823.7
R^2 = 0.83	R^2 = 0.93

The '±' indicates the standard error of the mean.

However, incubation of fecal samples with 5-fold excess levels of tryptophan relative to 5-HTP did not prevent the conversion of 5-HTP to 5-HI (Figure 4B). Therefore, despite the differential substrate affinity, these findings and findings from 5-HTP incubation with the human fecal samples (Figures 2C and 4B) illustrate that high levels of tryptophan do not prevent the conversion of 5-HTP.

Several genera of the gut bacteria, including *Escherichia*, *Fusobacterium*, and *Bacteroides*, have been reported to contain gene encoding a TnaA enzyme (Lee (2010)). To search for other intestinal bacteria that harbor this enzyme, the protein sequence (WP_001295247.1) from *E. coli* BW25113 was used as a query to search the U.S. National Institutes of Health Human Microbiome Project (HMP) protein database. The proteins were considered homologous when the minimal identity percentage was above 30 % and query cover was above 90 %. This analysis identified several

genera containing TnaA; *Escherichia*, *Fusobacterium*, *Bacteroides*, *Aeromonas*, *Clostridium*, *Klebsiella*, *Providencia* and *Propionibacterium* (Figure 3E).

To confirm the *in silico* analysis, a comprehensive screening of gut-associated bacterial strains containing TnaA enzyme was performed. Among these bacteria, members of the normal gut bacteria, *Fusobacterium nucleatum* subsp. *animalis* and *Fusobacterium simiae* displayed a similar ability to fully metabolize 5-HTP into 5-HI after 24 h of anaerobic incubation (Figures 3F and 3G). Moreover, the gut isolate *E. coli* DSM 11250 demonstrates production of 5-HI (about 30 µM) after 20 min of incubation at 37°C, which was 6-fold higher than the laboratory strain *E. coli* BW25113 (about 5 µM). Altogether, these results show that TnaA is the enzyme involved in 5-HTP conversion to 5-HI, and this function is encoded by genomes of a variety of gut bacterial species that are abundantly present in both the small and large intestine (Jaglin, *et al.*, *Front. Neurosci.* 2018, 12, 216).

Example 3 - Bacterial production of 5-HI dependent on microbiota composition and pH

To determine the possible cause of variation in bacterial conversion of 5-HTP into 5-HI in humans (Figures 1A and 1B), the microbial 16S rRNA profiling of the human fecal samples were checked. Of the main differences in the microbial relative abundance among the three classes of Converters was the *tnaA*-encoding genera of gut microbiota, which decreased from 18.7 % in the High Converters, to 10.6 % in the Intermediate Converters, to 6.2 % in the Non-Converters (Figures 5A and 6). The presence of *tnaA*-encoding bacteria in Non-Converters suggested that the TnaA enzyme is present but is inactive, presumably due to different environmental conditions in the tested samples. Strikingly, the levels of *Bifidobacterium* genera increased with decreasing levels of *tnaA*-encoding

gut bacteria, from 1.1 % in the High Converters, to 9.7 % in the Intermediate Converters, to 36.5 % in the Non-Converters (Figures 5A and 6). These data suggest that the bacterial production of 5-HI from 5-HTP is dependent on the microbiota composition.

To confirm that the presence of high abundance of *Bifidobacterium* in the samples of Non-Converters resulted in inhibiting the conversion of 5-HTP by TnaA enzyme, *F. nucleatum* subsp. *animalis* was incubated, which fully converted 5-HTP to 5-HI (Figure 3F) anaerobically with either 50 % or 10 % of bifidobacterial suspension of *Bifidobacterium breve* and 5-HTP conversion to 5-HI was analyzed using HPLC-ED/UV. Remarkably, 5-HI production was abolished when *F. nucleatum* subsp. *animalis* was incubated with 100 µM 5-HTP in the presence but not in the absence of *B. breve* (Figures 5B-5D). It was expected that *Bifidobacterium breve* would change the pH of the growth media, which might affect the TnaA activity, which is strongly dependent on the pH conditions (Kim *et al.*, *Arch. Pharm. Res.* 1995, 18, 351-355). Indeed, the pH measurements revealed that *Bifidobacteria* present in the fusobacterial suspension decreases the pH of the solution from 7.3 before the start of the incubation to pH= 5.29, 5.34, and 7.14 after 24 h of incubation with 50 %; 10 % and 0 % *B. breve*, respectively (Figures 5B-5D). Taken together, the results indicate an inhibitory effect of *Bifidobacteria* on the microbial conversion of 5-HTP through the reduction of the pH, which affects the optimal growth of bacteria as well as the tryptophanase activity.

To further confirm that the acidic pH is responsible for abolishing the production of 5-HI, *Fusobacterium nucleatum* subsp. *animalis* were incubated anaerobically with 5-HTP and the pH of the growth media was adjusted to pH = 5.3. Samples were collected after 24 hrs and were analyzed using HPLC-ED/UV. Indeed, no production of 5-HI was seen further confirming that the TnaA activity is inhibited in low pH.

Example 4 - 5-HI accelerates GI motility in vivo

Being the bacterial metabolite of 5-HTP, that has been recently shown to accelerate the gut motility (Israelyan (2019)), 5-HI was tested for its ability to also affect gut motility *in vivo*. To this end, 30 mg/kg of 5-HI was orally administered to Wild-type Groningen (WTG) adult male rats (n = 10). First, baseline measurements of total gut transit time (TGTT) were performed in all rats. Baseline TGTT measurements showed a natural variation among the WTG rats, similar to what exists among humans. Subsequently, the rats were randomly appointed to either vehicle (10 % sucrose) group or 5-HI-treated group, and TGTT was measured in both groups. Remarkably, the 5-HI-treated group had significantly decreased TGTT compared to the baseline measurements before treatment (Figure 7A). Moreover, the defecation frequency was significantly increased in the 5-HI-treated group compared to the vehicle group (Figure 7B), without affecting food intake or changes in body weight (Figure 7C). Together, the *in vivo* data suggest that 5-HI stimulates the GI motility in rats.

Example 5 - 5-HI is a potent stimulator of the colonic gut contractility

Presumably, 5-HI can exert its stimulatory effect on gut motility observed *in vivo* through its stimulation of serotonin (5-HT) release from the EC cells or its action on enteric neurons, or smooth muscles underlying the wall of the gut. To test these hypotheses, the effect of 5-HI on 5-HT release in RIN14B cells, a model cell line of EC cells, was evaluated (Nozawa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 2009, 106, 3408-3413). When RIN14B cells were stimulated with 100 μM 5-HI (concentration of 5-HI employed was based on the 5-HI levels detected in the fecal samples from healthy subjects (Figure 1A)), induction of 5-HT release was significantly increased (330 ± 12 nM) compared to the control (Figure 8A).

In parallel, the ability of 5-HI to translocate through the intestinal tissue was observed (Figure 9A), as well as cross the first pass metabolism, which was indicated by its detection in urine samples of rats after administration of 30 mg/kg 5-HI (3.5 ± 1.3 μM). These data suggest that 5-HI can reach the GI motility control system and induce gastrointestinal contractions via a second mechanism besides its stimulation of 5-HT production. Thus, an *ex vivo* organ bath system was used (Jepersen *et al.*, *J. Vis. Exp.* 2015, 1-9), where dissected proximal colonic tissues with intact mucosa from untreated WTG rats were cut to approximately 3 mm rings and suspended in an organ bath, and a pressure transducer and data-acquisition software displayed the measurement of tension generated by the smooth muscle of intestinal walls as described before (Muller *et al.*, *Cell* 2014, 158, 300-313). Focus was placed on the colon since it was observed that the small intestinal transit lasted only about 15 min in average (data not shown), while the large intestinal transit ranged from 10 hrs to 24 hrs (Figure 9A).

When 100 μM 5-HI was applied to the colonic tissue to test the 5-HI effect on colonic contractility, a significant increase in the contractility was observed compared to the baseline (quantification of the data is further described under *Examples*; Figure 8B). Next, a dose-response curve of 5-HI was performed and EC_{50} was determined to be 37 μM (Figure 8C), confirming that 5-HI will produce its maximum effect at the selected dose (100 μM).

To further investigate whether 5-HI can augment the stimulatory effect of 5-HT on colonic contractility, 100 μM 5-HI was applied sequentially with 10 μM 5-HT to the colonic tissue in the organ bath system. 5-HT concentration was based on previous reports showing levels of 5-HT in the mucosa from variety of animal models and human specimens (Bertrand (2010)). Interestingly, when applied after 10 min of the 5-HT exposure (this timing is used in all the following organ bath experiments), the addition of

100 μM 5-HI elicited significantly higher amplitude in the tissue contractility compared to the 5-HT induced response (Figure 8D). Stimulation of the 5-HT_3 and 5-HT_4 receptors, the main 5-HT receptors involved in the gut motility, by 5-HT on IPANs causes ACh release, resulting in smooth muscle contractions (Sikander *et al.*, *Clin. Chim. Acta* 2009, 403, 47-55). To test whether the augmented amplitude enhancement of the maximum 5-HT response upon addition of 5-HI was due to the action of 5-HI on the 5-HT_3 or 5-HT_4 receptors, the 5-HT_3 antagonist ondansetron (1 μM) or the 5-HT_4 antagonist SB-207266 (1 μM) were added to the colonic tissue to completely abolish the maximum 5-HT response (Tuladhar *et al.*, *Br. J. Pharmacol.* 2000, 131, 1716-1722; Wardle *et al.*, *Br. J. Pharmacol.* 1996, 118, 665-670). Addition of either ondansetron or SB-207266 sequentially with 5-HT or 5-HI, respectively, resulted in a complete inhibition of 5-HT-, but not the 5-HI-induced response (Figures 8E, 8F, 9B, and 9C), suggesting that 5-HI does not work via 5-HT_3 nor 5-HT_4 receptors to stimulate the colonic contractions.

To further investigate whether 5-HI facilitates its action via receptors on either enteric nerves or smooth muscle, 1 μM tetrodotoxin (TTX) (Wegener *et al.*, *FASEB J.* 2006, 20, 1260-1262), a neurotoxin that prevents axonal transmission by blocking sodium ion transport (Gershon *et al.*, *Br. J. Pharmacol. Chemother.* 1967, 29, 259-279), or 1 μM hexamethonium, a nicotinic cholinergic antagonist often referred to as the prototypical ganglionic blocker, was added to the colonic tissue. Addition of both TTX and hexamethonium had a complete inhibitory effect on the response of 5-HT (Figures 9D and 9E), but did not alter the response of 5-HI (Figures 8G and 9F), indicating that the receptors by which 5-HI exerts its effect on gut contractility are located on the smooth muscle, as is the case for the ACh response (Gershon (1967)).

Next, the neurotransmitter ACh (50 μM) was applied to the tissue to induce a maximum increase in the intestinal smooth muscle tone (Tezuka

et al., *Exp. Gerontol.* 2004, 39, 91-100), which has been long recognized as a key component in gut motility (Lentle *et al.*, *Neurogastroenterol. Motil.* 2013, 25, 931-942). Intriguingly, addition of 100 μM 5-HI significantly intensified the amplitude of the ACh-induced contractility in the colonic tissue (Figure 8H). The ACh response is mainly exerted through muscarinic acetylcholine receptor subtypes 2 (M2R) and 3 (M3R) located on the smooth muscle cells in the GI tract (Hansen (2003)). To test whether 5-HI exhibits its action via either M2R or M3R, AQ-RA 741 (1 μM) or DAU 5884 hydrochloride (100 nM), selective antagonists of the M2R or M3R, respectively (Doods *et al.*, *Eur. J. Pharmacol.* 1991, 192, 147-152; Di Patrizio *et al.*, *Am. J. Physiol. -Regul. Integr. Comp. Physiol.* 2015, 309, R805-R813) were applied sequentially with 5-HI or ACh to the tissue. As expected, the ACh-induced response was inhibited by the addition of either the M2R or M3R antagonist (Figures 9G and 9H). In contrast, there was a significant increase in the 5-HI-induced response when M3R was inhibited (Figure 7I), while the addition of M2R antagonist did not inhibit the 5-HI-induced response (Figure 7J), suggesting that 5-HI exerts its excitatory effect on the colonic smooth muscles through other receptors.

Typically, the stimulation of muscarinic receptors by ACh causes the opening of a variety of cationic channels (*e.g.*, non-selective Ca^{2+} channels (NSCCs), specifically the interconnected transient receptor potential channel 4 (TRPC4) and 6 (TRPC6) (Tsvilovskyy *et al.*, *Gastroenterology* 2009, 137, 1415-1424) and L-type voltage-dependent Ca^{2+} channels (L-VDCCs) (Wegener (2006))) in the smooth muscle cells of the GI tract, thereby producing Ca^{2+} influx followed by muscle contraction. To test whether NSCCs or L-VDCCs are involved in the 5-HI-induced gut contractility, ACh was applied sequentially with 5-HI followed by the addition of selective antagonists of TRPC4 and TRPC6, ML 204 (1 μM) and SAR 7334 (1 μM), respectively (Tsvilovskyy (2009)). Only the ACh-induced response, but not the 5-HI-induced response, was inhibited (Figures 9I and

9J), respectively. Next, 5-HI was applied sequentially with 1 μM nifedipine (Tsai *et al.*, *J. Ethnopharmacol.* 2012, 142, 694-699), a non-selective antagonist of L-VDCCs. Remarkably, the 5-HI-induced response was completely abolished with the addition of nifedipine (Figure 8K), suggesting that 5-HI elicits its response via L-VDCCs. Moreover, nifedipine is thought to block the contractile apparatus of the smooth muscle (Zhang *et al.*, *Neurogastroenterol. Motil.* 2010, 22). Therefore, to additionally verify that 5-HI is acting via L-VDCCs, 100 μM of ATP, which causes contraction mainly by the release of Ca^{2+} from intracellular Ca^{2+} stores, was added to the colonic tissue to validate an independent contractile agent in the presence of nifedipine. Indeed, colonic contractility induced by ATP was only partially inhibited by nifedipine compared to when ATP was not present (Figure 9K).

Finally, to recover the spontaneous contractions of the colonic tissue after the nifedipine treatment (nifedipine was washed away from the tissue in the organ bath), only 5-HI but not ACh were able to restore the tissue to the baseline contractions compared (Figure 8L) strongly showing that 5-HI to be a potent stimulant of the colonic contractility. Collectively, these results show that 5-HI mediates its effect on gut contractility via two distinct mechanisms: 1) stimulation of the release of 5-HT from EC cells, and 2) induction of L-VDCCs on the smooth muscle cells of the GI tract, which in turn, increases intracellular Ca^{2+}.

By combining a suite of *in vitro* biochemical, culture-based, and organ bath assays, microbiota sequencing, *in vivo* animal experiments, and human intervention study, the potency of 5-HI was identified, a product of gut microbial conversion of the dietary supplement and antidepressant 5-HTP, to accelerate GI motility indirectly via stimulation of 5-HT release from EC cells, and directly via activation of L-type calcium channels located on the colonic smooth muscle cells.

Metabolites with similar chemical structures, such as indole and

indole derivatives, have large effects on host (Bansal *et al.*, *Proc. Natrl. Acad. Sci.* 2010, 107, 228-233; Chimerel *et al.*, *Cell Rep.* 2014, 9, 1202-1208; Medvedev *et al.*, *Biol. Targets Ther.* 2007, 1, 151-162; Biersack *et al.*, *Curr. Drug Targets* 2012, 13, 1705-1719), thus it is expected that 5-HI will also influence host physiology (Bae *et al.*, *Biol. Pharm. Bull.* 2010, 33, 550-555; Cadenas *et al.*, *Free Radic. Res. Commun.* 1989, 6, 11-17; Kumar *et al.*, *Cell Host Microbe* 2020, 28, 1-13). Of note, 5-HI is often wrongly associated with 5-hydroxyoxindole (Lee (2010); Lee *et al.*, *Trends Microbiol.* 2015, 23, 707-718), which is an oxindole with a hydroxyl group at position 5 of the indole ring, and is detected in blood, plasma, and the brain (Crumeyrolle-Arias *et al.*, *J. Neurosci. Res.* 2008, 86, 202-207).

The primary outcome is that a wide variety of small and large intestinal bacteria possessing tryptophanase (TnaA) activity, typically converting tryptophan to indole, can efficiently convert 5-HTP into 5-HI in the intestinal lumen, with variability in the conversion levels (Figures 1A, 3F, and 3G). The variation in the production of 5-HI in the human fecal samples was associated with high relative abundance of *Bifidobacteria* in the human fecal samples (Figure 5A), which is consistent with previous data showing that *Bifidobacterium spp.* isolated from healthy individuals inhibited the TnaA activity of *E. coli* or total human intestinal microbiota (Park *et al.*, *Arch. Pharm. Res.* 1998, 21, 149-159). Similarly, TnaA activity and indole levels are reported to be decreased in the infant fecal samples, which are dominated by *Bifidobacteria* compared to fecal samples from adults (Ishibashi *et al.*, *Malays. J. Nutr.* 1997, 3, 149-159). The inhibition effect of *Bifidobacteria* on TnaA is due to the ability of *Bifidobacteria* to decrease the pH of the bacterial or fecal suspensions (Figures 5B-5D), which is consistent with previous studies where TnaA activity is shown to be dependent on pH (Kim (1995)). Moreover, the stringent control of the TnaA enzyme expression likely plays a role in the variability in the conversion levels of 5-HTP among the tested bacterial strains. In *E. coli,* for example,

tnaA expression is strictly controlled via various regulatory networks, including catabolite repression and transcription attenuation mechanisms (Gong *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 2001, 98, 8997-9001; Botsford *et al.*, *J. Bacteriol.* 1971, 105, 303-312). Catabolite repression of TnaA have been shown to be differently affected by inclusion of various carbon sources in the growth medium and by aerobiosis (Botsford (1971)).

Deciphering the mechanism of 5-HI stimulatory effect on gut motility observed in WTG rats (Figure 7), the inventors surprisingly demonstrated that 5-HI significantly increases 5-HT release from ECs (Figure 8A). ECs are found on the mucosal surface and release 5-HT into the gut wall in response to mechanical stimuli or nutrients (Kim *et al.*, *J. Clin. Invest.* 2001, 108, 1051-1059; Lund *et al.*, *Mol. Metab.* 2018, 11, 70-83). The released 5-HT stimulate IPANs protruding to the mucosa, which results in various gastrointestinal reactions, such as peristaltic reflex (Hansen (2003)). The results that 5-HI stimulates 5-HT release from EC cells adds another piece of evidence that gut microbiota-derived metabolites affect 5-HT release and are involved in influencing the host gut motility (Yano (2015); Reigstad (2015); Lund (2018)).

The data shows that 5-HI is able to increase the stimulatory effect of 5-HT and this effect is not via stimulation of 5-HT_3 and 5-HT_4 receptors, or its action via neuronal action potentials (Figures 8E-8G and 9F). Kooyman *et al.*, have shown that 5-HI, when used as a chemical to mimic the aromatic moiety of 5-HT, could modulate 5-HT-induced desensitization of the 5-HT_3 receptor-mediated inward current in murine neuroblastoma cells (Kooyman *et al.*, *Br. J. Pharmacol.* 1994, 112, 541-546). The observed 5-HI effect occurred via a competitive or non-competitive interaction, at low (10 mM) or high (10-50 mM) concentrations, respectively. These concentrations are 100-500 times higher than the concentration employed in the present study (100 μM), which was based on the 5-HI levels detected in the fecal samples from healthy subjects (Figure 1A) and the EC_{50} value

(Figure 8C). The differences in concentrations of 5-HI used by Kooyman *et al.* and in the present study may explain the discrepancy in the results. Moreover, the biophysical properties of the 5-HT_3 receptors expressed in the murine neuroblastoma cells used in the study of Kooyman *et al.* could widely differ from those expressed in the rat colonic tissue (Lummis *et al.*, *J. Biol. Chem.* 2012, 287, 40239-40245). The findings which show that 5-HI does not have stimulatory effect on 5-HT_3 receptors, is further supported by the application of ondansetron, a selective antagonist of 5-HT_3 receptors, TTX and hexamethonium, which both blocks neuronal transmission (Figures 8E, 9D, and 9F).

The increased 5-HI induced smooth muscles contraction upon inactivation of the M3R (Figure 8I) is most likely because when activated, M3R, the main receptor mediating contractions in the smooth muscle (Uchiyama *et al.*, *J. Smooth Muscle Res.* 2004, 40, 237-247), leads to inactivation of VDCCs followed by continuous decrease in the number of VDCCs available for Ca^{2+} discharge (Unno (2005); Unno *et al.*, *J. Physiol.* 1995, 484, 567-581). Accordingly, less Ca^{2+} can enter via the L-VDCCs (Wegener (2006)). Through its direct activation of the L-VDCCs, 5-HI was able to restore the entry of Ca^{2+} (Figure 8K). This is also supported by 5-HT release experiment (Figure 8A), where EC cells are endowed with L-VDCCs and 5-HT release largely depends on Ca^{2+} influx into the cells (Racké *et al.*, *Behav. Brain Res.* 1995, 73, 83-87). L-VDCCs are also known as voltage-gated ion channels and are densely expressed in the smooth muscle cells of the GI tract (Wegener (2006); Chen *et al.*, *Physiol. Genomics* 2007, 31, 492-509). Specifically, a subtype of L-VDCCs, L-type $Ca_v1.2$ Ca^{2+} channel, was described as essential for precise functioning of gut contractility in intestinal smooth muscle (Wegener (2006)). Moreover, altered expression or function of L-VDCCs in the gut was shown to cause GI motility disorders, including constipation (Zhang (2010); Beyder *et al.*, *Therap. Adv. Gastroenterol* 2012, 5, 5-21; Liu *et al.*, *Gastroenterology* 2001,

120, 480-489). Indeed, L-VDCCs agonists may serve to restore intracellular Ca^{2+} dependent motility to the constipated colon by activating the Ca^{2+} channel (Liu (2001)). This claim is further strengthened by the results where the oral administration of 5-HI reduces TGTT in WTG rats (Figure 7A).

The inventors arrived at a model for the mechanisms by which 5-HI is expected to accelerate intestinal motility depicted in Figure 10.

The newfound understanding of the formation of 5-HI by a wide range of TnaA-harboring bacteria will guide how therapeutic communities of microorganisms can be designed in which the production of 5-HI can be genetically specified. However, despite the fact that 5-HI maybe present at micromolar concentration in the gut of individuals who administer 5-HTP, its levels can vary greatly among individuals (from 0.1 to 13.2 μM). Against this backdrop and similar to *Escherichia coli* heat-stable enterotoxin, from which the recently approved GI motility drug linaclotide was derived (McWilliams *et al.*, *Drugs* 2012, 72, 2167-2175), delivering 5-HI microbial metabolite as a targeted drug into the human gut to stimulate gut contractility may represent a significant advance towards precision treatment of diseases, where constipation is a risk factor.

Materials and methods to Examples 1-5 (Section A)

Study participants and fecal specimen collection

The study protocol was evaluated by the ethical committee of the University of Groningen Medical Center. Eighteen volunteers (8 male and 10 female) between the ages 20 and 40 were recruited. Participants were excluded if they had used antibiotics, diarrhea inhibitors, laxatives, proton pump inhibitors, or had any gastrointestinal discomfort within the last three weeks. The study coordinator met with each eligible participant to review the consent and study details. All subjects signed the confidentiality

and consent form.

A plastic collection container, relief container, and gloves were provided to each subject for fecal collection. Subjects collected the feces and delivered it immediately to the study coordinator who delivered it to the laboratory for processing. One part of the fecal specimen was homogenized in Liquid Amies medium containing 20 % glycerol (Table 2), snap-frozen and stored in -80 °C. The second part was only snap-frozen without any additives in liquid nitrogen and stored in -80 °C.

Table 2. Constituents of Liquid Amies with 20 % medium.

Component	**Concentration**
Sodium chloride	3.00 g/L
Potassium chloride	0.20 g/L
$CaCl_2$	0.10 g/L
$MgCl_2$	0.10 g/L
KH_2PO_4	0.20 g/L
Na_2HPO_4	1.10 g/L
Sodium thioglycolate	1.00 g/L
85 % glycerol	235.29 mL/L

Fecal specimen incubation

Fecal samples were suspended in an Enriched Beef Broth (EBB) based on SHIME medium (Auchtung *et al.*, *Microbiome* 2015, *3*(42)) (Table 3) (5 % w/v). Samples were incubated anaerobically (10 % H_2, 10 % CO_2, 80 % N_2) in a Don Whitley Scientific DG250 Workstation (LA Biosystems, Waalwijk, The Netherlands) at 37 °C overnight prior to the addition of 100 µM 5-hydroxy-L-tryptophan (5-HTP; H9772, Sigma). Subsequently, samples containing 5-HTP were further incubated anaerobically at 37 °C for 6 h and 24 h, prior to HPLC-UV analysis.

Table 3. Constituents of EBB medium.

Component	**Conc. (g/L)**	**Vitamin solution (1000x)**	**Conc. (g/L)**	**Trace elements (1000x)**	**Conc. (g/L)**
Glucose	2.000	D-biotin	0.0020	EDTA	1.000
NaCl	0.080	D-pantothenic acid	0.0100	$ZnSO_4.7H_2O$	0.178
K_2HPO_4	5.310	Ca^{2+} nicotinamide	0.0050	$MnSO_4.H_2O$	0.452
KH_2PO_4	2.650	Vitamin B12	0.0005	$FeSO_4.7H_2O$	0.100
$NaHCO_3$	0.400	Thiamine HCl	0.0040	$CoSO_4.7H_2O$	0.181
Beef extract	5.000	*p*-aminobenzoic acid	0.0050	$CuSO_4.5H_2O$	0.010
Yeast extract	3.000	Riboflavin	0.0050	H_3BO_3	0.010
Peptone	0.600	Folic acid	0.0020	$Na_2MoO_4.2H_2O$	0.010
$CaCl_2$	0.008	Pyridoxal-5-phosphate	0.0100	$NiSO_4.6H_2O$	0.111
$MgSO_4$	0.008	Vitamin K1	0.0005		
Cysteine	0.500				
Hemin	0.005				

HPLC-ED/UV analysis and sample preparation

A volume of 1 mL of ice-cold methanol was added to 0.25 mL bacterial cell suspensions and fecal samples suspensions and stored at -20 °C until further use. Cell and protein precipitates were removed by centrifugation at 20,000 × g for 10 min at 4 °C. Supernatant was transferred to a new tube and the methanol fraction was evaporated in a Savant speed-vacuum dryer (SPD131, Fisher Scientific, Landsmeer, The Netherlands) at 60 °C for 2 h. The aqueous fraction was reconstituted to 1 mL with MilliQ. Samples were filtered and injected into the HPLC system; For *E. coli* BW25113 *tnaA* mutant experiment and *Fusobacterium* strains screening experiment, HPLC with electrochemical detection was used (Jasco AS2059 plus autosampler, Jasco Benelux, Utrecht, The Netherlands; Knauer K-1001 pump, Separations, H. I. Ambacht, The Netherlands; Dionex ED40 electrochemical detector, Dionex, Sunnyvale, USA, with a glassy

carbon working electrode (DC amperometry at 1.0 V, with Ag/AgCl as reference electrode), for every other experiment HPLC with UV detection (280 nm) was used (Waters 2695 Alliance Separations module, Milford, MA, USA; UV6000LP Detector, Thermo Fisher Scientific, Waltham, MA, USA). Samples were analyzed on a C18 column (Kinetex 5 µm, C18 100 Å, 250 × 4.6 mm, Phenomenex, Utrecht, The Netherlands) using a gradient of water/methanol with 0.1 % formic acid (0-10 min, 95-80 % H_2O; 10-20 min, 80-5 % H_2O; 20-23 min 5 % H_2O; 23-31 min 95 % H_2O). Data recording and analysis were performed using Chromeleon software (version 6.8 SR13).

Oral 5-HTP administration (human study)

The study protocol was evaluated by the ethical committee of the University of Groningen Medical Center. Five out of the 18 tested subjects who participated in the fecal samples donation, were randomly selected and asked to orally administer 5-HTP tablets (Swanson Health Products, Fargo, ND, USA) (50 mg 5-HTP per tablet) daily for one week, as recommended by the manufacturer. The study coordinator met with each participant to review the consent and study details. All subjects signed the confidentiality and consent form. Volunteers collected fecal samples on Day 0 (control, no 5-HTP ingested), Day 1 (first ingestion of 5-HTP tablet), Day 3 and Day 8 (one day after last 5-HTP was ingested), respectively. A plastic collection container, relief container and 5-HTP tablets were provided to each subject for fecal collection on specified days. Subjects collected the feces and delivered it immediately to the study coordinator who delivered it to the laboratory for fecal samples extraction of metabolites. The rest of the sample was stored in -80 °C.

Fecal samples extraction of metabolites

After fecal specimen collection, part of the sample was weighed and MilliQ was added (1:5 w/v). Solution was homogenized thoroughly. Next, 250 µL of this solution was transferred to 1 mL of 100 % ice-cold methanol. Samples were stored at -20 °C until analysis by HPLC-UV.

DNA extraction and 16S rRNA gene profiling

DNA extraction was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research) according to manufacturer's instructions. Illumina 16S rRNA gene amplicon libraries were generated and sequenced at BaseClear (Leiden, The Netherlands). In short, barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. 10-25 ng genomic (g)DNA was used as template for the first PCR with a total volume of 50 µL using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers appended with Illumina adaptor sequences. PCR products were purified and the size of the PCR products were checked on Fragment analyzer (Advanced Analytical) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer (Advanced Analytical) and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. The raw sequencing data produced was processed removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FASTQC

quality control tool version 0.10.0. The Illumina paired reads were merged into single reads (so-called pseudoreads) through sequence overlap, after removal of the forward and reverse primers. Chimeric pseudoreads were removed and the remaining reads were aligned to the RDP 16S gene databases. Based on the alignment scores of the pseudoreads, the taxonomic depth of the lineage is based on the identity threshold of the rank; Species 99 %, Genus 97 %, Family 95 %, Order 90 %, Class 85 %, and Phylum 80 %.

Bacteria

Escherichia coli DH5a and BL21 were routinely grown aerobically in Luria-Broth (LB) at 37 °C with continuous agitation. Other strains listed in Table 4 were grown either aerobically at 37 °C with continuous agitation or anaerobically at 37 °C in an EBB unless otherwise noted. Bacteria were inoculated from -80 °C stocks and grown overnight. Before the experiment, cultures were diluted 1:100 in fresh medium from overnight cultures. 5-HTP was supplemented during the lag or stationary phase depending on the experiment. Growth was followed by measuring the optical density (OD) at 600 nm in a spectrophotometer (UV1600PC, VWR International, Leuven, Belgium).

Table 4. Bacterial strains.

Bacterial strains	**Genotype**	**Reference**
Escherichia coli DH5α	F–; endA1; glnV44; thi-1; recA; relA1; gyrA96; deoR; nupG; purB20; φ80dlacZΔM15; Δ(lacZYA-argF)U169; hsdR17(rκ–mκ+); λ–	[1]
Escherichia coli BL21 (DE3)	E. coli str. B; F– ; ompT; gal; dcm; lon; hsdSB(rB– mB–); λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) ; [malB+]K-12(λS)	[2]
Escherichia coli BW25113	lacI+;rrnBT14; ΔlacZWJ16; hsdR514 ΔaraBADAH33; ΔrhaBADLD78; rph-1;	[3]

	Δ(araB–D)567; Δ(rhaD– B)568; ΔlacZ4787(::rrnB-3)	
Escherichia coli BW25113 *ΔtnaA*	lacI+;rrnBT14; ΔlacZWJ16; hsdR514 ΔaraBADAH33; ΔrhaBADLD78; rph-1; Δ(araB–D)567; Δ(rhaD– B)568; ΔlacZ4787(::rrnB-3); Δ*tnaA*	Molecular Systems Biology Group, University of Groningen
Escherichia coli DSM 11250		DSMZ

1: Grant *et al.*, *Proc. Natl. Acad. Sci. U.S.*A. 1990, 87, 4645-4649

2: Wood *et al.*, *J. Mol. Biol.* 1966, 16, 118-133

3: Datsenko *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 2000, 97, 6640-6645

Table 4. Continued.

Fusobacterium nucleatum susp. *animalis* DSM 19679		DSMZ
Fusobacterium simiae (gut isolate)		Department of Medical Microbiology, University of Groningen
Bifidobacterium breve DSM 20213		Stratingh Institute for Chemistry, University of Groningen

Fusobacterium nucleatum subsp. *animalis* DSM 19679 and *Bifidobacterium breve* DSM 20213 was grown anaerobically at 37 °C in an EBB. *Fusobacterium nucleatum* subsp. *animalis* and *B. breve* was first restreaked on Fastidious Anaerobe Agar supplemented with 5 % sheep blood or on MRS agar, respectively, and grown overnight before inoculation to liquid EBB culture. Before the experiment, 10 mL of *B. breve* was centrifuged and cells were washed in fresh 10 mL EBB. From the 100 % *B. breve* suspension, 50 % and 10 % of *B. breve* suspension was prepared. To

each of the suspensions, 1% of the *F. nucleatum* subsp. *animalis* culture was inoculated. Also, *F. nucleatum* subsp. *animalis* was inoculated 1:100 to fresh medium, which served as a control. To each of the suspensions, 100 µM 5-HTP or sterile MilliQ was supplemented. pH of the cultures was measured and 0.25 mL of each culture was taken for HPLC-UV analysis at t = 0 h and t = 24h.

Cloning and heterologous gene expression

Tryptophanase from *E. coli* BW25113 ($TnaA_{E.coli\ BW25113}$, accession: CP009273) was amplified using Phusion High-Fidelity DNA polymerase and primers listed in Table 5. Amplified *tnaA* gene was cloned in pET15b, resulting in pBW002 (Table 6). The plasmid was maintained in *E. coli* DH5α, verified by Sanger sequencing and transformed to *E. coli* BL21 (DE3). Overnight culture was diluted to OD_{600} = about 0.8.

Table 5. Primers.

Primers	**5'-Sequence-'3**	**Target**
bw156	CGCCTCGAGATGGAAAACTTTAAACATCTCCCTGAAC	*E. coli* BW25113 $TnaA_{E.coli\ BW25113}$
bw157	CGCGGATCCTTAAACTTCTTTAAGTTTTGCGGTGAAG	*E. coli* BW25113 $TnaA_{E.coli\ BW25113}$

Table 6. Plasmids.

Plasmids used	**Gene-insert**	**Reference**
pET15b		Novagen
pBW002	pET15b-$TnaA_{E.coli\ BW25113}$	

Protein translation was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, 11411446001, Roche Diagnostics) and culture was incubated overnight at 18 °C. The cells were directly used for protein isolation. Cell pellets were resuspended in 1/25th of buffer A (300 mM NaCl; 10 mM imidazole; 50 mM NaH_2PO_4, pH = 7.4) containing 0.2 mg/mL lysozyme (105281, Merck) and 2 µg/mL DNase (11284932001, Roche Diagnostics), and incubated for at least 10 min on ice before sonication (10 cycles of 15 s/on with 30 s cooling at 7 microns amplitude) using Soniprep-150 plus (Beun de Ronde, Abcoude, The Netherlands). Cell debris were removed by centrifugation at 20,000 x g for 20 min at 4 °C. The 6 x his-tagged protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) agarose matrix (30250, Qiagen). Cell free extracts were loaded on 0.5 mL Ni-NTA matrixes and incubated on a roller shaker for 2 h at 4 °C. The Ni-NTA matrix was washed three times with 1.5 mL buffer B (300 mM NaCl; 20 mM imidazole; 50 mM NaH_2PO_4, pH = 7.4) before elution with buffer C (300 mM NaCl; 250 mM imidazole; 50mM NaH_2PO_4, pH = 7.4). Imidazole was removed from purified protein fraction using Amicon Ultra centrifugal filters (UFC505024, Merck) and washed three times and reconstituted in buffer D (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH = 7.4). Protein concentration was measured spectrophotometrically (Nanodrop 2000, Isogen, De Meern, The Netherlands) using predicted extinction coefficient and molecular weight from Protein Molecular Weight tool (http://www.bioinformatics.org/sms/prot_mw.html).

Enzyme kinetics

L-Tryptophan and 5-HTP degradation by purified tryptophanase from *E. coli* BW25113 was tested by measuring indole or 5-HI formation, respectively, as previously described (Sasaki-Imamura (2010)). Reactions were performed in biological triplicates using L-tryptophan substrate ranges from 0 to 14.58 mM and 5-HTP substrate ranges from 0 to 12 mM. Enzyme

kinetics were performed in 50 mM potassium phosphate buffer containing 0.1 mM PLP (pyridoxal-5-phosphate, P9255, Sigma) and 20 or 200 nM enzyme at pH = 7.4. The reaction mixture was prewarmed for 5 min at 37 °C and was initiated by adding an enzyme solution and was terminated afterwards by the addition of 100 µL of 1 M HCl, following the addition of 100 µL of Kovac's reagent for indoles (60983, Sigma, The Netherlands). The supernatant was examined spectrophotometrically at 540 nm. The amounts of indole and 5-HI formed in each reaction were calculated from a standard curve. Michaelis-Menten kinetic curves were fitted using GraphPad Prism 7.

Bioinformatics

Tryptophanase amino acid sequence from *E. coli* BW25113 (NCBI accession: WP_001295247.1) was locally BLASTed against the protein sequences from the NIH HMP project (Bioproject ID 43021). All BLASTp hits were validated and only gut bacterial strains protein sequences were selected. The protein sequences were aligned in MEGA-X (Kumar *et al.*, *Mol. Biol. Evol.* 2018, 35, 1547-1549) using the Muscle algorithm with default settings. To determine which model to use for the tree estimation, model prediction was executed and the maximum-likelihood tree was constructed without Bootstrap and with partial deletion. The tree was exported in Newick format and colored using iTOL (Letunic *et al.*, *Nucleic Acids Res.* 2019, 47, W256-W259).

Rats and total gut transit time measurements

All animal procedures were approved by the Groningen University Committee of Animal experiments (approval number: AVD1050020197786), and were performed in adherence to the NIH Guide for the Care and Use of Laboratory Animals.

Sixteen male adult wild-type Groningen (WTG) rats (Groningen breed, male, age: 19-23 weeks) housed 3-5 animals/cage had *ad libitum*

access to water and food (Altromin 1414 mod. - NL_141005) in a temperature (21 ± 1 °C) and humidity-controlled room (45-60 % relative humidity), with a 12 h light/dark cycle (lights off at 1:00 p.m.). These outbred rats are very frequently used in behavioral studies due to the high inter-individual variation (Koolhaas *et al.*, *J. Vis. Exp.* 2013, 1-7), thus resembling, to some extent, the human inter-individual variation. On three occasions over a period of one week, rats were taken from their social housing cage in the beginning of the dark-phase cycle, and put in an individual training cage (L x W x H = 25 x 25 x 40 cm) containing wire-mesh, without bedding, food or water. Ten minutes after transfer to these cages, rats were given a drinking pipette with a 2.5 mL sucrose solution (10 % w/v). On two additional training occasions, rats were placed in metabolic cages, where carmine red test was conducted, and were trained as described above. Over the course of training, all rats were trained to drink the sucrose solution avidly. On the 8th occasion, the 10 % sucrose solution was used as a vehicle for the 1.2 % carmine red (C1022, Sigma) and 0.5 % methylcellulose (M0512, Sigma) mixture. Rats were given 1.2 mL of the carmine red solution to measure total gut transit time before and after the treatment of 5-HI, for both groups, 5-HI-treated group as well as vehicle group. 5-HI treated group were given daily 30 mg/kg of 5-HI (H31859, Sigma) in drinking pipette with a 2.5 mL sucrose solution (10 % w/v) for a period of 11 days. 10 % Sucrose solvent was used only for *in vivo* experiment as described before (Van Kessel (2019)). 30 mg/kg of 5-HI was chosen based on previous report (Mannaioni *et al.*, *Br. J. Pharmacol.* 2003, 138, 245-253). Fecal pellets were monitored every 30 min for the presence of carmine red in the fecal pellet. Total time taken for the appearance of the first red pellet was recorded as the total gut transit time.

5-HT release experiment

RIN14B cells (ATCC; (cat number: CRL-2059 LOT: 64037693) were seeded in 12-well plates at the rate of 2 x 10^5 cells/1 mL in RPMI1640 containing 10 % FBS/well and cultured for 72 h. The medium was removed before washing the cells with HBSS ($+Ca^{2+}$, $+Mg^{2+}$) containing 0.1 % BSA and 2 µM fluoxetine (F132, Sigma) (HBSS-S). The HBSS-S was again removed and replaced with 0.25 mL HBSS-S containing 100 µM 5-HI or only 0.25 mL HBSS-S, after which the solution was incubated further for 30 min at 37 °C. The supernatants were collected and stored at -20 °C until 5-HT measurement using an enzyme immunoassay (EIA) kit (Abnova).

Organ-bath experiments / rat colonic tissue ex vivo assay

Wild-type Groningen (WTG) male rats were sacrificed and a proximal colon was immediately removed and washed in 1X PBS and placed in 0.7 % NaCl solution. Approximately 3 mm rings were cut and were placed in an organ bath (Tissue Bath Station with SSL63L force transducer, Biopac Systems Inc. Varna, Bulgaria) filled with Krebs-Henseleit solution (NaCl, 7.02 g/L; KCl, 0.44 g/L; $CaCl_2.2H_2O$, 0.37 g/L; $MgCl_2.6H_2O$, 0.25 g/L; $NaH_2PO_4.H_2O$ 0.17 g/L; Glucose, 2.06 g/L; $NaHCO_3$, 2.12 g/L) gassed with Carbogen gas mixture (5 % CO_2, balanced with O_2) at 37 °C. At the beginning of the experiment, tension of the intestine of 0.5-1 g was obtained by adjusting the stretcher. Under these conditions, colonic rings were equilibrated for at least 45-60 min with replacement of Krebs-Henseleit solution approximately every 15 min. Sequentially, 10 µM serotonin (5-HT) (H9523, Sigma) or 50 µM acetylcholine (ACh) (A2661, Sigma) was added to induce a specific contractile response, and it was followed by addition of 100 µM 5-HI (H31859, Sigma). All the studied compounds added to the intestinal tissue in the organ bath were dissolved in MilliQ unless otherwise noted. To perform the tests with antagonists for the receptors involved in the gut motility, either 1 µM ondansetron (5-HT_3 antagonist; O3639,

Sigma); 1 μM SB-207266 (5-HT_4 antagonist; SML1349, Sigma); 1 μM tetrodotoxin (sodium ion transport blocker on the enteric neurons; TTX; 1078/1, Tocris Bioscience); 1 μM hexamethonium bromide (a nicotinic cholinergic antagonist; 4111, Tocris Bioscience); 1 μM AQ-RA 741 (muscarinic receptor 2 antagonist (M2R); 2292, Tocris Bioscience); 100 nM DAU 5884 hydrochloride (M3R antagonist; 2096; Tocris Bioscience); 1 μM ML204 (transient receptor potential channel 4 (TRPC4) antagonist; 4732, Tocris Bioscience); 1 μM SAR 7334 (TRPC6 antagonist; 5831; Tocris Bioscience); 100 μM ATP (Duchema); or 1 μM nifedipine (L-type Ca^{2+} channels antagonist; 1075; Tocris Bioscience) was applied sequentially with either ACh, 5-HT or 5-HI. As control, 0.1 % DMSO (solvent of AQ-RA 741, ML 204 and nifedipine) or 20 mM citrate buffer (solvent of TTX) was applied prior to addition of mentioned compounds to the tissue to check for any change in contractions. Each treatment lasted for about 10 min. Data was recorded BioPac Student Lab 4.1 (Build: Feb 12, 2015). Quantitative analysis of the organ bath recordings was performed in BioPac Student Lab 4.1, where each 10 min recording segment was selected and FFT analysis was done with following settings: data were padded with zeros, mean was removed, and magnitude was displayed with linear transform, signal was processed using Hamming window. Afterwards, the maximum amplitudes of the dominant frequencies obtained from FFT analysis were selected and analyzed in GraphPad Prism 7. The illustrative contractions (5 min) segments data were extracted from BioPac Student Lab 4.1 and analyzed in GraphPad Prism 7.

Statistical analysis and (non-)linear regression models

All statistical tests and (non-)linear regression models were performed using GraphPad Prism 7. For enzyme kinetics with 5-HTP as substrate, the non-linear Michaelis-Menten regression model was used. For enzyme kinetics with tryptophan as substrate, the non-linear substrate

inhibition kinetic model was used. For pairwise and two independent group comparison unpaired *T*-test was used, while for multiple group comparison one-way-ANOVA or RM 2-way ANOVA was performed followed by a Fisher's LSD test. For *ex vivo* organ-bath measurements, the Wilcoxon matched-pairs (before/after) signed rank test was used. Data are presented as mean ± SEM and $p < 0.05$ was considered statistically significant. The (n) refers to the number of individuals, rats or rats' tissues used for each experiment. The number of rats for *in vivo* experiment was based on previous study (Van der Wulp *et al.*, *J. Pediatr. Gastroenterol. Nutr.* 2012, 55, 457-462) to achieve power of 80 % with α of 0.05. Specific test, significance and (n) number are indicated in the figure legends.

Section B

Example 6 - Analogs of 5-hydroxyindole

Fifteen analogs of 5-HI, as well as 5-HI, were screened for their effect on rat colonic contractility. These compounds (100 μM) were applied to rat colonic tissue in an *ex vivo* model (see *Organ-bath experiments* in *Materials and methods to Examples 1-5 (Section A)* for the method). From the screened compounds, 5-HI (Sigma Aldrich, H31859), 6-hydroxyindole (6-HI; Fluorochem, 023442), 4-hydroxyindole (4-HI; Sigma Aldrich, 219878), 5-methoxyindole (5-MI; TCI, M0731), 7-hydroxyindole (7-HI; Sigma Aldrich, CDS005198), and 5-aminoindole (5-AI; Sigma Aldrich, 307203) were able to enhance the rat colonic contractility. Indole (Sigma Aldrich, W259306), indole-3-carboxaldehyde (Sigma Aldrich, 129445), (1*H*-indol-3-yl)methanamine (Fluorochem, 078831), and 5-hydroxyindole-3-acetic acid (5-HIAA; Sigma Aldrich, H8876) showed inhibitory effect on the colonic contractility when compared to the baseline. Indole-3-acetic acid (Sigma Aldrich, I3750), 5-hydroxyoxindole (Sigma

Aldrich, CDS004194), 5-hydroxy-2-carboxylic acid (Sigma Aldrich, 418608), indole-2-carboxylic acid (TCI, I0332), and 1-methylindole (TCI, M0561) did not show any effect.

It was established whether the substituent hydroxyl group of 5-HI and/or the position of the hydroxyl would be important for enhancement of the stimulating effect on rat colonic contractility. Thereto, the molecules were grouped with different positions of the hydroxyl (4-HI, 5-HI, 6-HI, and 7-HI) or with different substituents at the 5-position (5-MI and 5-AI).

The six compounds were tested *ex vivo* on rat intestinal tissue (*Organ-bath experiments; see above)* and the amplitude, and frequency of contractions were measured and compared to control (no treatment). The half maximal effective concentration (EC_{50} values) were obtained and ranged from 33.5 μM for 5-MI, 41.4 μM for 5-HI, 57.5 μM for 4-HI, 116.4 μM for 6-HI, 158 μM for 7-HI, to 382 μM for 5-AI (Figure 11). The dose response curves provided an opportunity for the comparison of individual EC_{50} values and thus compare each molecule's effect on colonic contractility.

EC_{50} values of the molecules with different positions of the hydroxyl were compared (Figure 12) and EC_{50} values of molecules with different substituents at the 5-position were compared (Figure 13). Interestingly, there is no significant difference observed between the EC_{50} values of 4-HI, 5-HI, and 6-HI (Figure 12). However, the EC_{50} value of 5-AI is significantly increased compared to the EC_{50} values of 5-HI and 5-MI (Figure 13). These results show that when oxygen (*e.g.*, in 5-HI and 5-MI) is replaced with nitrogen (*e.g.*, in 5-AI), the EC_{50} value increases significantly, suggesting that the oxygen in the hydroxyl group is more important than the position of the hydroxyl group.

Example 7 - 5-HI and 5-HI analogs stimulate colonic contractility

The effects of 5-HI, 6-HI, 4-HI, 5-MI, 7-HI, 5-EI, and 5-AI were separately tested on rat colonic tissue *ex vivo* at an average maximal effective concentration of 100 μM for 15 min. Compared to the appropriate baseline, all analogs enhanced the rat colonic contractility, but to a different extent (Figure 14).

Potassium chloride (KCl) is the natural contracting agent within the gut. Thus, it was considered that compounds that cause a stimulatory effect within the range of the maximal KCl effect are safe to be applied *in vivo*. 100 μM of 5-HI, 6-HI, 4-HI, 5-MI, or 5-AI was applied subsequently with KCl (30 mM).

The results in Figure 15 show that 5-HI, 5-MI, and 5-AI have an activity similar to that of KCl. On the other hand, 6-HI and 4-HI exceeded the maximum KCl response with over 50 % and 150 %, respectively.

Claims

1. A method for the treatment of a condition associated with reduced gut motility, comprising administering to a subject in need thereof a pharmaceutically effective amount of 5-hydroxyindole (5-HI) or an analog thereof having the capability to increase gut motility.

2. The method according to claim 1, wherein the 5-HI or analog thereof is administered orally.

3. The method according to claim 1 or 2, comprising administering 5-HI.

4. The method according to claim 3, wherein the condition is constipation, preferably constipation associated with colorectal cancer or Parkinson's disease.

5. The method according to any one of claims 1-4, wherein 5-HI or analog thereof is administered in a dosage range of 0.1-100 mg/kg body weight, preferably 1-50 mg/kg body weight, more preferably 20-30 mg/kg body weight.

6. The method according to any one of claims 1-5, wherein the analog is a compound of formula (I),

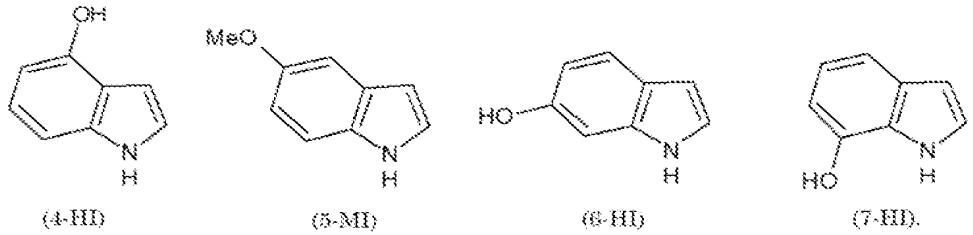
(I),

wherein

R^1 is hydrogen and is at the 4-, 6- or 7-position; or

R^1 is ethyl or methyl and is at the 4-, 5-, 6-, or 7-position.

7. The method according to any one of claims 1-6, wherein the analog is selected from



(4-HI) (5-MI) (6-HI) (7-HI).

8. 5-Hydroxyindole (5-HI) or an analog thereof for use in the treatment of a condition associated with reduced gut motility, wherein a pharmaceutically effective amount of 5-HI or analog thereof having the capability to increase gut motility is administered to a subject in need thereof.

9. 5-HI or analog thereof for use according to claim 8, wherein the 5-HI or analog thereof is administered orally, preferably in a dosage range of 0.1-100 mg/kg body weight, preferably 1-50 mg/kg body weight, more preferably 20-30 mg/kg body weight.

10. 5-HI or analog thereof for use according to claim 8 or 9, wherein the condition is constipation, preferably associated with colorectal cancer or Parkinson's disease.

11. 5-HI or analog thereof for use according to any one of claims 8-10, wherein the 5-HI or analog thereof stimulates release of 5-hydroxytryptamine (serotonin) from enterochromaffin cells.

12. 5-HI or analog thereof for use according to any one of claims 8-11, wherein the 5-HI or analog thereof activates L-type voltage-dependent Ca^{2+} channels on colonic smooth muscle cells.

13. 5-HI for use according to any one of claims 8-12.

14. 5-HI analog for use according to any one of claims 8-12, wherein the analog is a compound of formula (I),

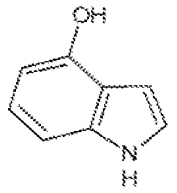

(I),

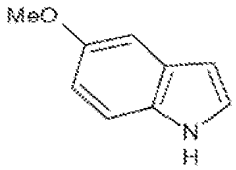
wherein

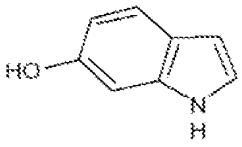
R^1 is hydrogen and is at the 4-, 6-, or 7-position; or

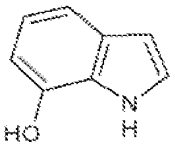
R^1 is methyl and is at the 4-, 5-, 6-, or 7-position.

15. 5-HI analog for use according to claim 14, selected from


(4-HI)

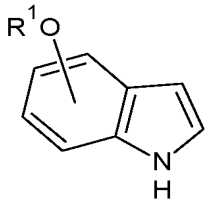

(5-MI)


(6-HI)


(7-HI).

16. 5-Hydroxyindole (5-HI) or an analog thereof, preferably as defined in claim 14 or 15, for use as an activator of gut motility or as a contractile agent.

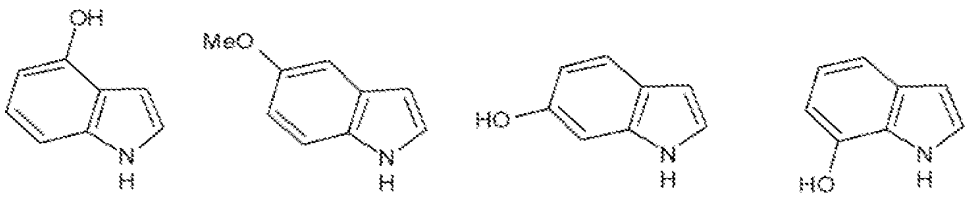
17. 5-Hydroxyindole (5-HI) analog for use as a medicament, wherein the analog is a compound of formula (I),


(I),

wherein

R^1 is hydrogen and is at the 4-, 6-, or 7-position; or
R^1 is methyl and is at the 4-, 5-, 6-, or 7-position.

18. 5-HI analog for use according to claim 17, selected from



(4-HI) (5-MI) (6-HI) (7-HI).

19. A pharmaceutical composition, preferably formulated for oral administration, comprising a 5-hydroxyindole (5-HI) analog as defined in claim 17 or 18, and a pharmaceutically acceptable vehicle, diluent or carrier.

20. A method for identifying an activator of gut motility, comprising:
i) contacting *in vitro* a 5-HI analog to be tested for activating gut motility with colonic tissue, and
ii) determining whether the 5-HI analog induces smooth muscle cell contractility.

21. A method for providing a compound having the capability to increase gut motility, comprising:
i) providing a library of 5-HI analogs, and
ii) testing members of the library for their *in vitro* capacity to activate L-type voltage-dependent Ca^{2+} channels.

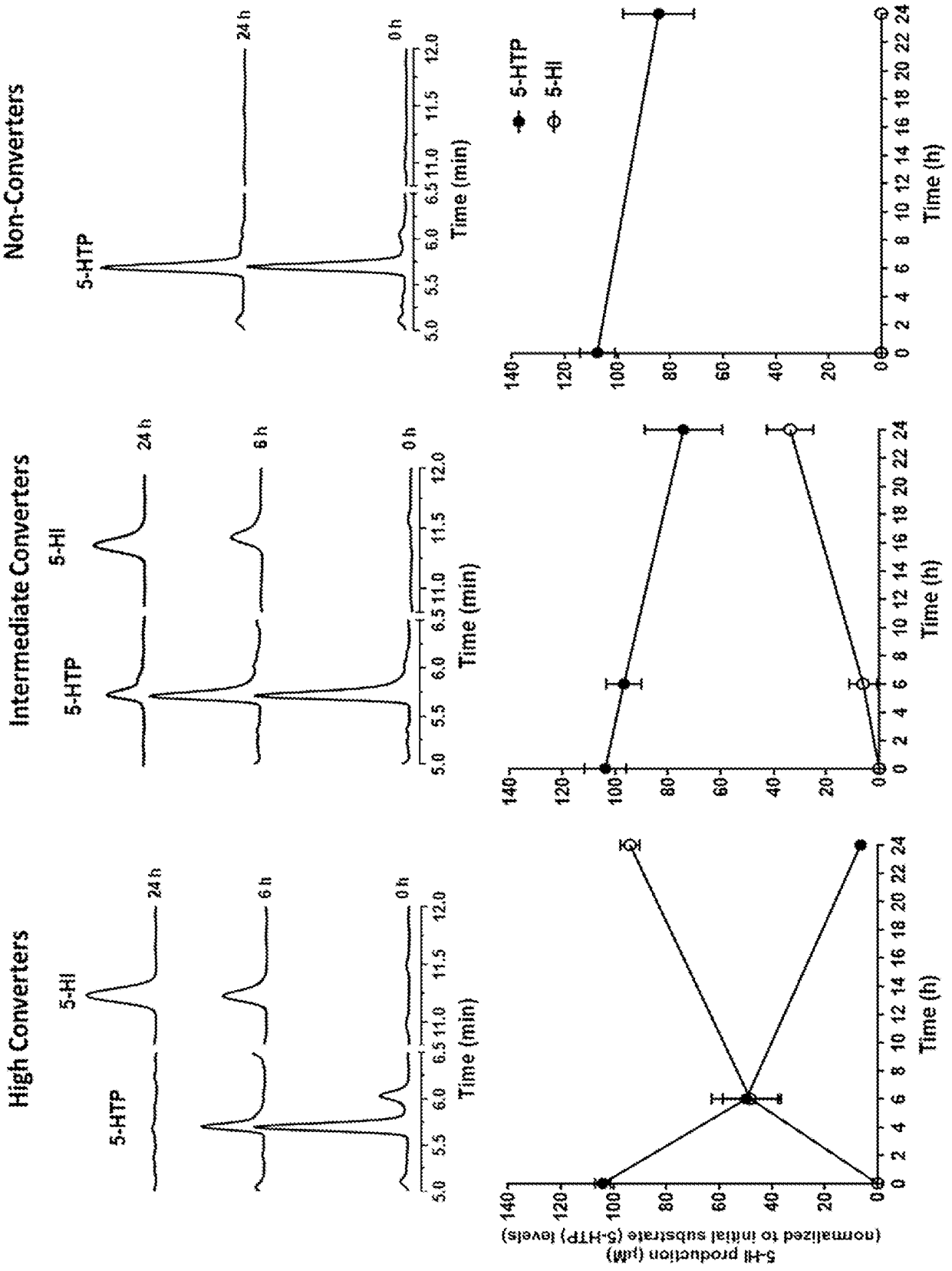

High Converters
Intermediate Converters
Non-Converters
5-HTP
5-HI
24 h
6 h
0 h
Time (min)
Time (h)
5-HI production (µM)
(normalized to initial substrate (5-HTP) levels)

Fig. 1A

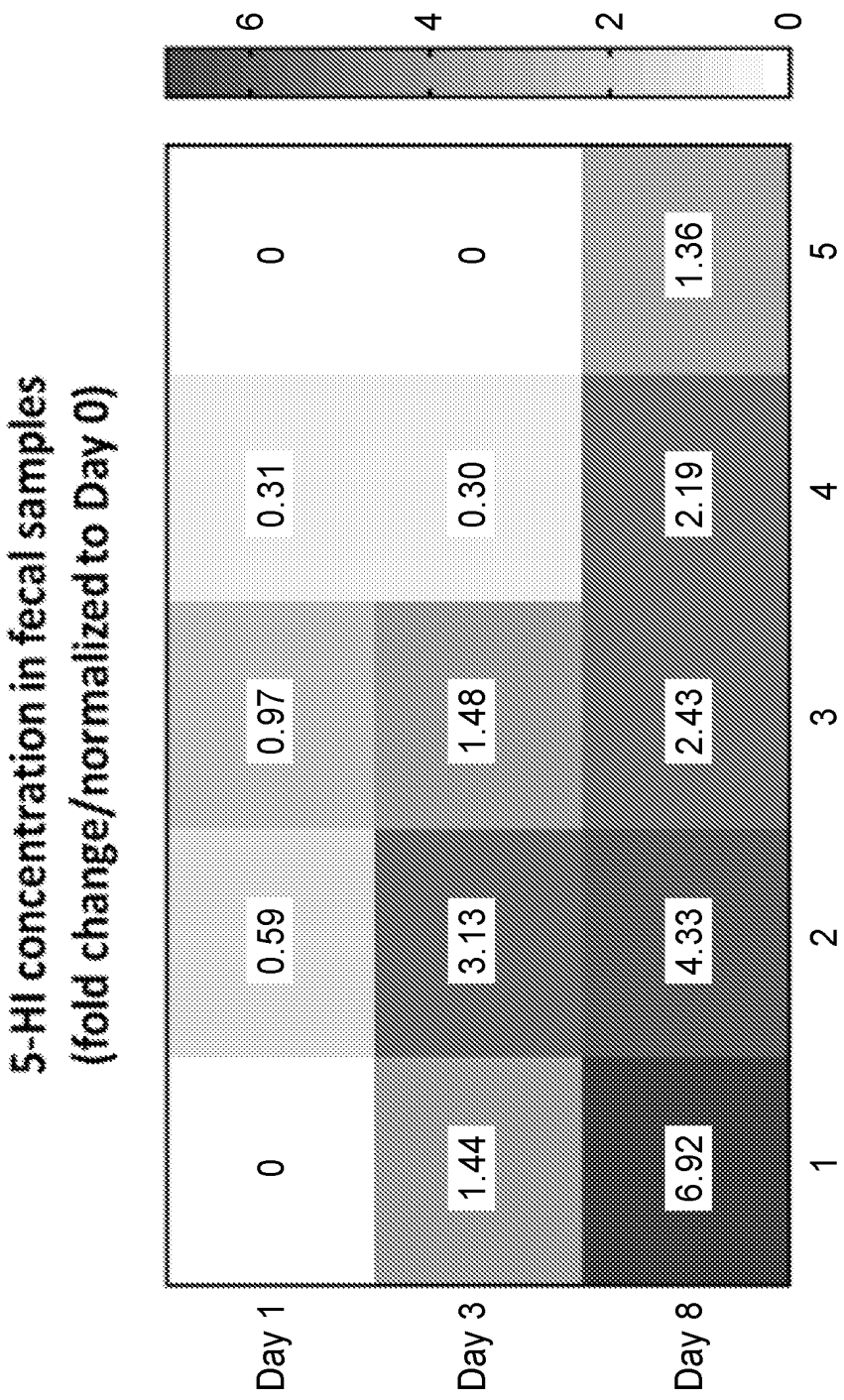

5-HI concentration in fecal samples
(fold change/normalized to Day 0)
6
4
2
0
Day 1
Day 3
Day 8
1
2
3
4
5
0
0.59
0.97
0.31
0
1.44
3.13
1.48
0.30
0
6.92
4.33
2.43
2.19
1.36

Fig. 1B

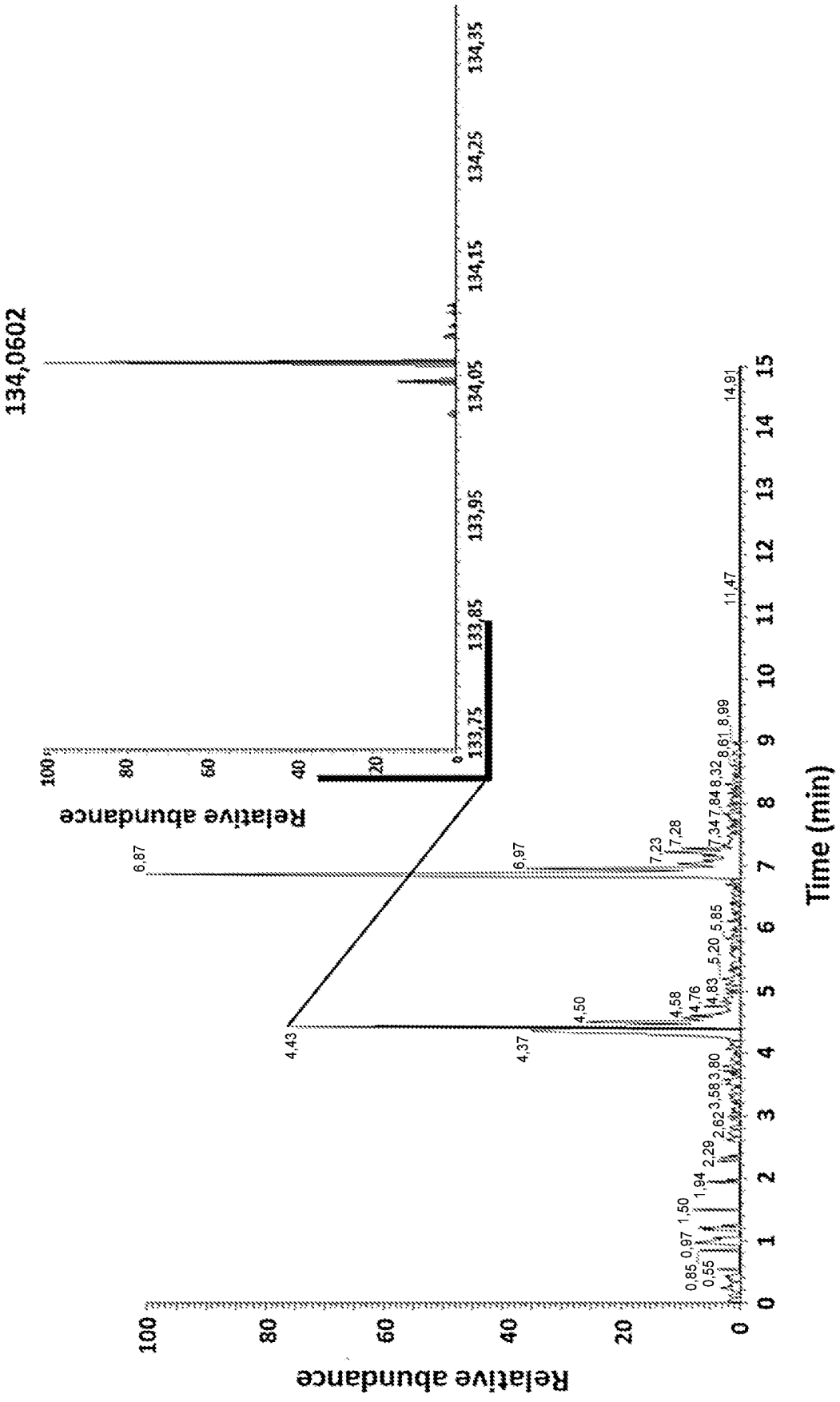


Fig. 2A

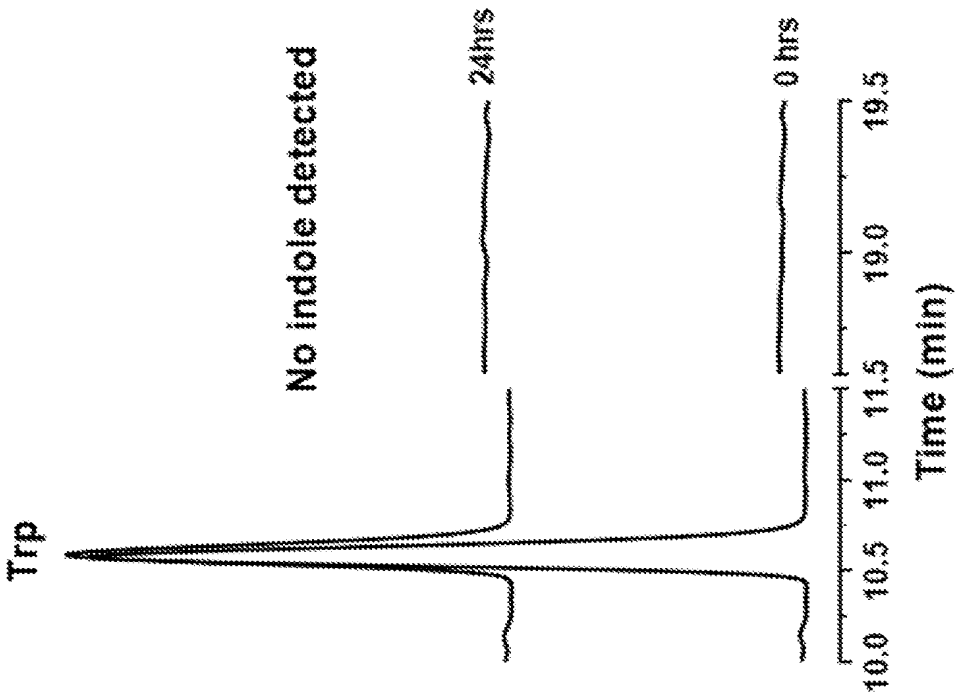

Trp
No indole detected
24hrs
0 hrs
10,0
10.5
11.0
11.5
19.0
19.5
Time (min)

Fig. 2C

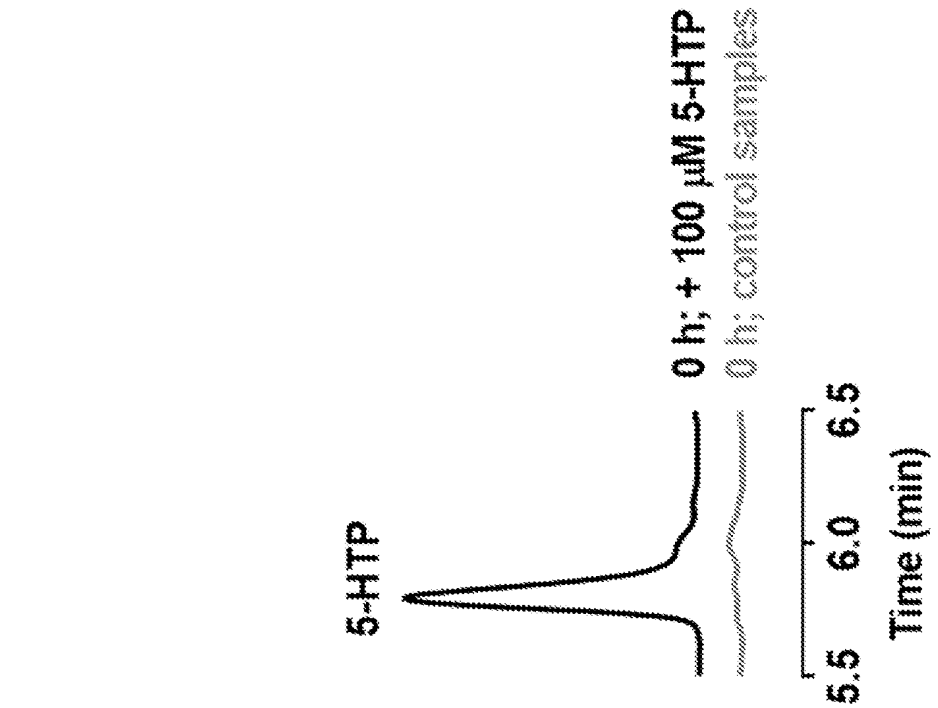

5-HTP
0 h; + 100 µM 5-HTP
0 h; control samples
5.5
6.0
6.5
Time (min)

Fig. 2B

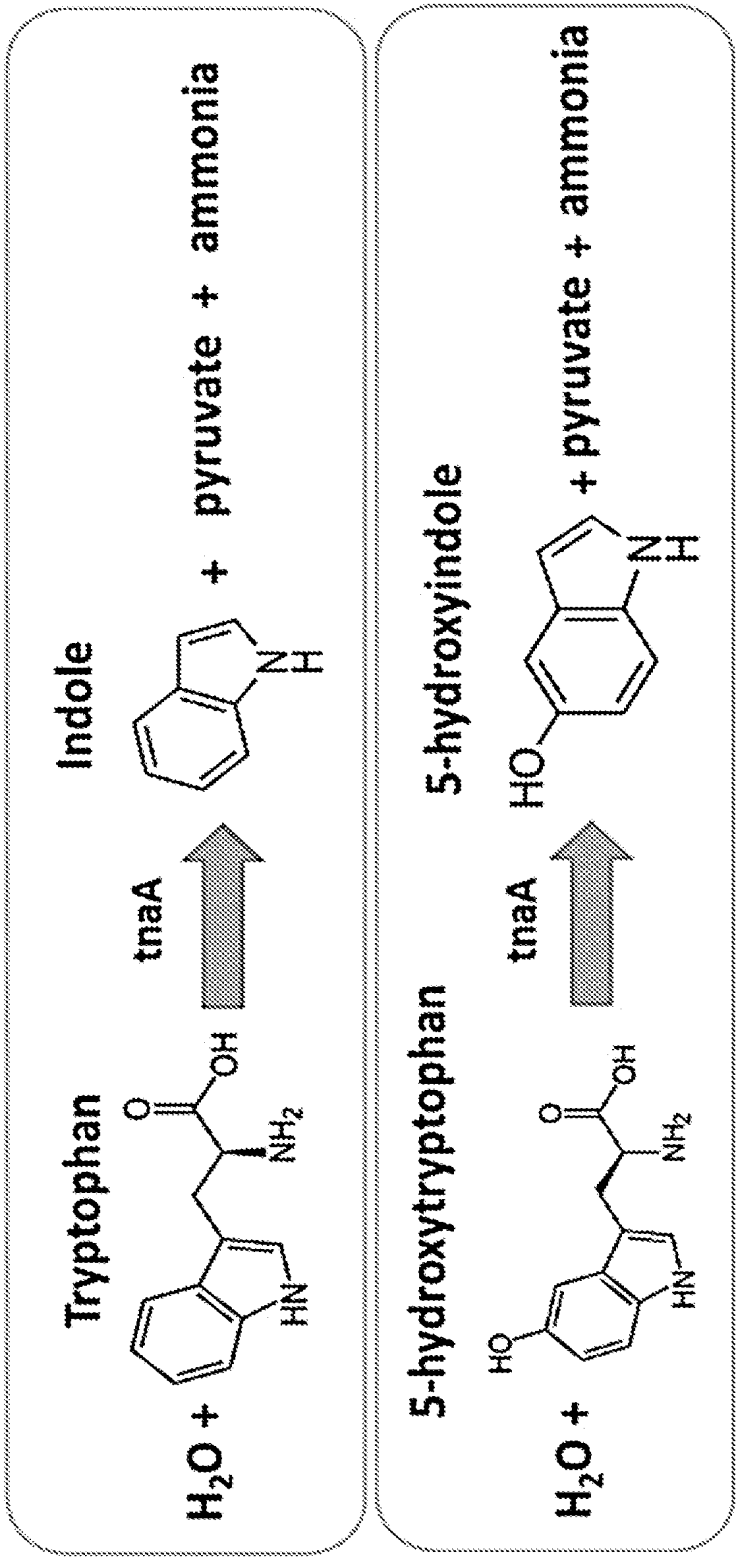

Tryptophan
tnaA
Indole
+ pyruvate + ammonia
H2O +
5-hydroxytryptophan
tnaA
5-hydroxyindole
+ pyruvate + ammonia
H2O +

Fig. 3A

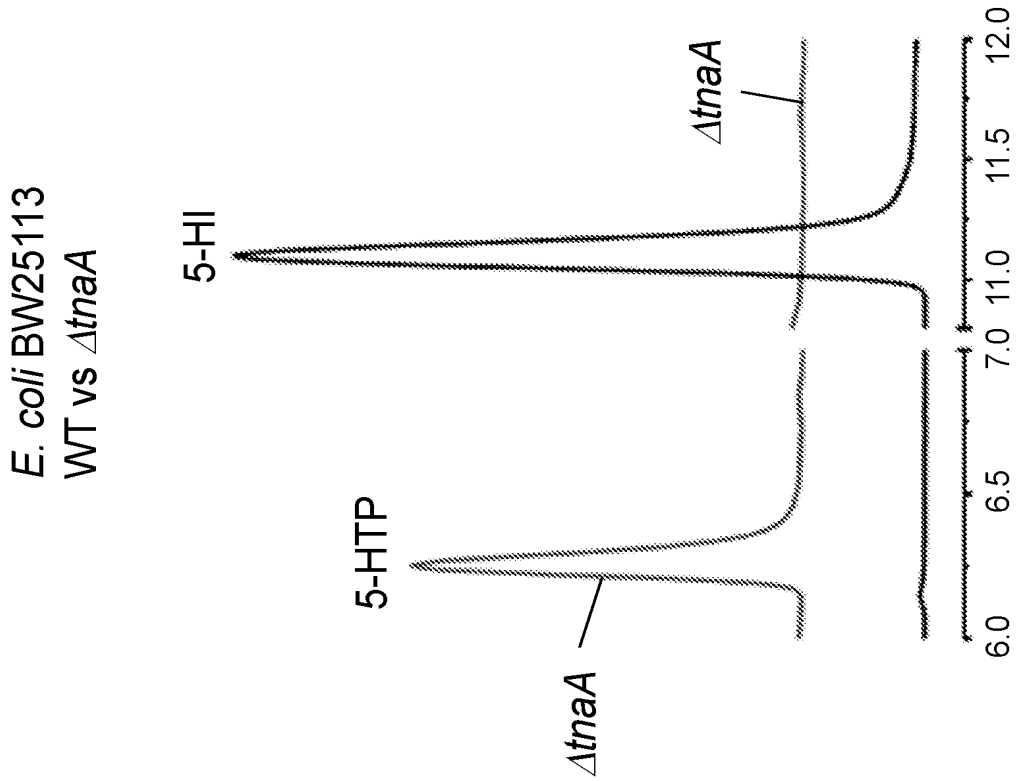

E. coli BW25113
WT vs ΔtnaA
5-HI
ΔtnaA
5-HTP
ΔtnaA
6.0
6.5
7.0
11.0
11.5
12.0
Time (min)

Fig. 3B

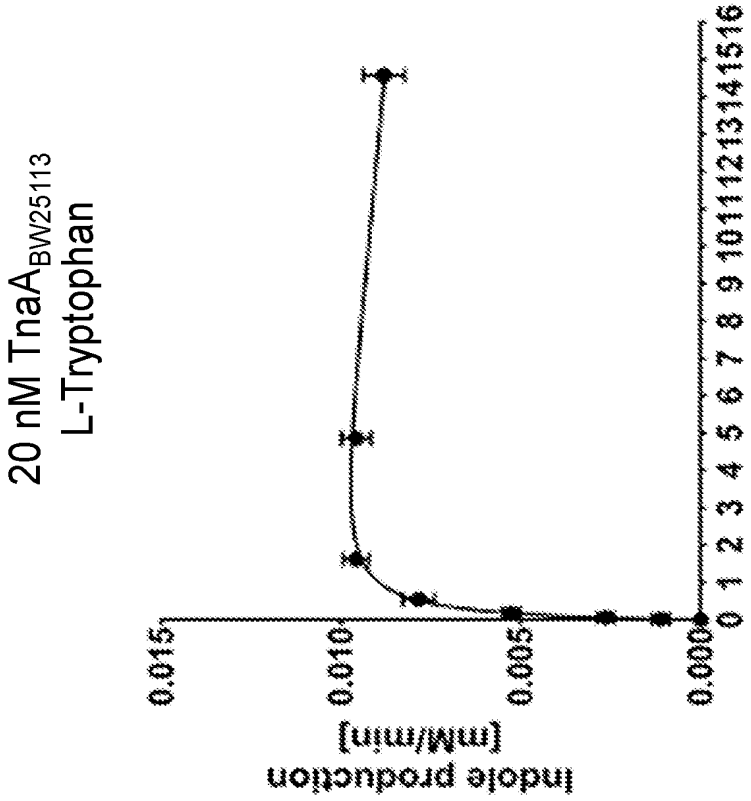

20 nM TnaA$_{BW25113}$
L-Tryptophan
Indole production
[mM/min]
0.015
0.010
0.005
0.000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
L-Trp [mM]

Fig. 3D

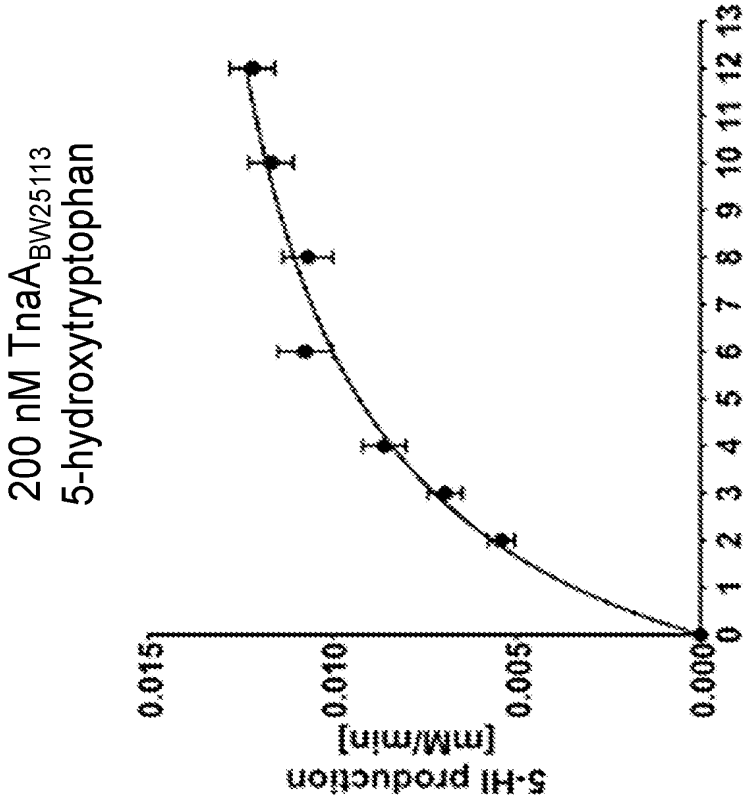

200 nM TnaA$_{BW25113}$
5-hydroxytryptophan
5-HI production
[mM/min]
0.015
0.010
0.005
0.000
0 1 2 3 4 5 6 7 8 9 10 11 12 13
5-HTP [mM]

Fig. 3C

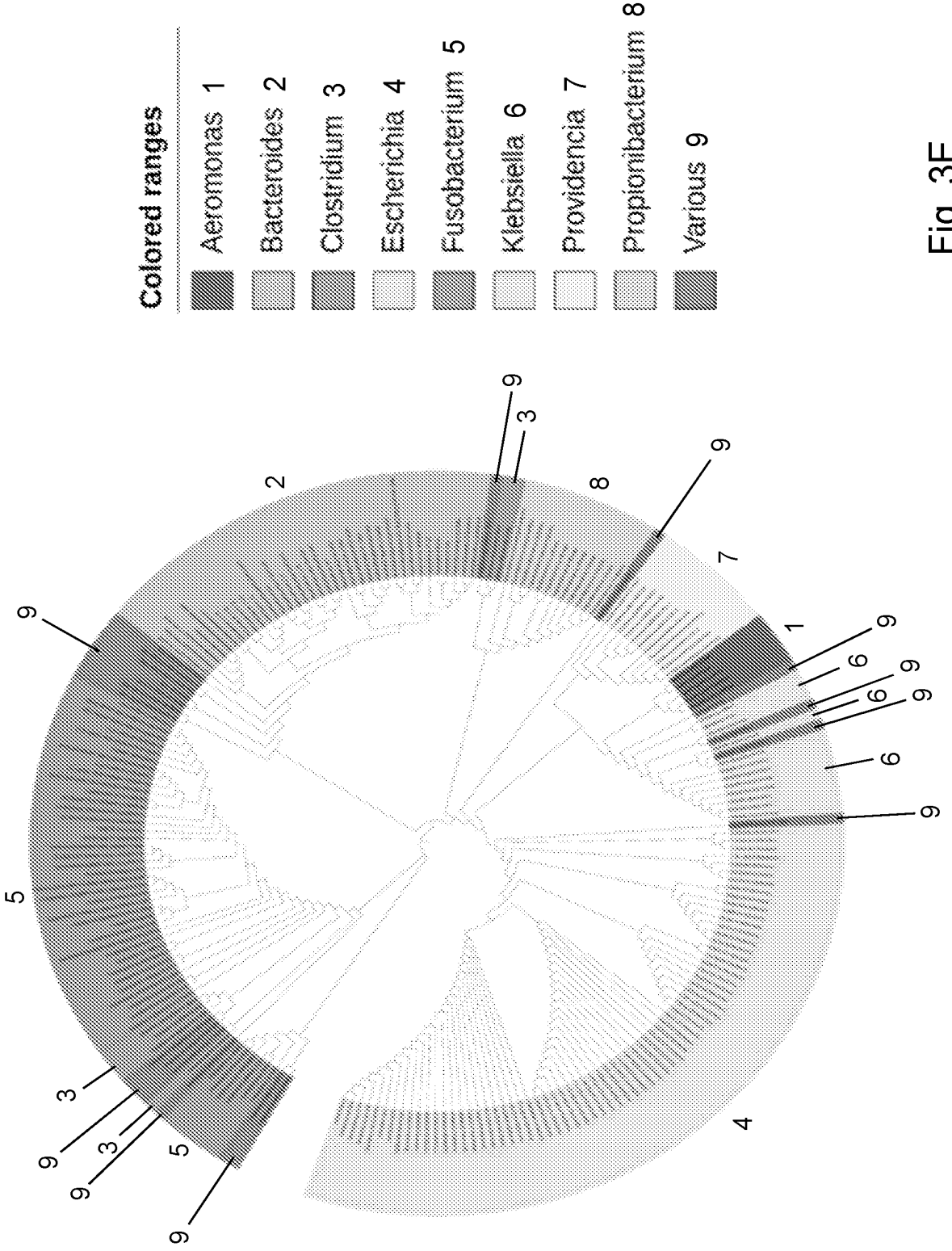

Colored ranges
Aeromonas 1
Bacteroides 2
Clostridium 3
Escherichia 4
Fusobacterium 5
Klebsiella 6
Providencia 7
Propionibacterium 8
Various 9

Fig. 3E

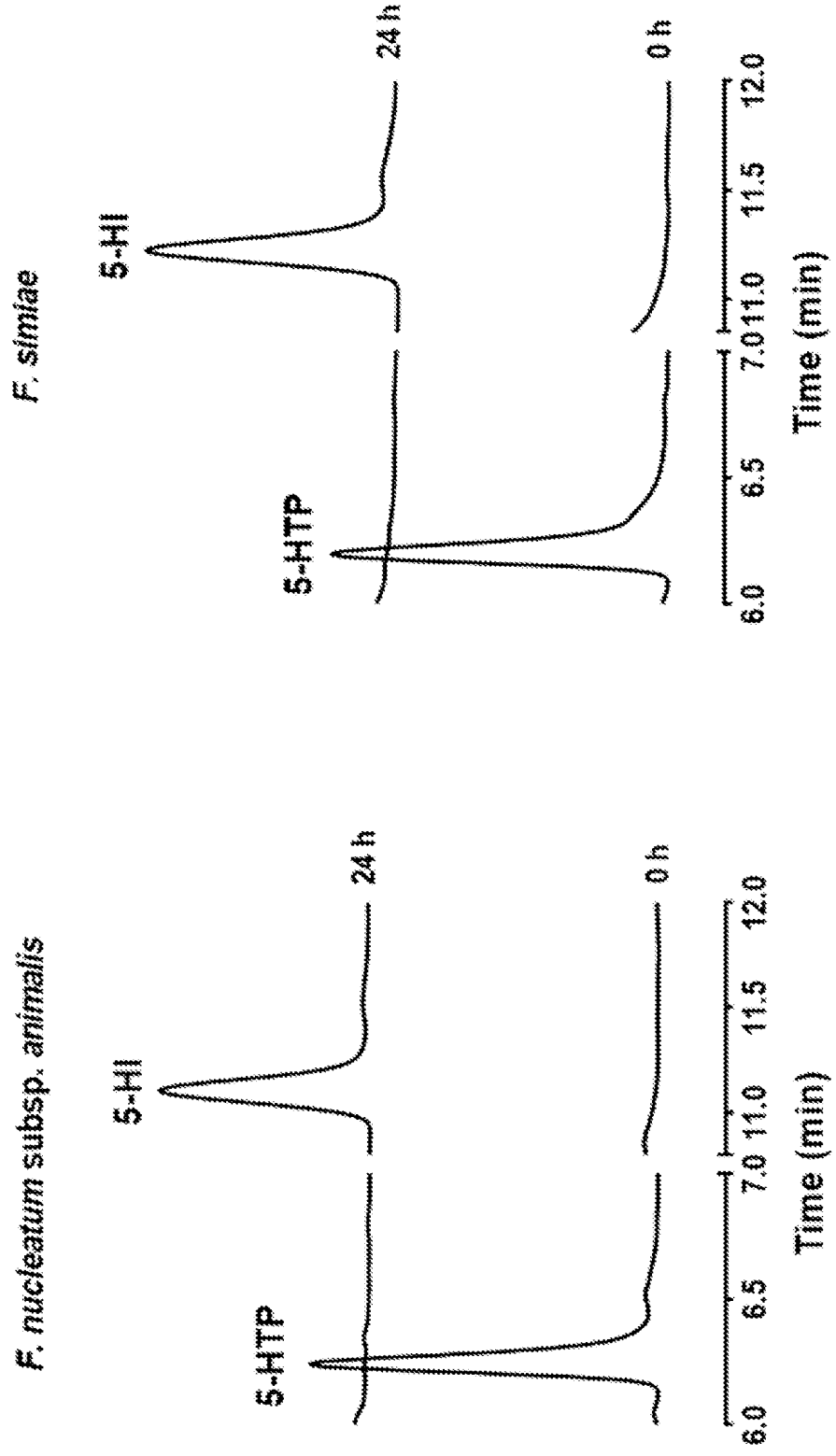

F. simiae
5-HI
24 h
5-HTP
0 h
6.0
6.5
7.0
11.0
11.5
12.0
Time (min)

Fig. 3G

F. nucleatum subsp. animalis
5-HI
24 h
5-HTP
0 h
6.0
6.5
7.0
11.0
11.5
12.0
Time (min)

Fig. 3F

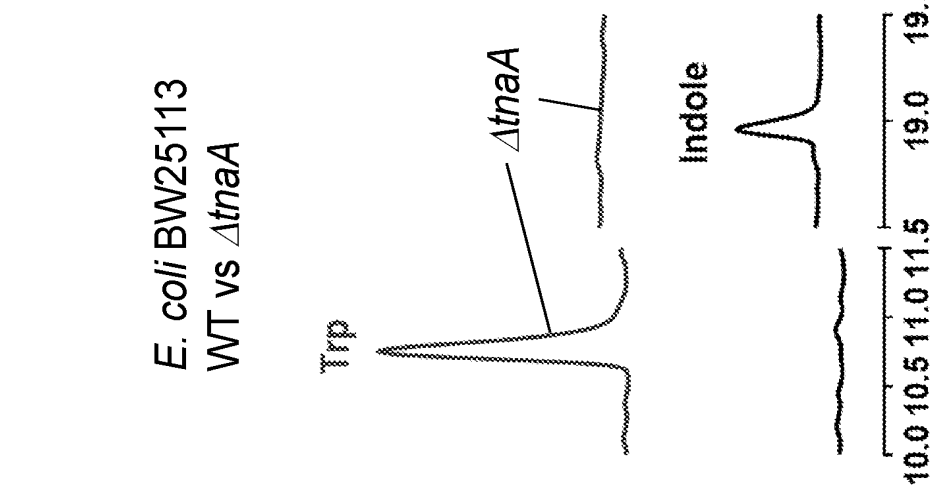

E. coli BW25113
WT vs ΔtnaA
Trp
ΔtnaA
Indole
10.0 10.5 11.0 11.5
19.0 19.5
Time (min)

Fig. 4A

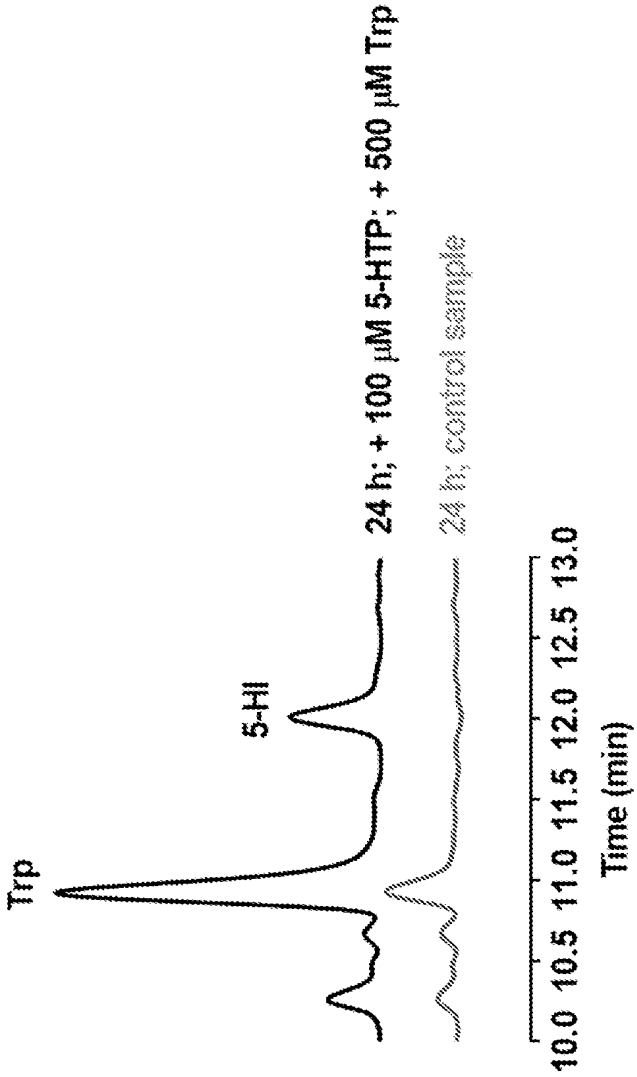

Trp
5-HI
24 h; + 100 µM 5-HTP; + 500 µM Trp
24 h; control sample
10.0 10.5 11.0 11.5 12.0 12.5 13.0
Time (min)

Fig. 4B

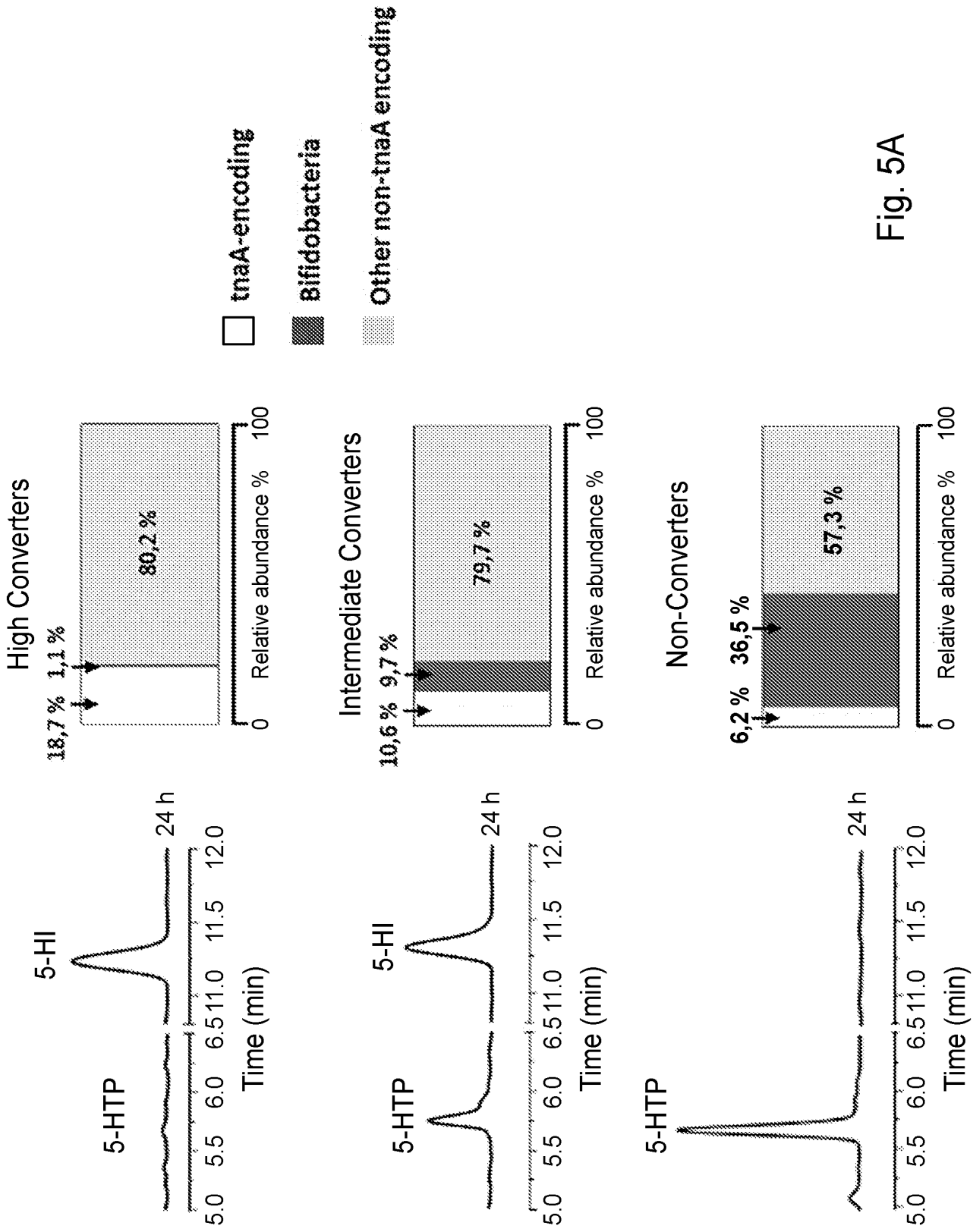

tnaA-encoding
Bifidobacteria
Other non-tnaA encoding
High Converters
18,7 %
1,1 %
80,2 %
0
Relative abundance %
100
5-HI
5-HTP
24 h
5.0 5.5 6.0 6.5 11.0 11.5 12.0
Time (min)
Intermediate Converters
10,6 %
9,7 %
79,7 %
0
Relative abundance %
100
5-HI
5-HTP
24 h
5.0 5.5 6.0 6.5 11.0 11.5 12.0
Time (min)
Non-Converters
6,2 %
36,5 %
57,3 %
0
Relative abundance %
100
5-HTP
24 h
5.0 5.5 6.0 6.5 11.0 11.5 12.0
Time (min)

Fig. 5A

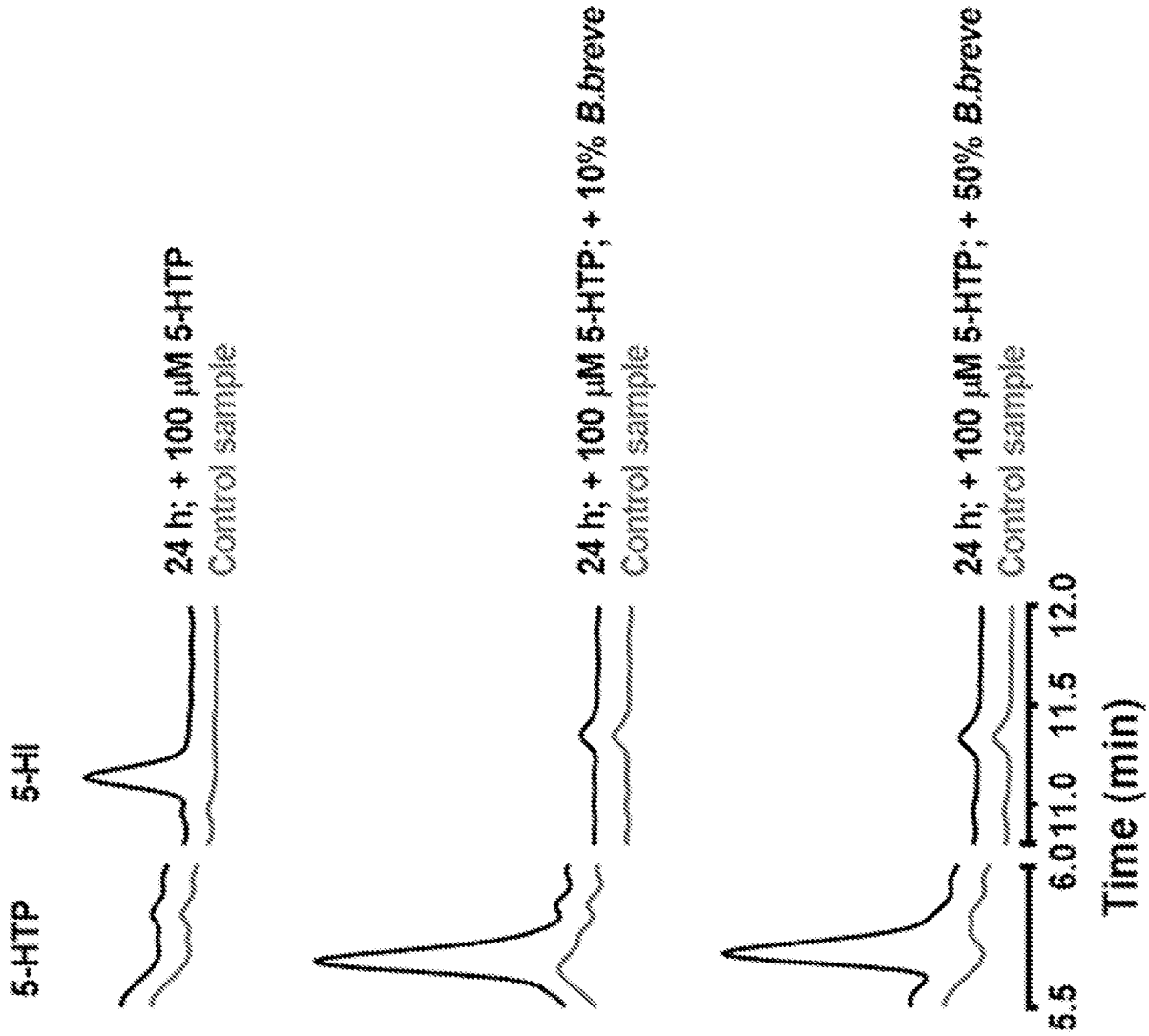


Fig. 5B

Fig. 5C

Fig. 5D

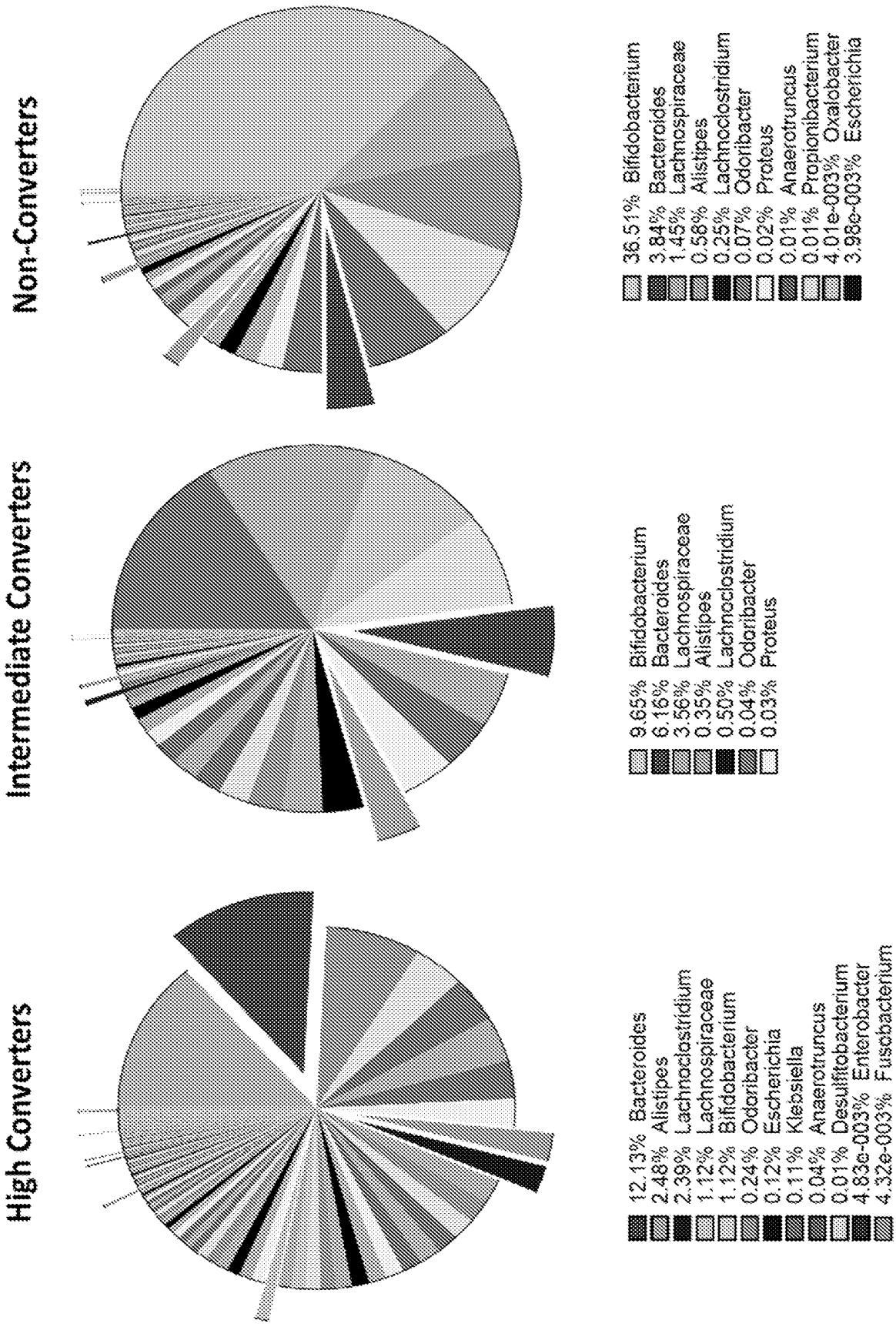

High Converters
12.13% Bacteroides
2.48% Alistipes
2.39% Lachnoclostridium
1.12% Lachnospiraceae
1.12% Bifidobacterium
0.24% Odoribacter
0.12% Escherichia
0.11% Klebsiella
0.04% Anaerotruncus
0.01% Desulfitobacterium
4.83e-003% Enterobacter
4.32e-003% Fusobacterium
Intermediate Converters
9.65% Bifidobacterium
6.16% Bacteroides
3.56% Lachnospiraceae
0.35% Alistipes
0.50% Lachnoclostridium
0.04% Odoribacter
0.03% Proteus
Non-Converters
36.51% Bifidobacterium
3.84% Bacteroides
1.45% Lachnospiraceae
0.58% Alistipes
0.25% Lachnoclostridium
0.07% Odoribacter
0.02% Proteus
0.01% Anaerotruncus
0.01% Propionibacterium
4.01e-003% Oxalobacter
3.98e-003% Escherichia

Fig. 6

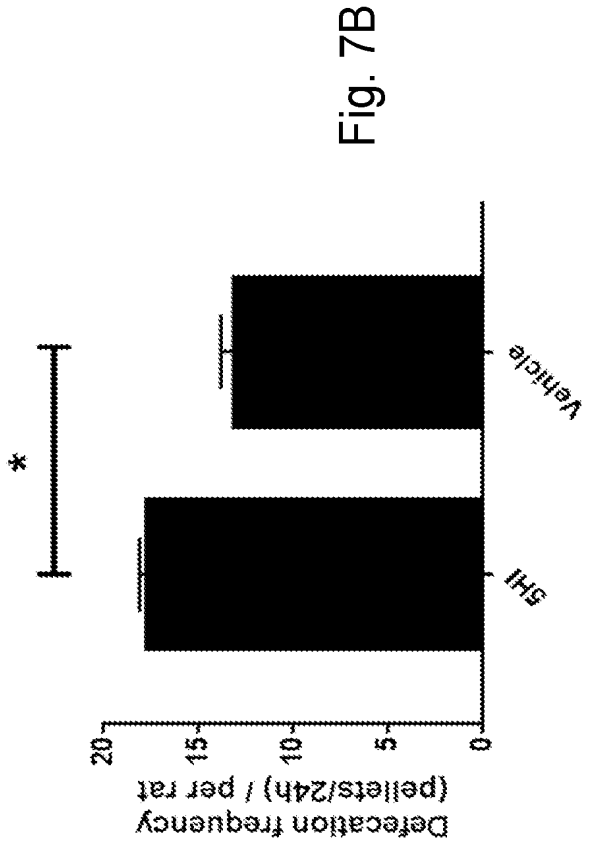


Fig. 7B

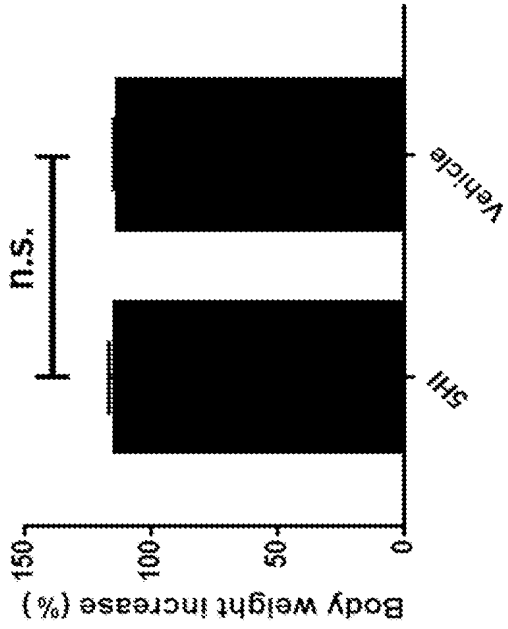


Fig. 7C

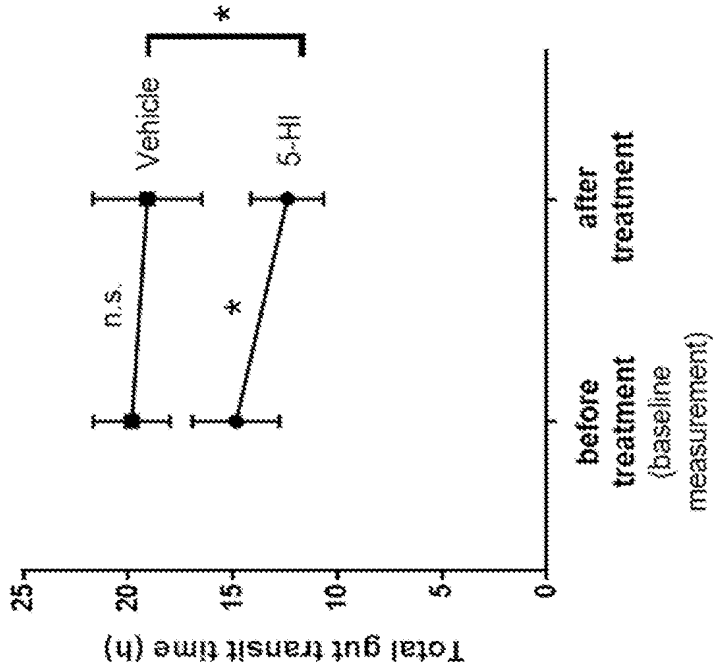


Fig. 7A

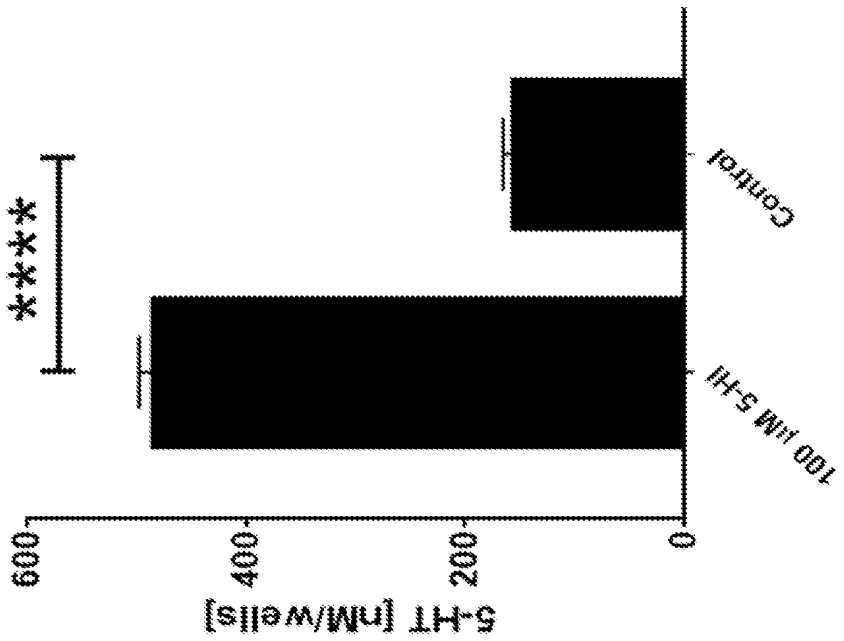


Fig. 8A

Fig. 8B

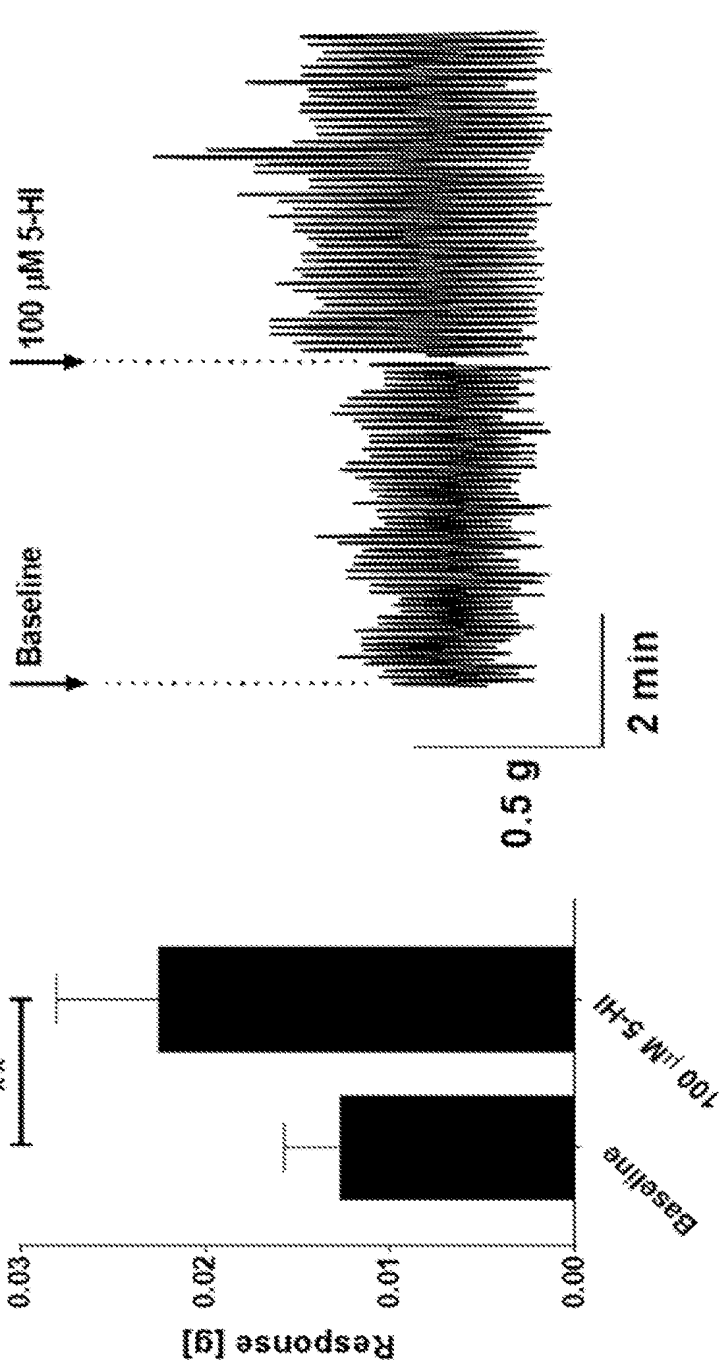
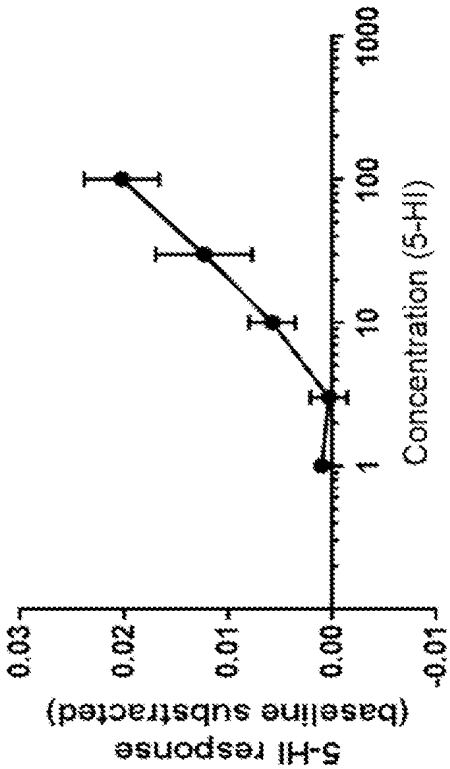

Response [g]
0.03
0.02
0.01
0.00
Baseline
100 µM 5-HI
**
0.5 g
2 min
Baseline
100 µM 5-HI

Fig. 8C


5-HI response
(baseline substracted)
0.03
0.02
0.01
0.00
-0.01
Concentration (5-HI)
1
10
100
1000

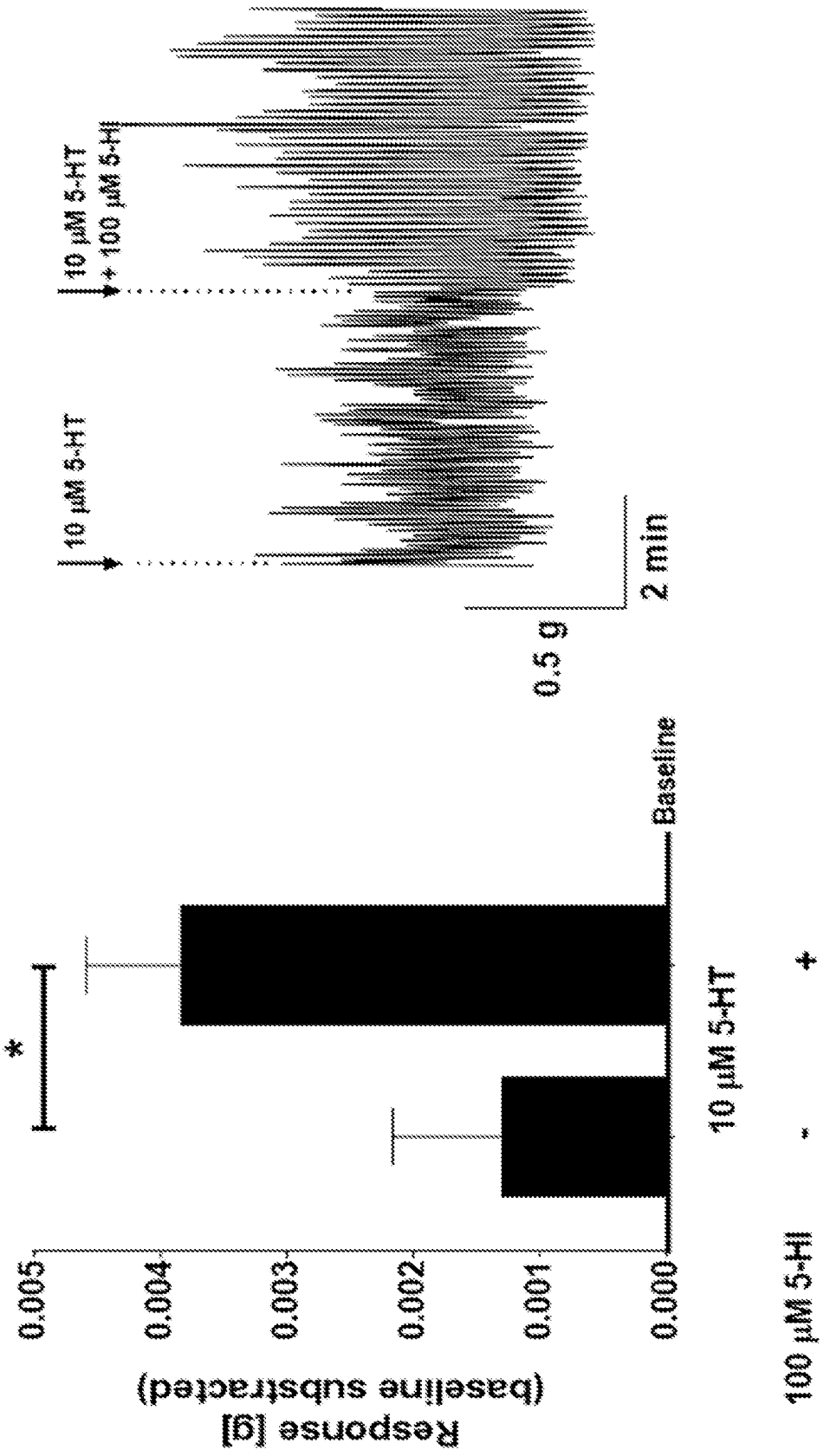

10 µM 5-HT
+ 100 µM 5-HI
10 µM 5-HT
0.5 g
2 min
Response [g]
(baseline substracted)
0.005
0.004
0.003
0.002
0.001
0.000
Baseline
*
10 µM 5-HT
100 µM 5-HI
-
+

Fig. 8D

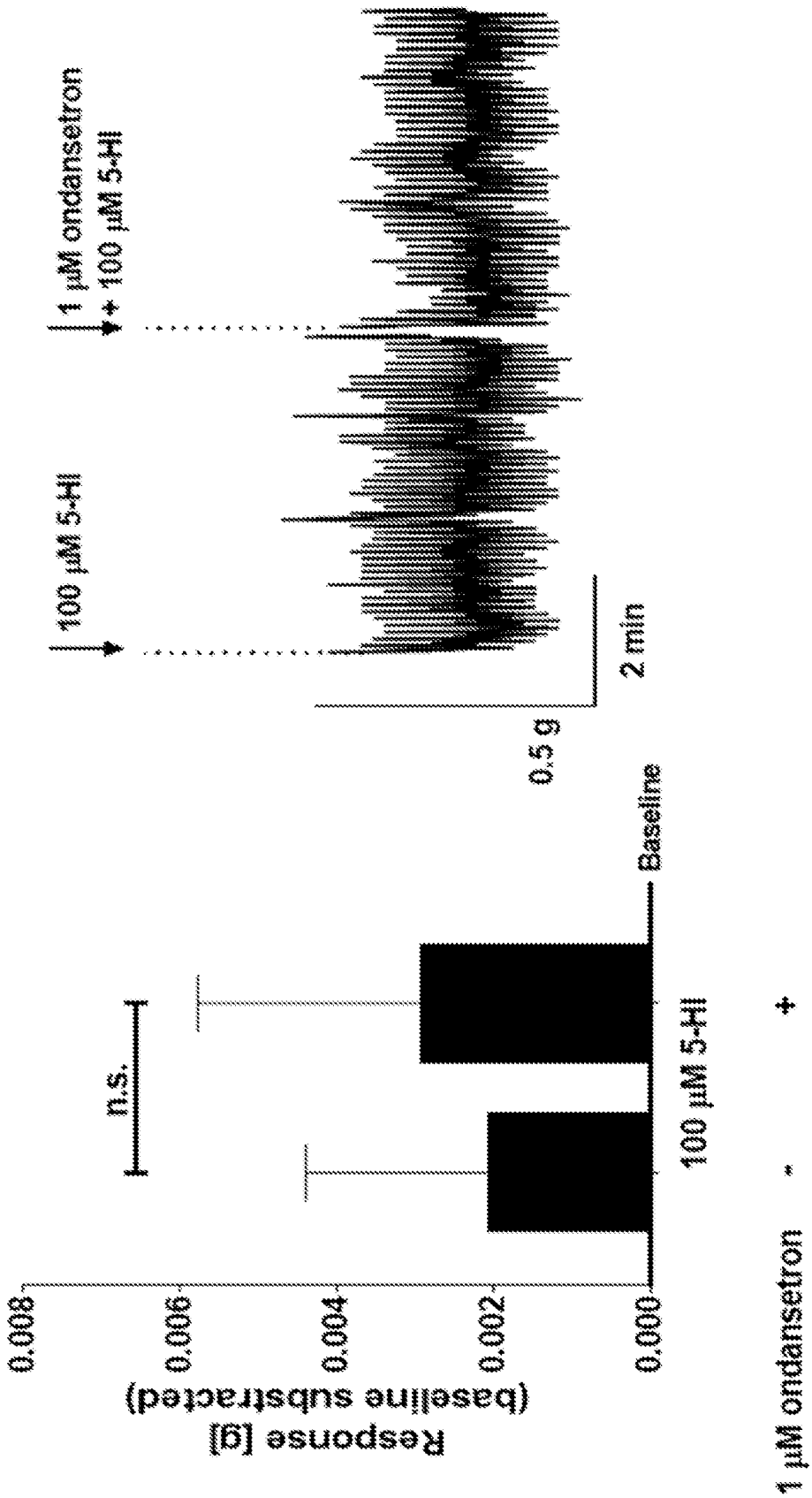

1 µM ondansetron
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.008
0.006
0.004
0.002
0.000
n.s.
Baseline
100 µM 5-HI
1 µM ondansetron
-
+

Fig. 8E

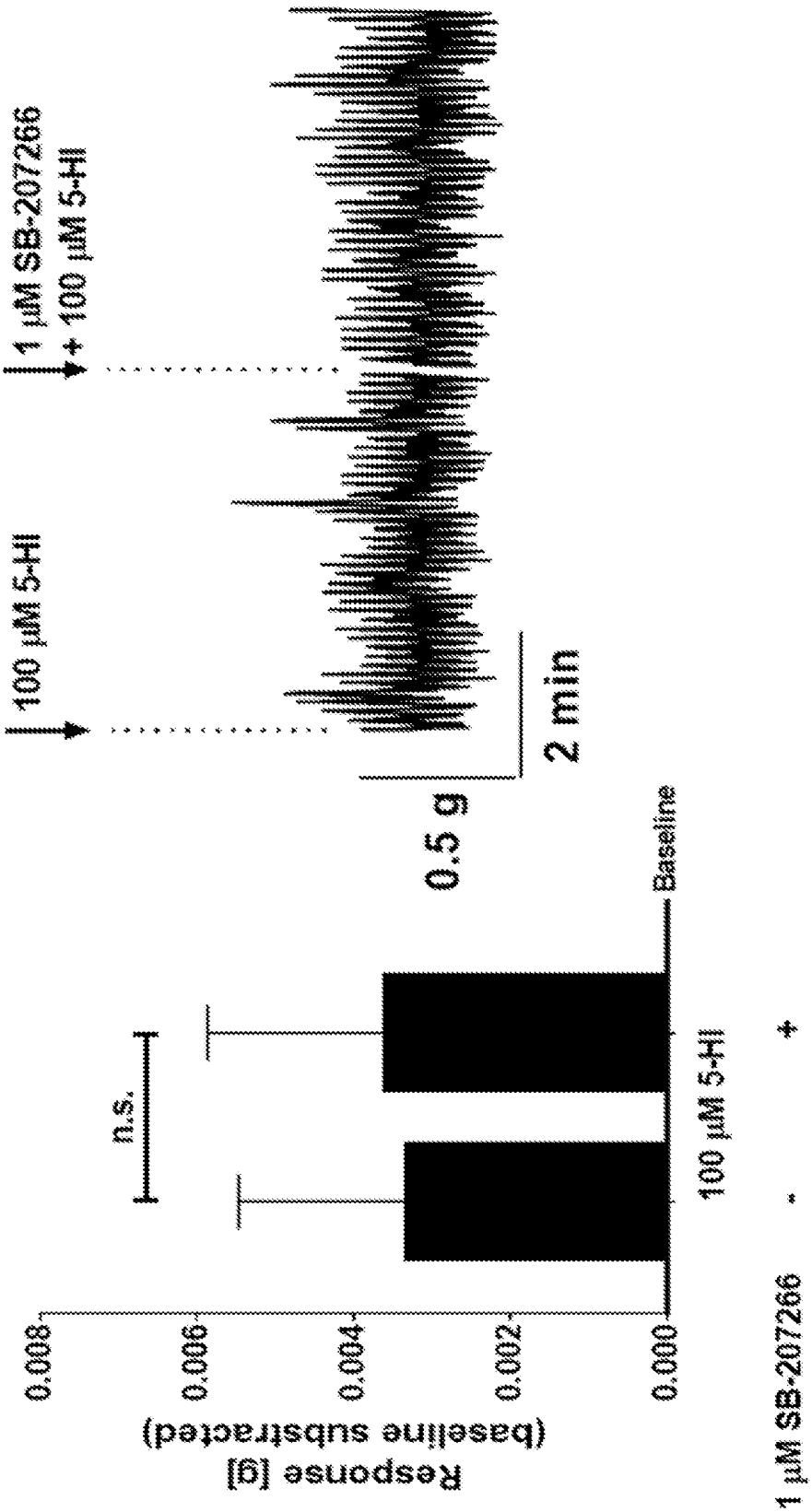

1 µM SB-207266
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.008
0.006
0.004
0.002
0.000
n.s.
Baseline
100 µM 5-HI
1 µM SB-207266
-
+

Fig. 8F

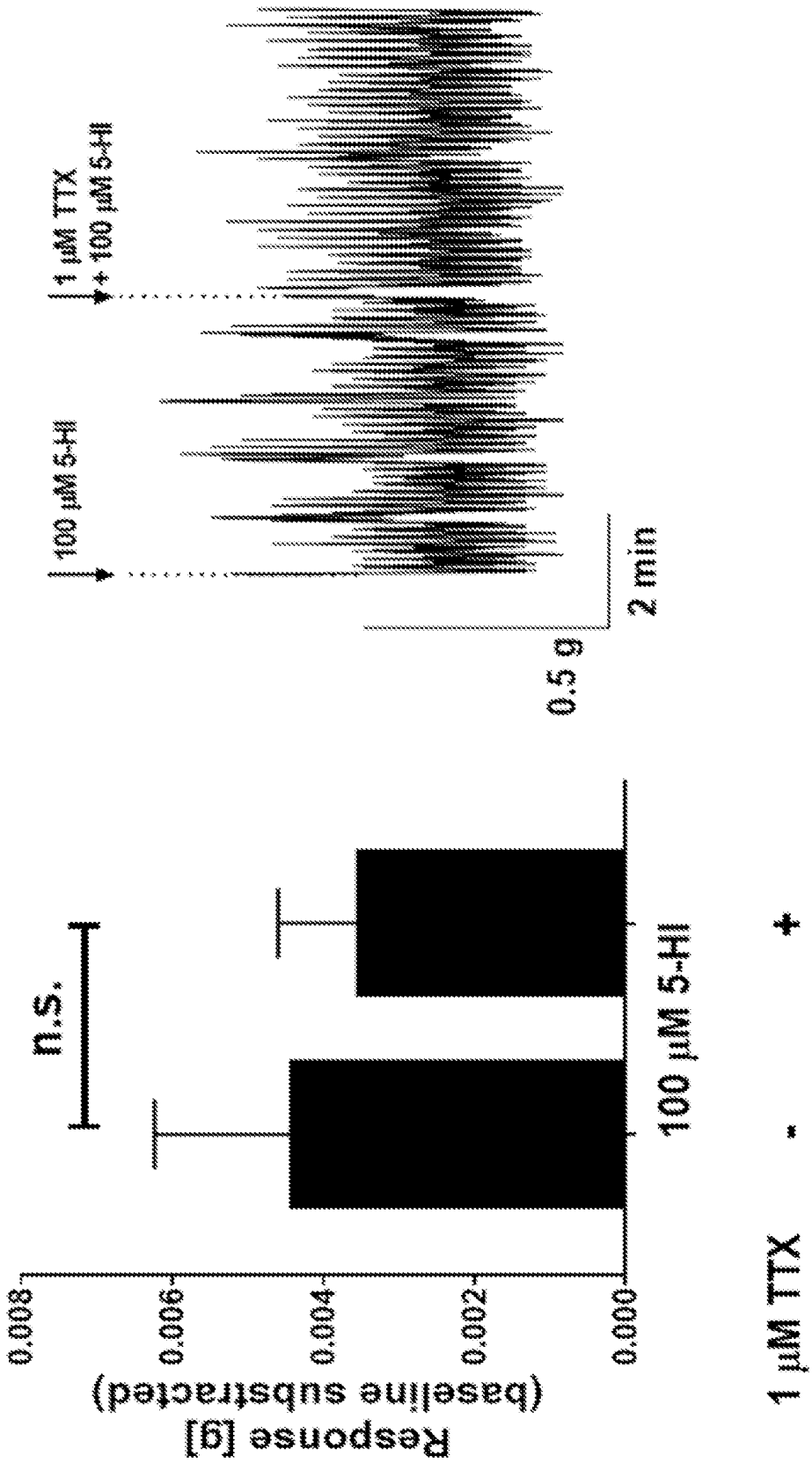

1 µM TTX
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.008
0.006
0.004
0.002
0.000
n.s.
100 µM 5-HI
1 µM TTX
-
+
+

Fig. 8G

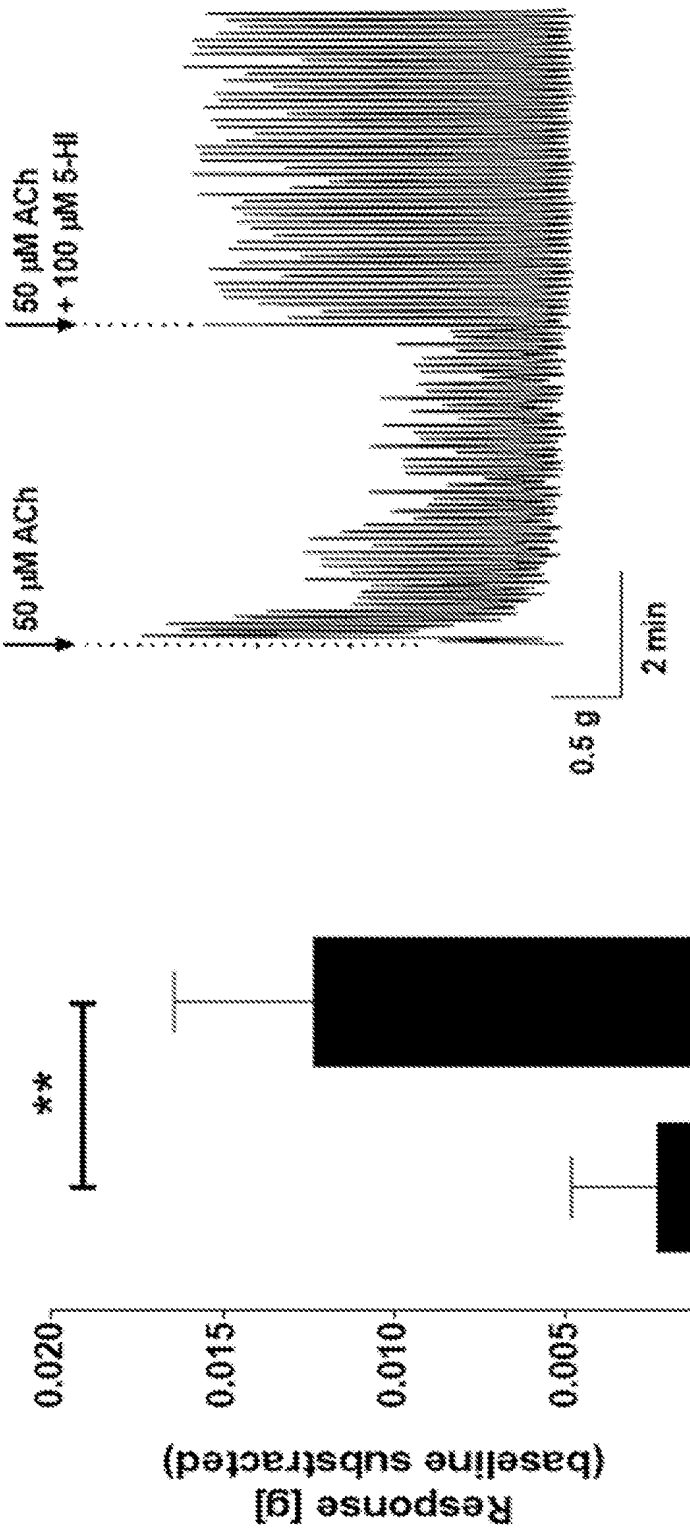

50 µM ACh
+ 100 µM 5-HI
50 µM ACh
0.5 g
2 min
Response [g]
(baseline substracted)
0.020
0.015
0.010
0.005
0.000
**
Baseline
50 µM Ach
100 µM 5-HI
-
+

Fig. 8H

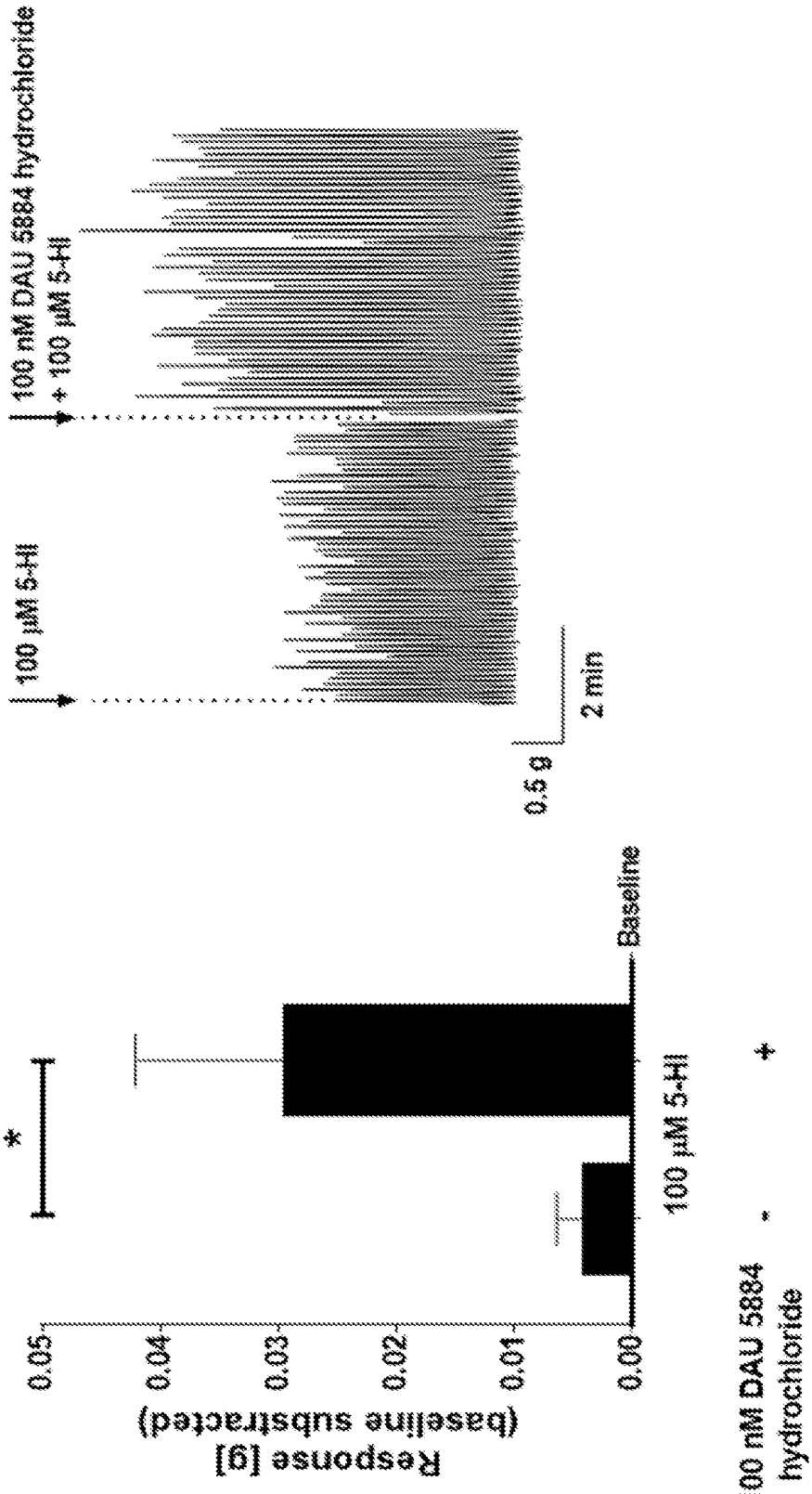

100 nM DAU 5884 hydrochloride
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.05
0.04
0.03
0.02
0.01
0.00
*
Baseline
100 µM 5-HI
100 nM DAU 5884 hydrochloride
-
+

Fig. 8I

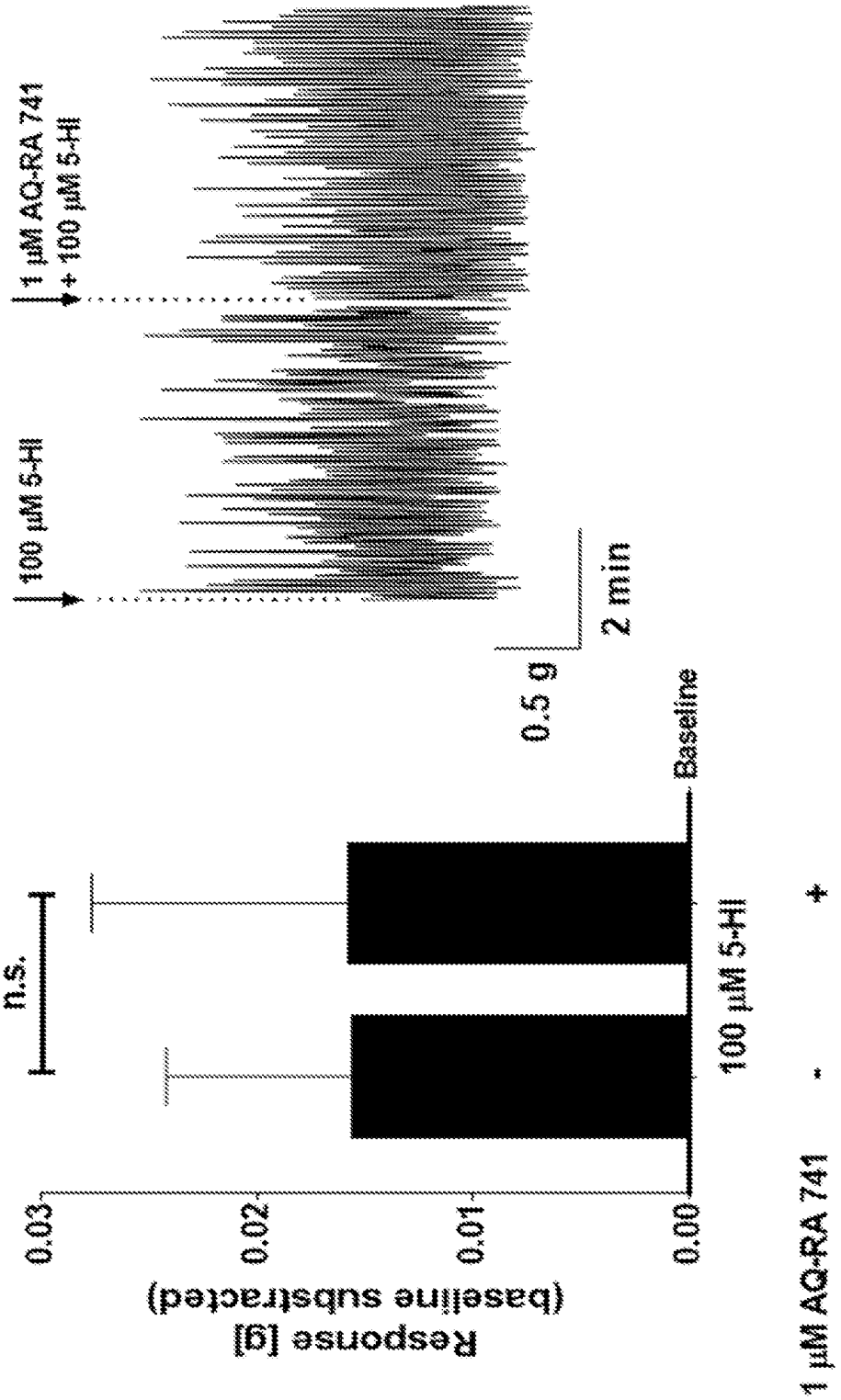

1 µM AQ-RA 741
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.03
0.02
0.01
0.00
n.s.
Baseline
100 µM 5-HI
1 µM AQ-RA 741
-
+

Fig. 8J

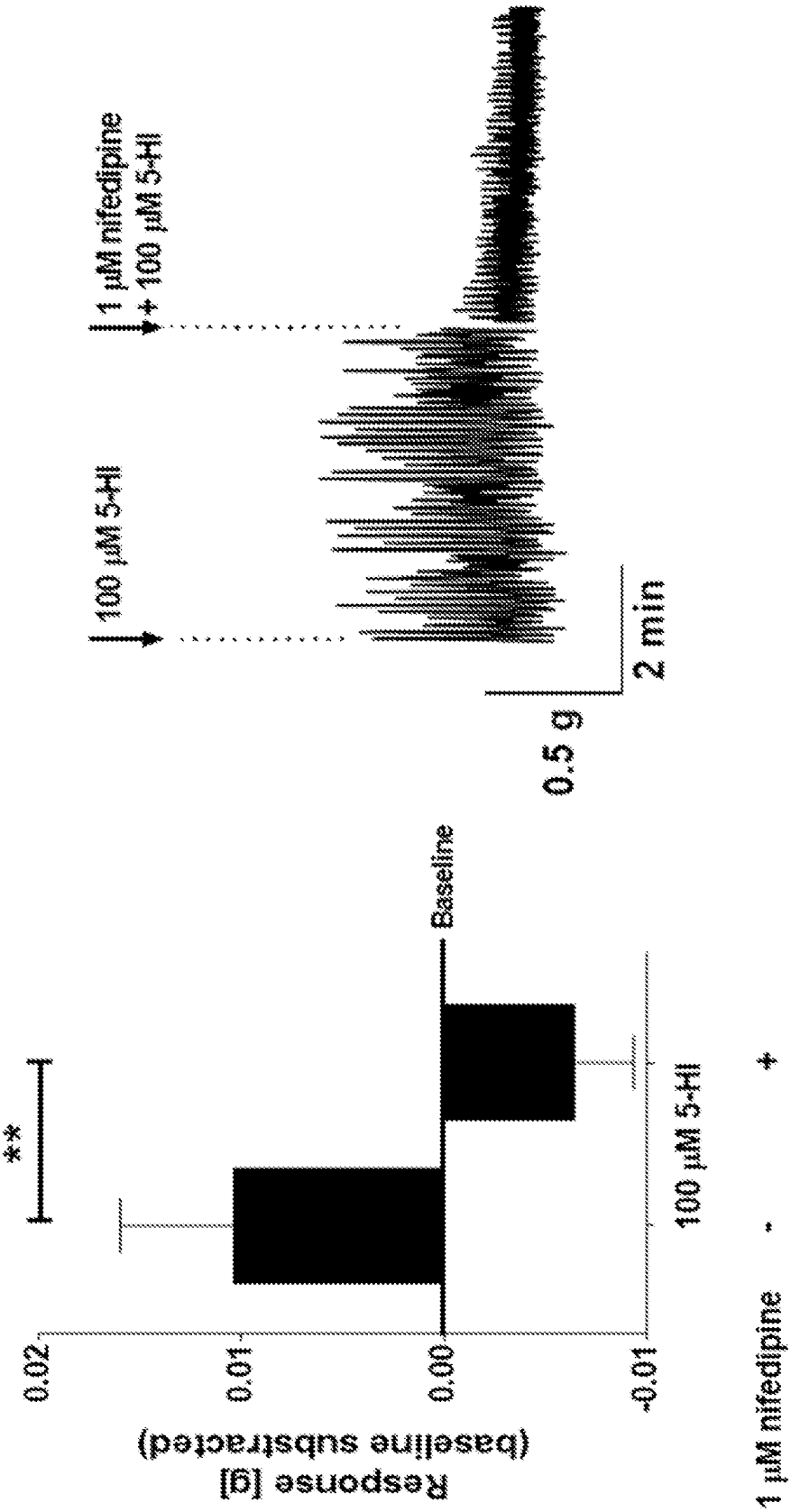

1 µM nifedipine
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Baseline
**
0.02
0.01
0.00
-0.01
Response [g]
(baseline substracted)
100 µM 5-HI
1 µM nifedipine
-
+

Fig. 8K

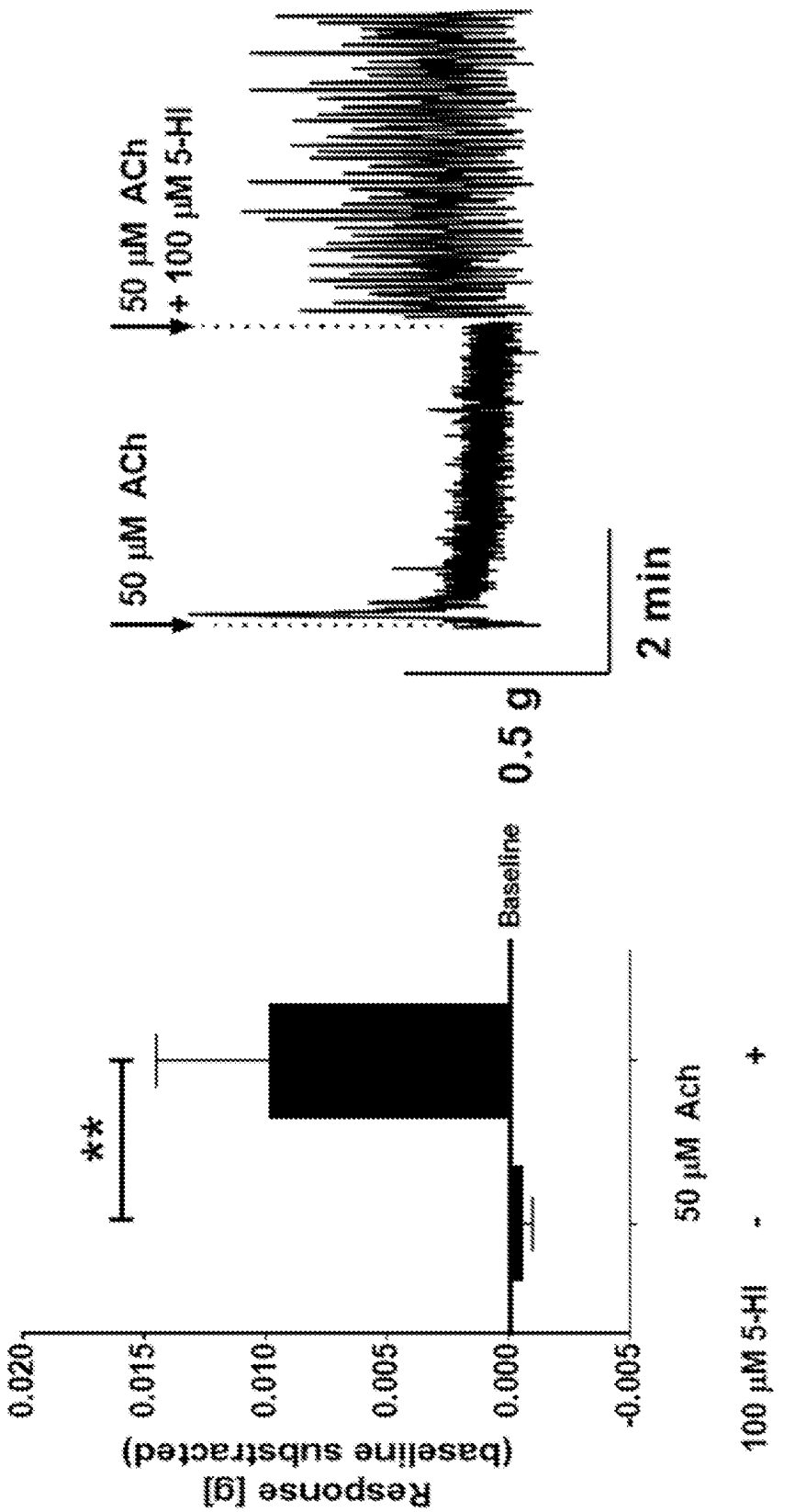

50 µM ACh
50 µM ACh
+ 100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.020
0.015
0.010
0.005
0.000
-0.005
**
Baseline
50 µM Ach
100 µM 5-HI
-
+

Fig. 8L

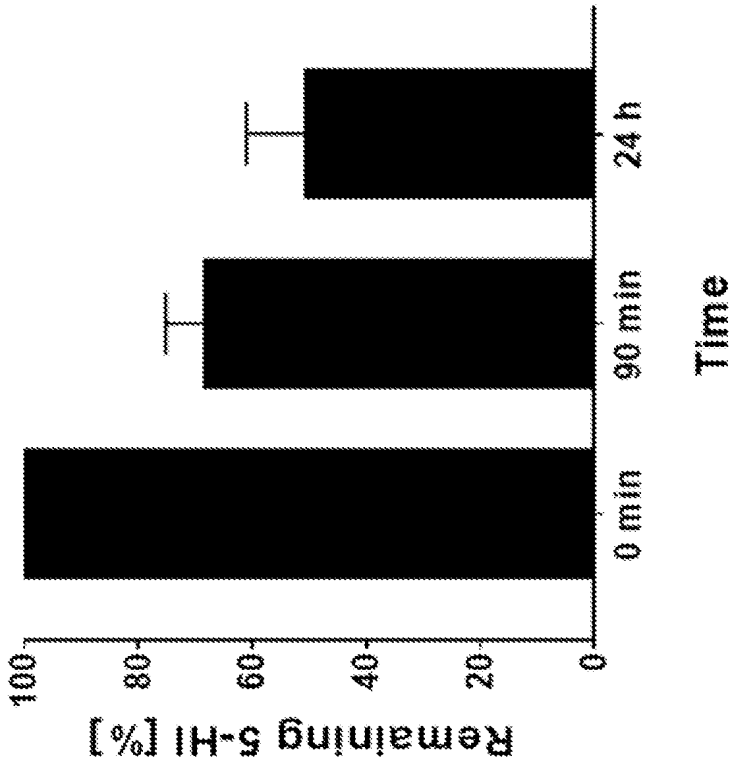


Fig. 9A

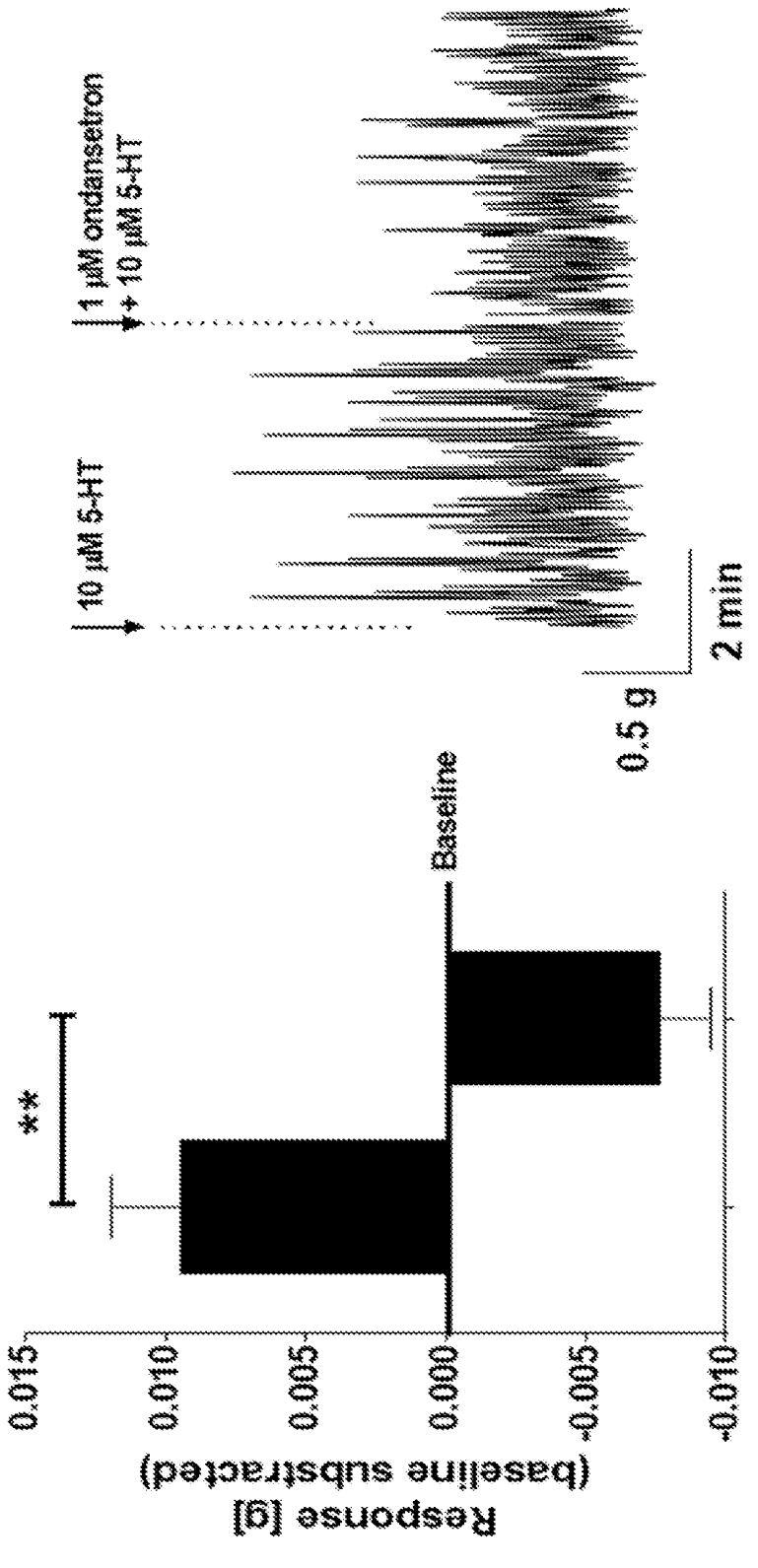

1 µM ondansetron
+ 10 µM 5-HT
10 µM 5-HT
0.5 g
2 min
Baseline
**
Response [g]
(baseline substracted)
0.015
0.010
0.005
0.000
-0.005
-0.010
1 µM ondansetron - +
10 µM 5-HT

Fig. 9B

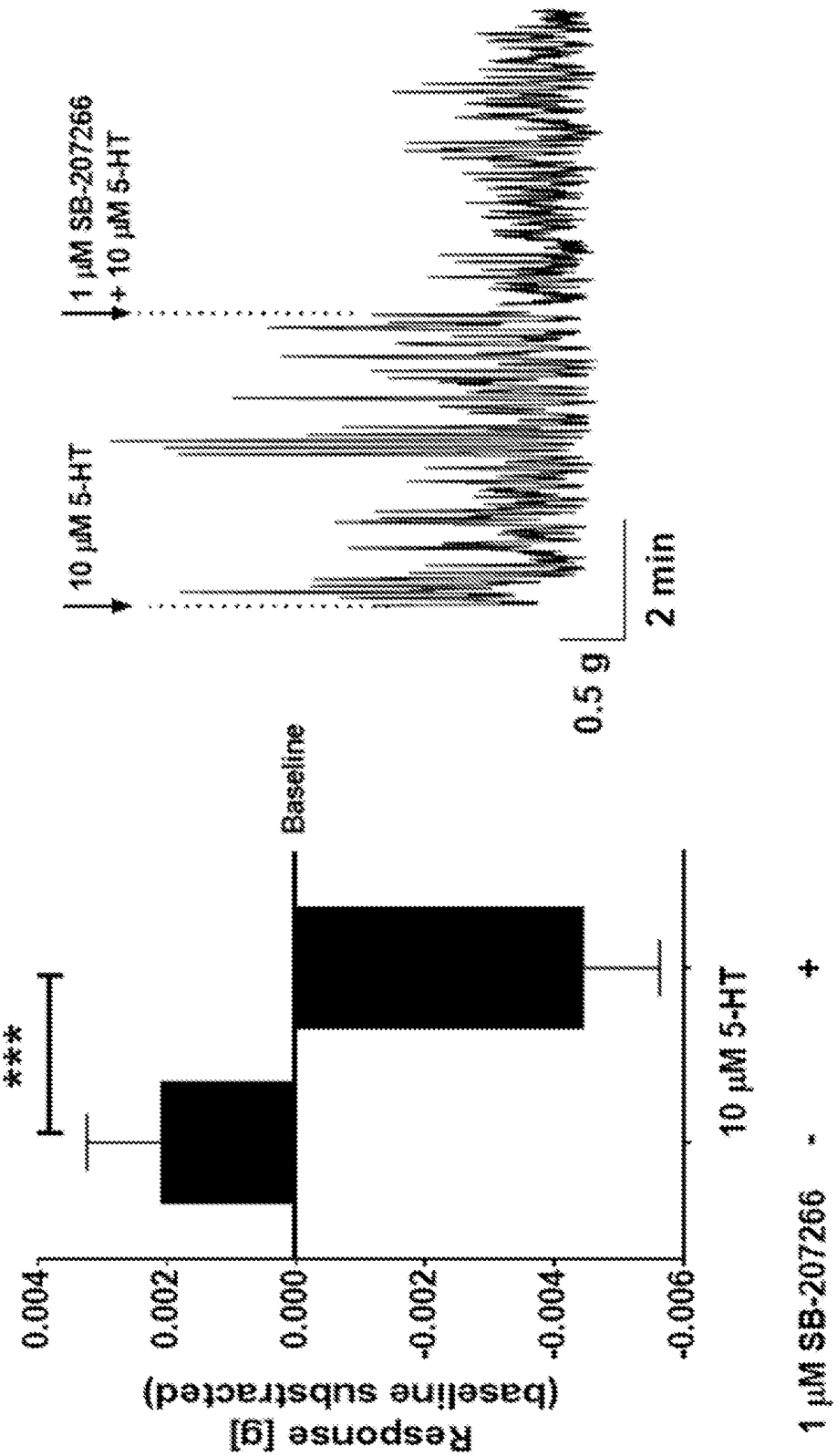

1 µM SB-207266
+ 10 µM 5-HT
10 µM 5-HT
0.5 g
2 min
Baseline
***
0.004
0.002
0.000
-0.002
-0.004
-0.006
Response [g]
(baseline substracted)
1 µM SB-207266 - +
10 µM 5-HT

Fig. 9C

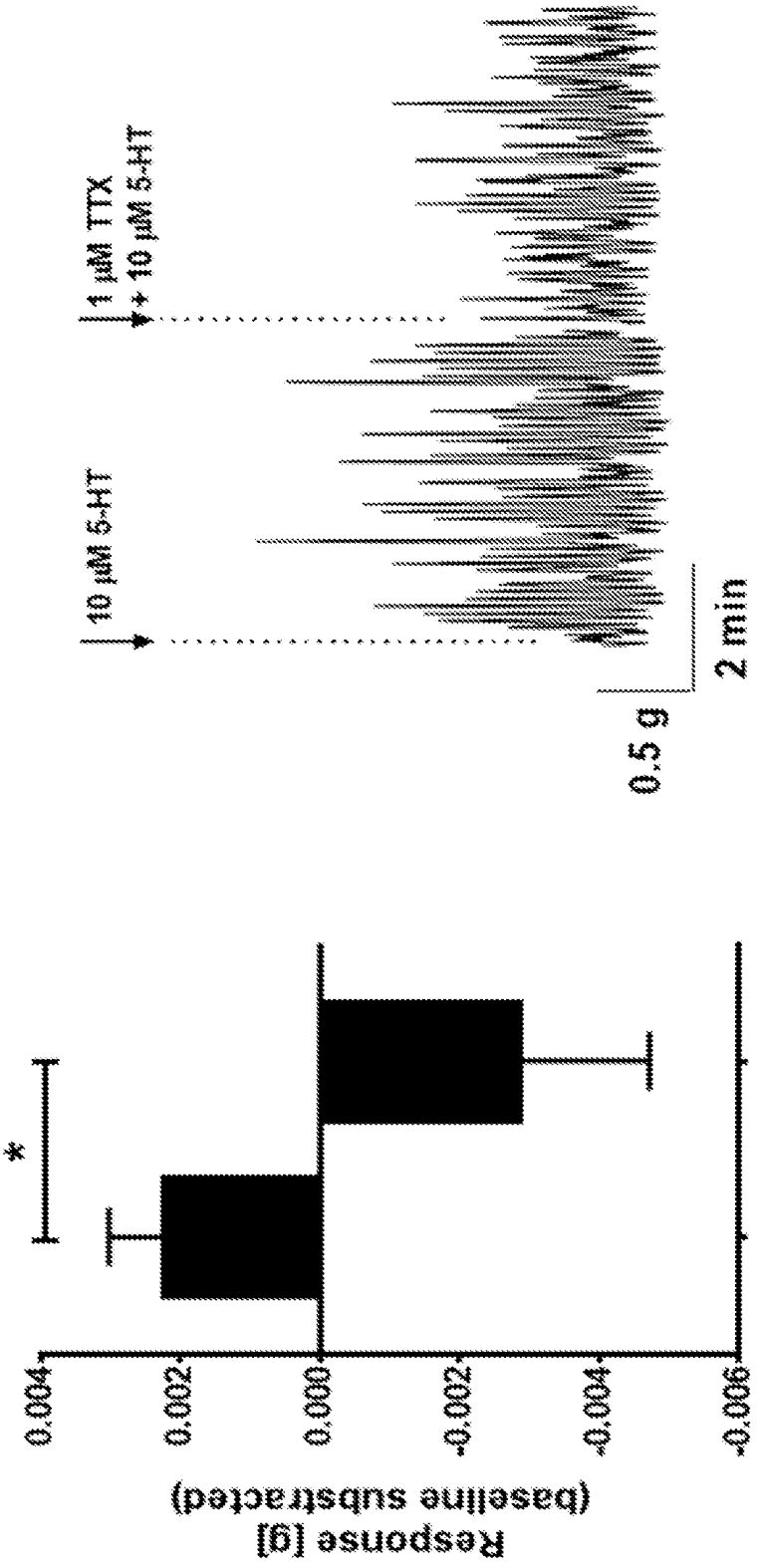

1 µM TTX
+ 10 µM 5-HT
10 µM 5-HT
0.5 g
2 min
Response [g]
(baseline substracted)
0.004
0.002
0.000
-0.002
-0.004
-0.006
*
1 µM TTX - +
10 µM 5-HT

Fig. 9D

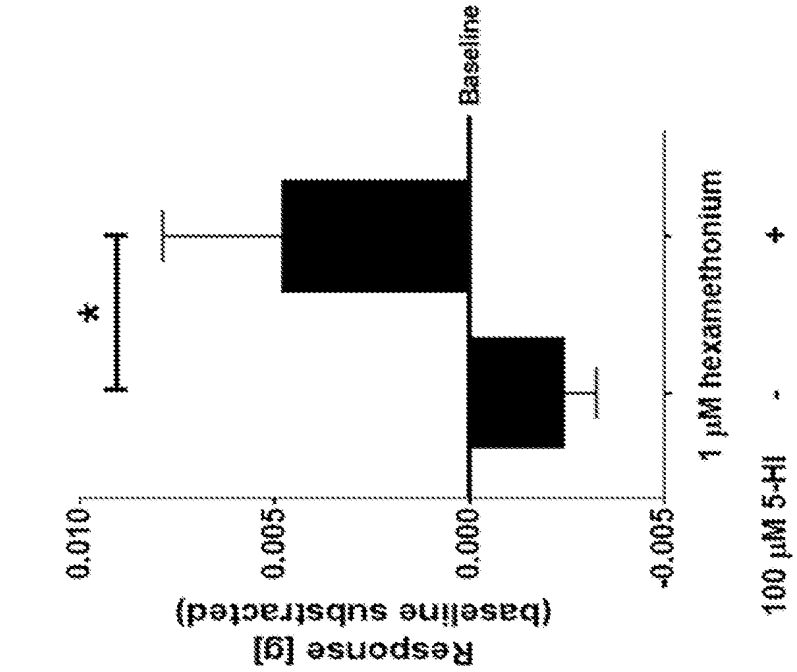

Response [g]
(baseline substracted)
0.010
0.005
0.000
-0.005
Baseline
*
100 µM 5-HT
1 µM hexamethonium
-
+

Fig. 9F

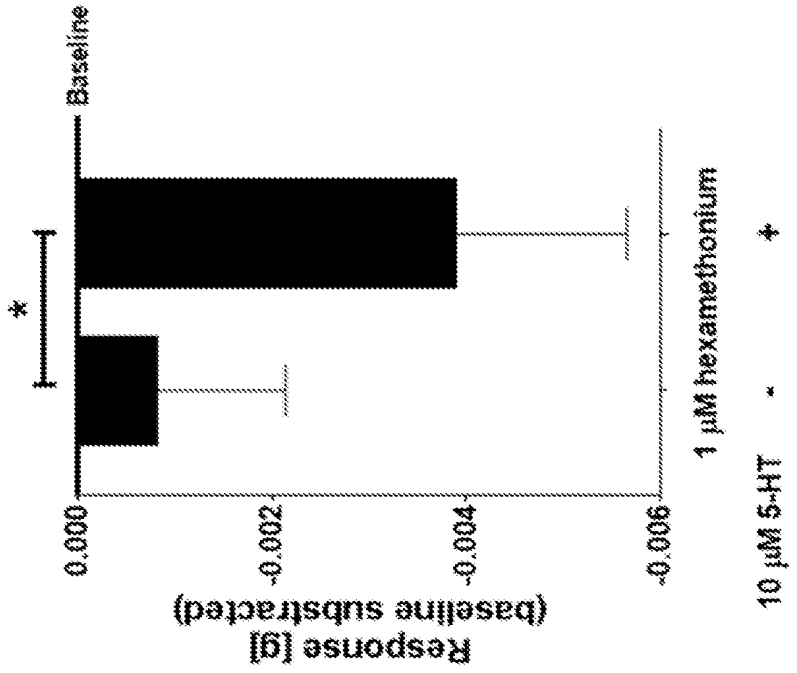

Response [g]
(baseline substracted)
0.000
-0.002
-0.004
-0.006
Baseline
*
10 µM 5-HT
1 µM hexamethonium
-
+

Fig. 9E

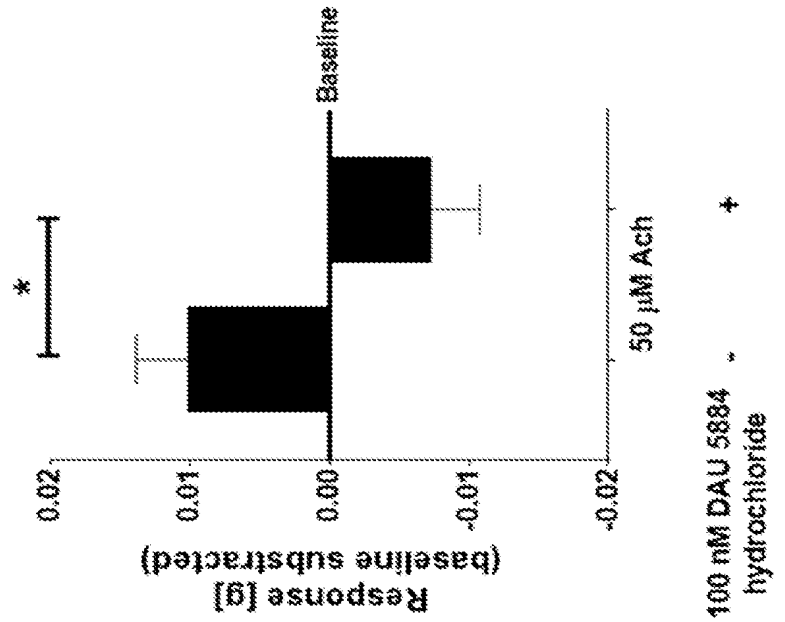

Response [g]
(baseline substracted)
0.02
0.01
0.00
-0.01
-0.02
Baseline
*
50 µM Ach
100 nM DAU 5884 hydrochloride
-
+

Fig. 9H

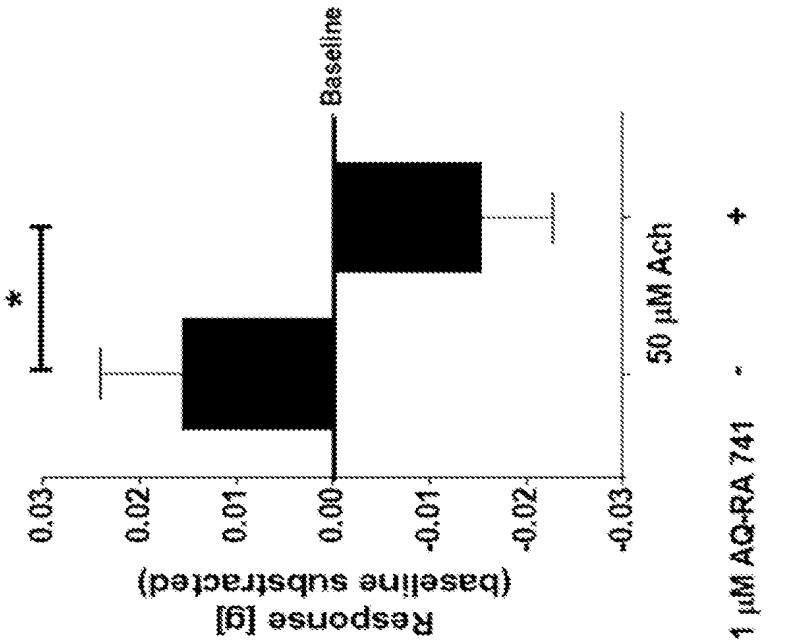

Response [g]
(baseline substracted)
0,03
0,02
0,01
0,00
-0,01
-0,02
-0,03
Baseline
*
50 µM Ach
1 µM AQ-RA 741
-
+

Fig. 9G

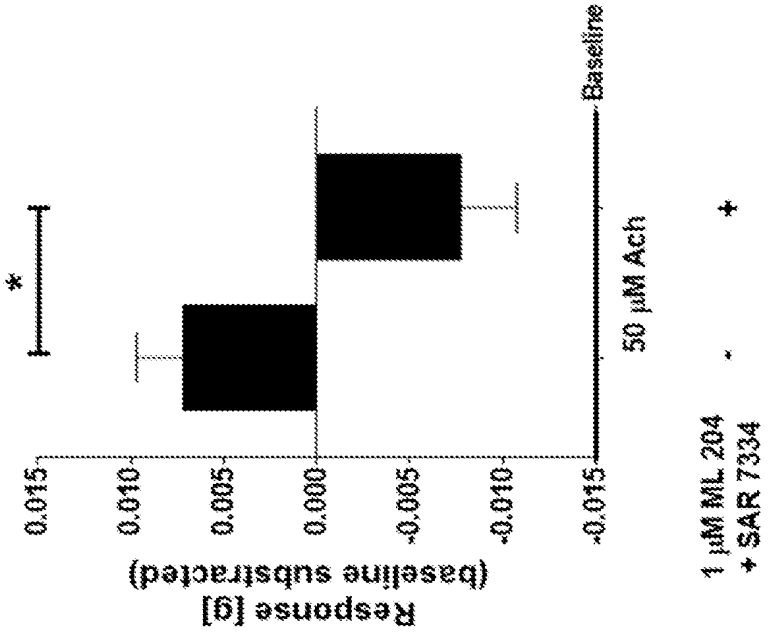

Response [g]
(baseline substracted)
0.015
0.010
0.005
0.000
-0.005
-0.010
-0.015
Baseline
50 µM Ach
1 µM ML 204
+ SAR 7334
*

Fig. 9I

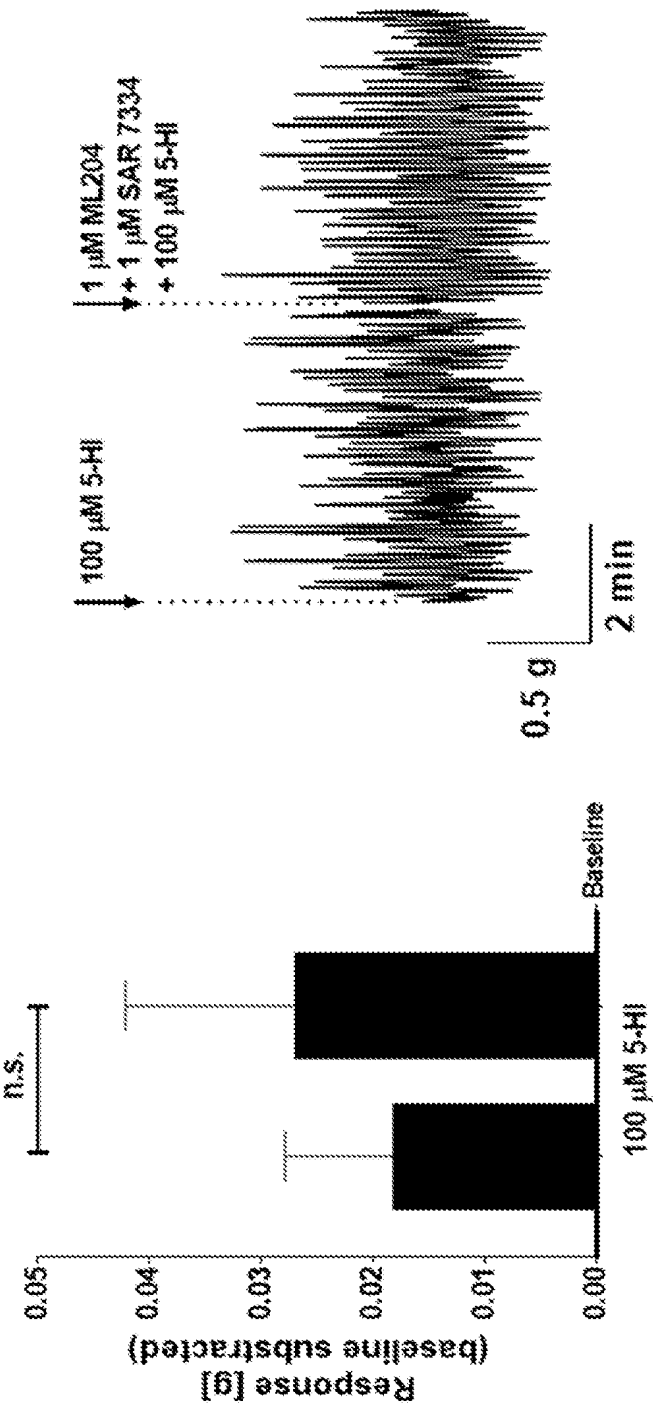

1 µM ML204
+ 1 µM SAR 7334
+ 100 µM 5-HI
100 µM 5-HI
0.5 g
2 min
Response [g]
(baseline substracted)
0.05
0.04
0.03
0.02
0.01
0.00
n.s.
Baseline
100 µM 5-HI
1 µM ML 204
+ SAR 7334
-
+

Fig. 9J

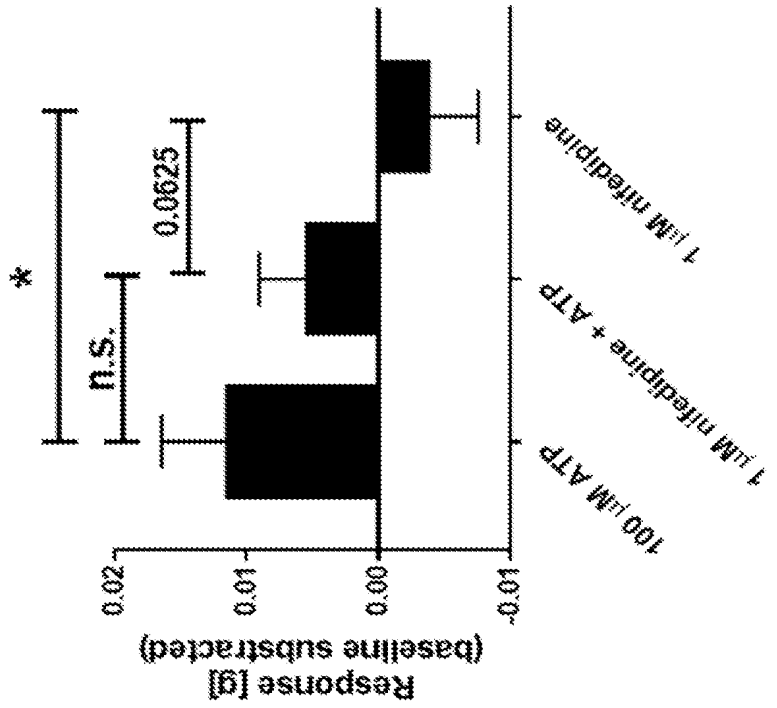

Response [g]
(baseline substracted)
0.02
0.01
0.00
-0.01
100 µM ATP
1 µM nifedipine + ATP
1 µM nifedipine
n.s.
0.0625
*

Fig. 9K

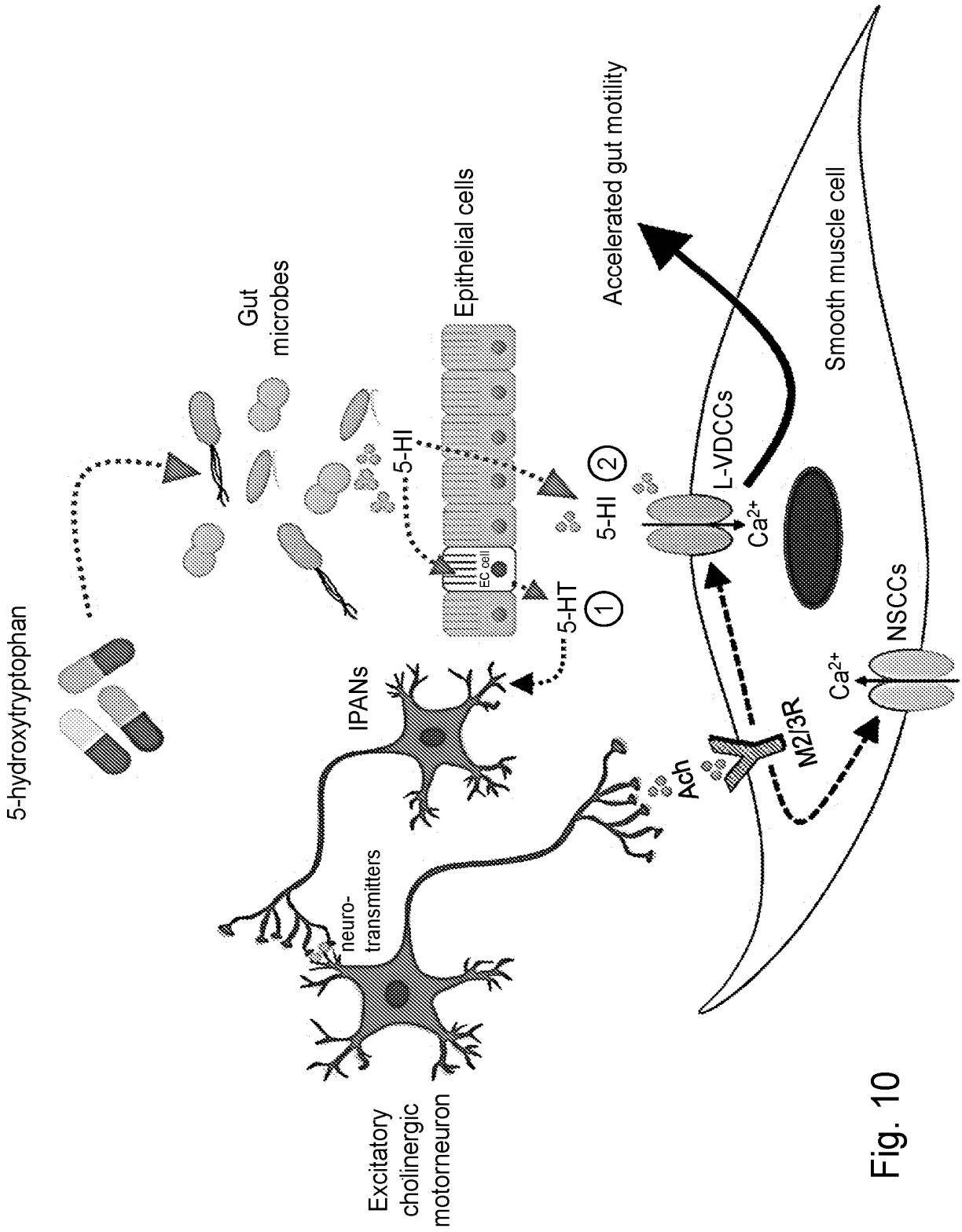

5-hydroxytryptophan
Gut microbes
Epithelial cells
Accelerated gut motility
Smooth muscle cell
5-HI
EC cell
5-HT
1
5-HI
2
L-VDCCs
Ca^{2+}
IPANs
neuro-transmitters
Ach
M2/3R
Ca^{2+}
NSCCs
Excitatory cholinergic motorneuron

Fig. 10

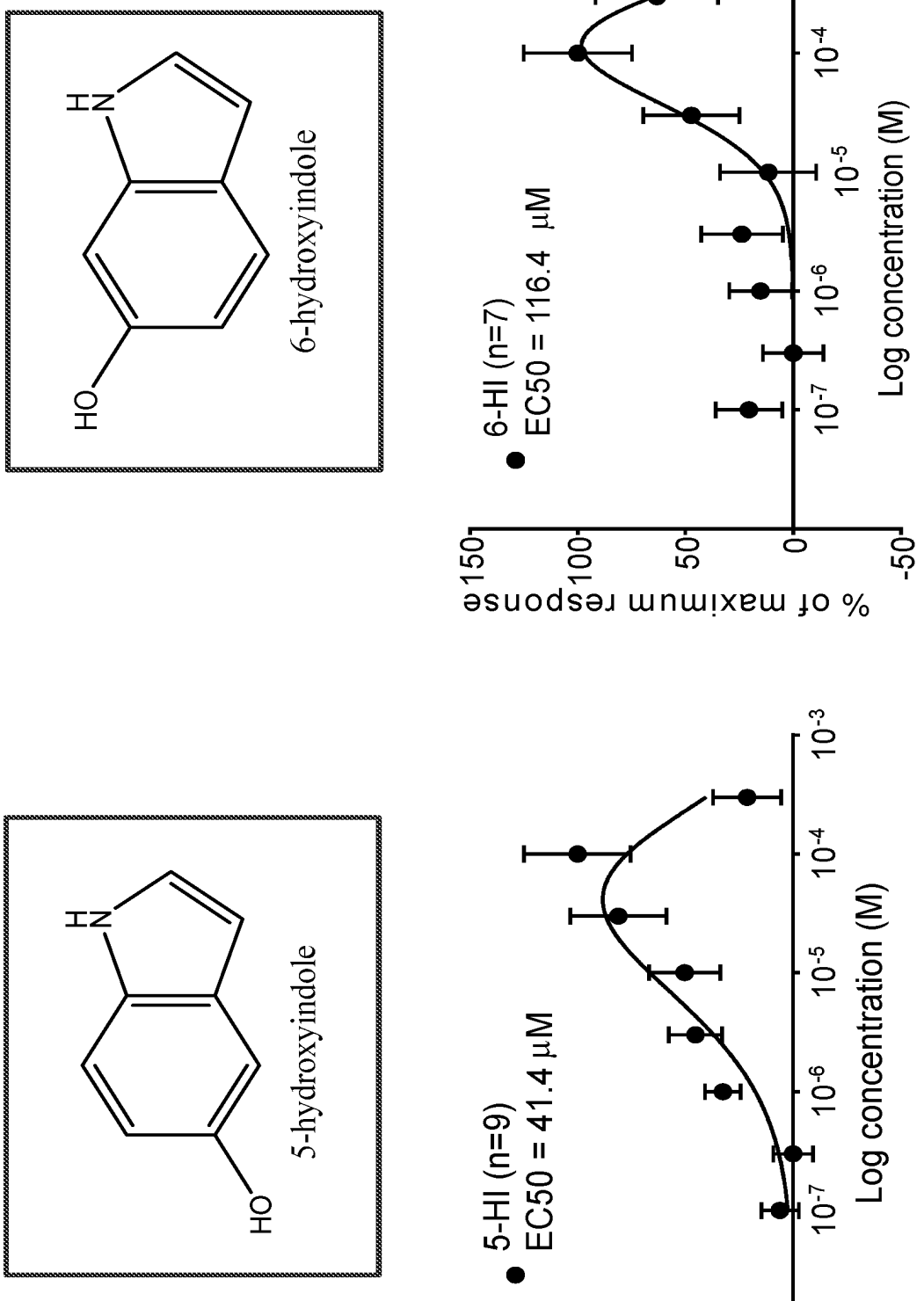
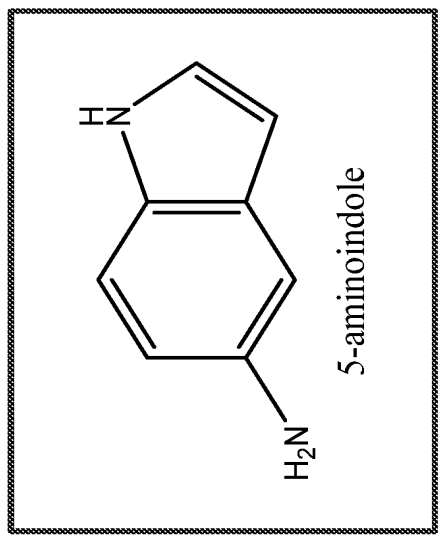
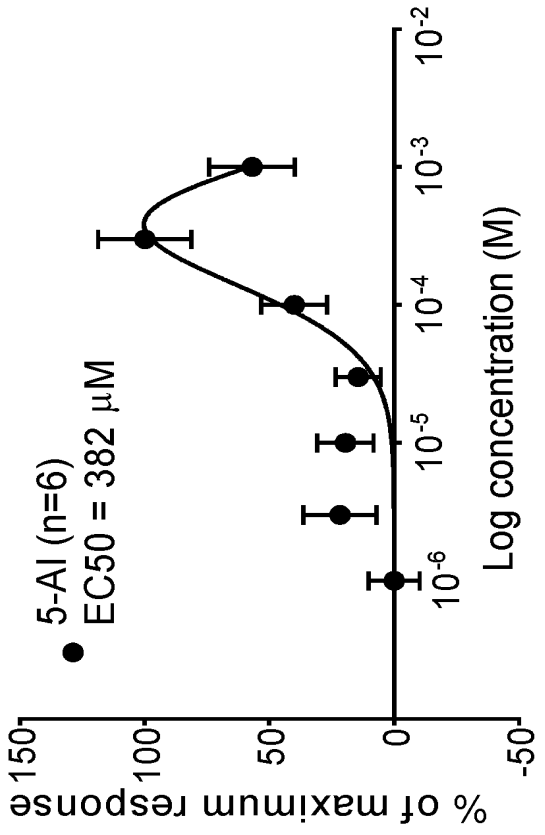
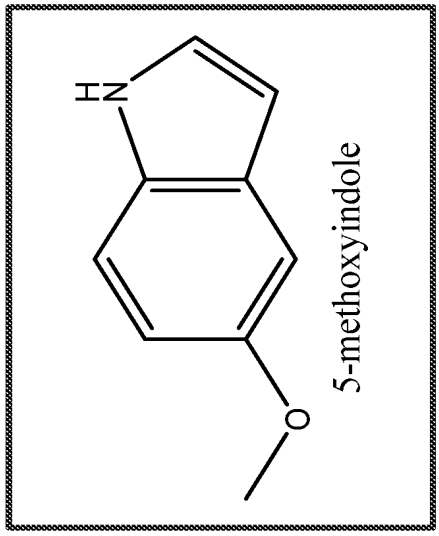

6-hydroxyindole
HO
N
H
6-HI (n=7)
EC50 = 116.4 µM
% of maximum response
150
100
50
0
-50
10-7
10-6
10-5
10-4
10-3
Log concentration (M)
5-hydroxyindole
HO
N
H
5-HI (n=9)
EC50 = 41.4 µM
% of maximum response
150
100
50
0
-50
10-7
10-6
10-5
10-4
10-3
Log concentration (M)

Fig. 11


5-aminoindole
H2N
HN


5-AI (n=6)
EC50 = 382 µM
% of maximum response
150
100
50
0
-50
10-6
10-5
10-4
10-3
10-2
Log concentration (M)


5-methoxyindole
O
HN

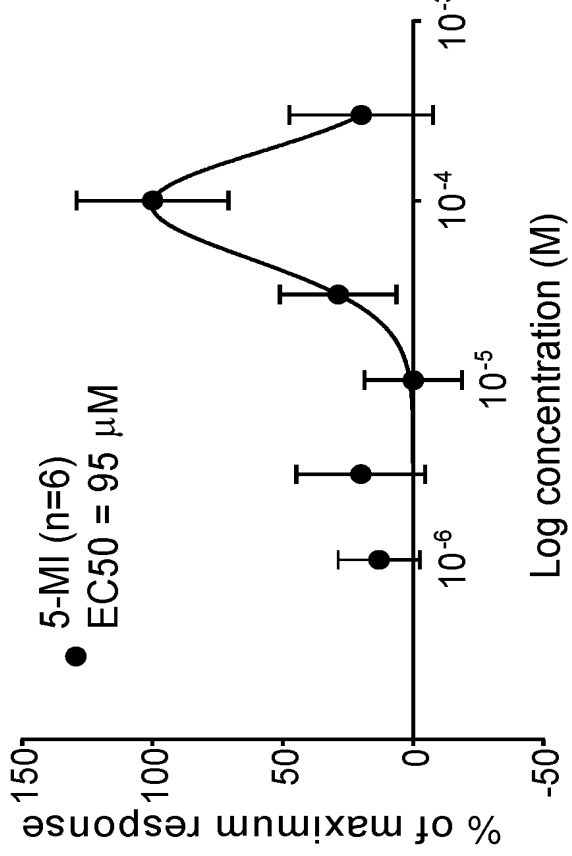
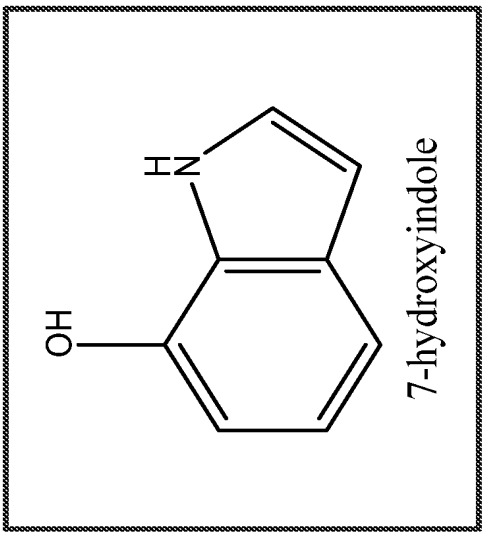
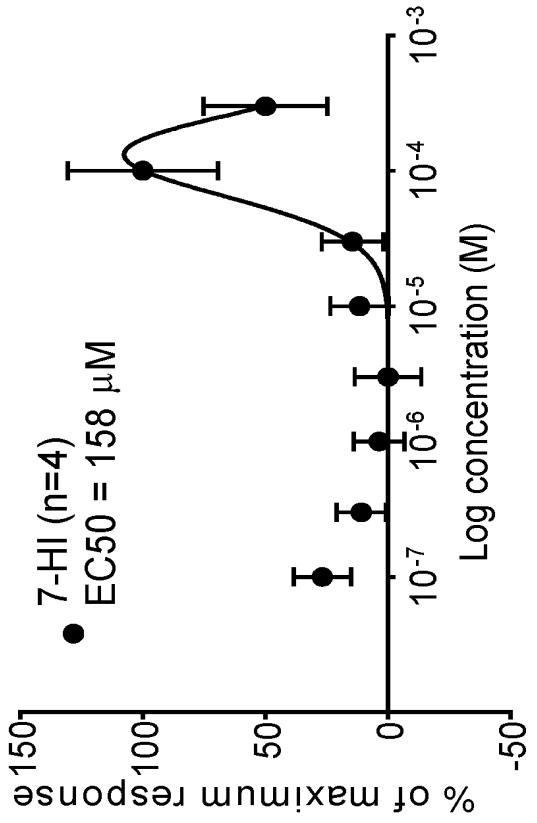
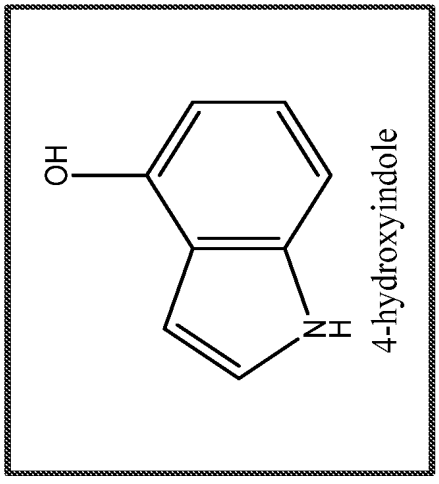

5-MI (n=6)
EC50 = 95 µM
% of maximum response
150
100
50
0
-50
10-6
10-5
10-4
10-3
Log concentration (M)

Fig. 11, Cont'd


OH
H
N
7-hydroxyindole


7-HI (n=4)
EC50 = 158 µM
% of maximum response
150
100
50
0
-50
10-7
10-6
10-5
10-4
10-3
Log concentration (M)


OH
H
N
4-hydroxyindole

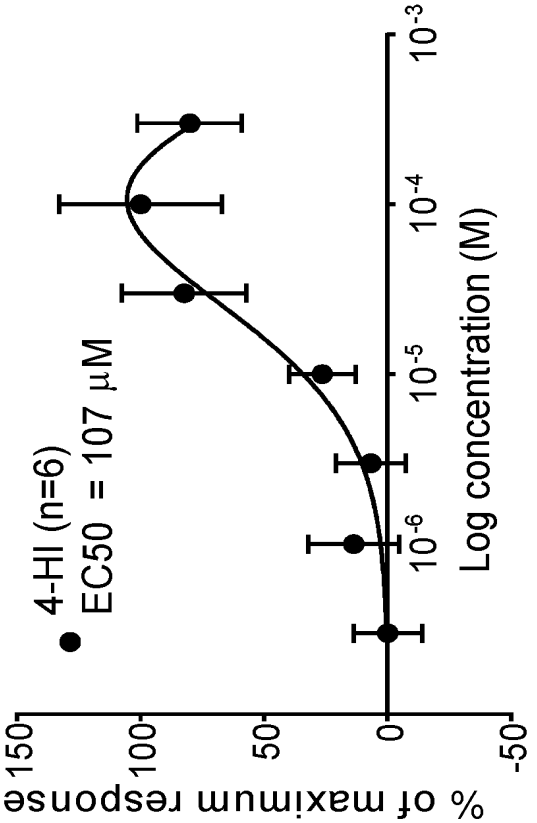

4-HI (n=6)
EC50 = 107 µM
% of maximum response
150
100
50
0
-50
10-6
10-5
10-4
10-3
Log concentration (M)

Fig. 11, Cont'd

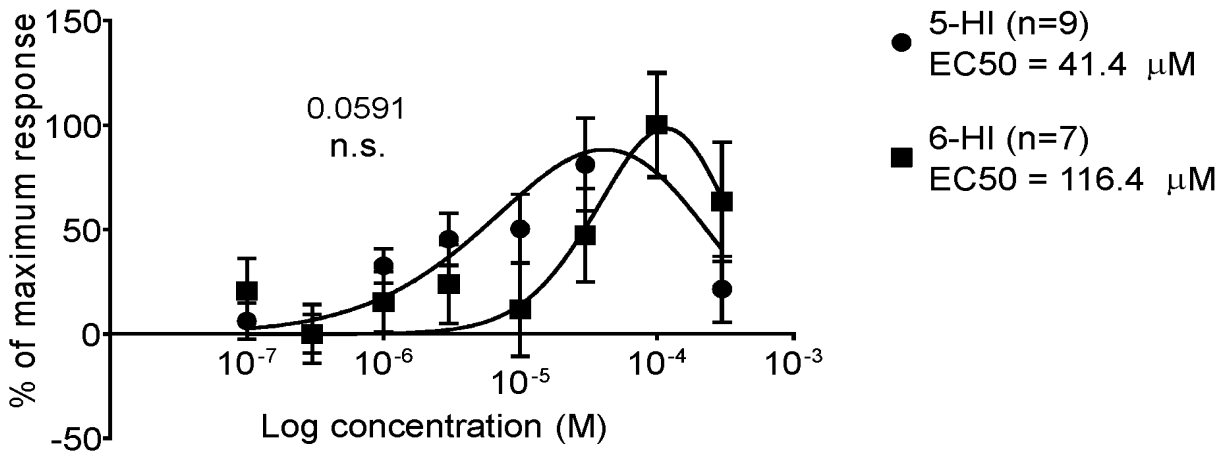


Fig. 12A

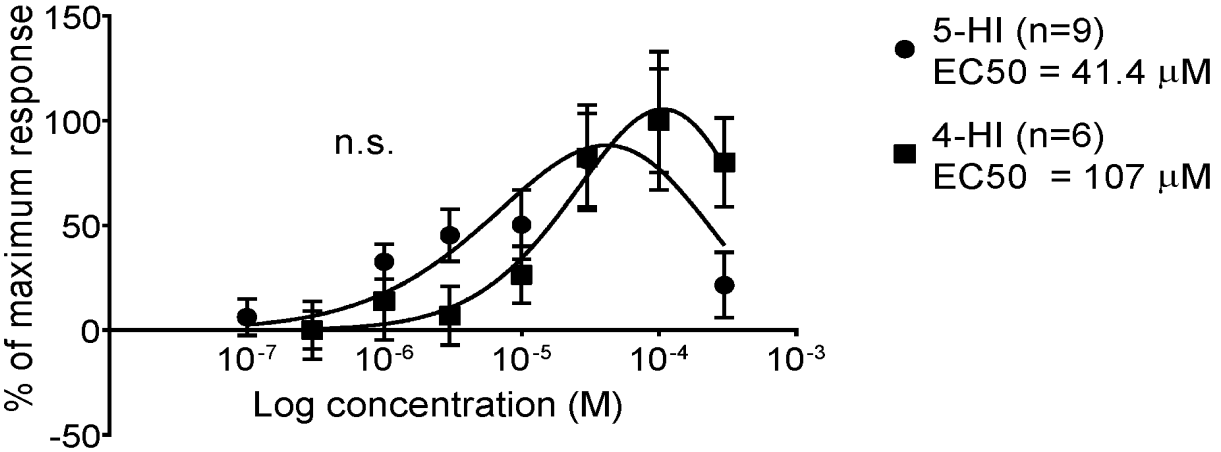


Fig. 12B

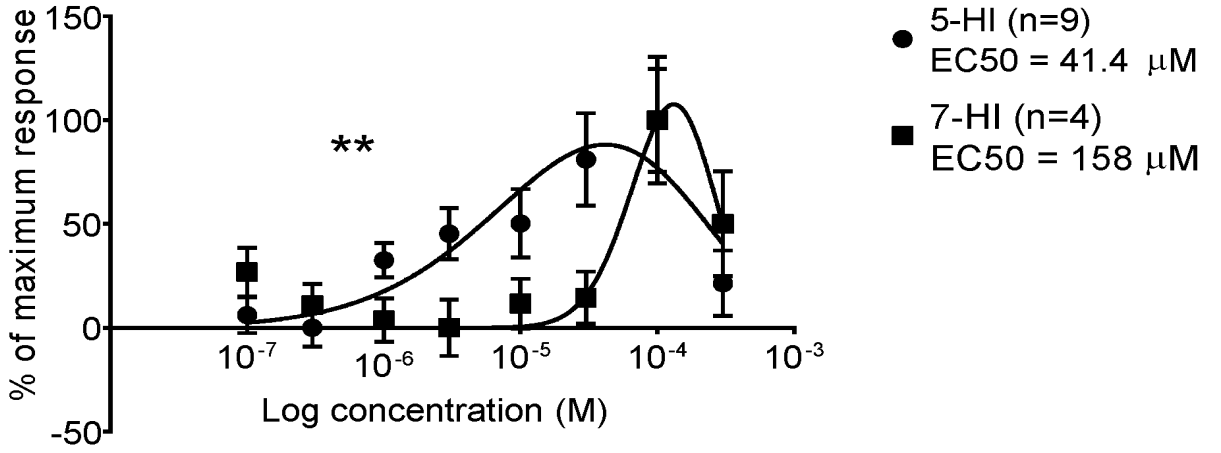


Fig. 12C

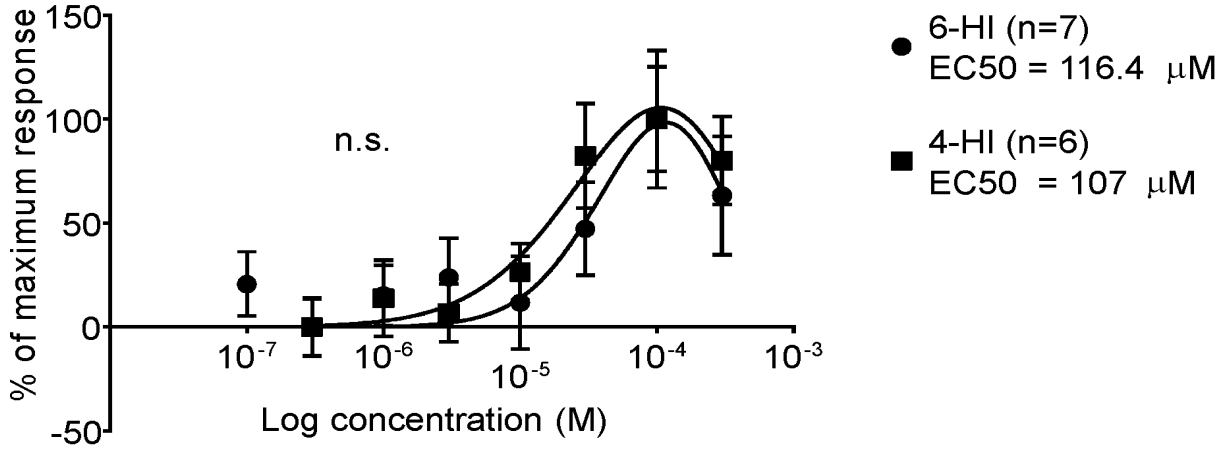


Fig. 12D

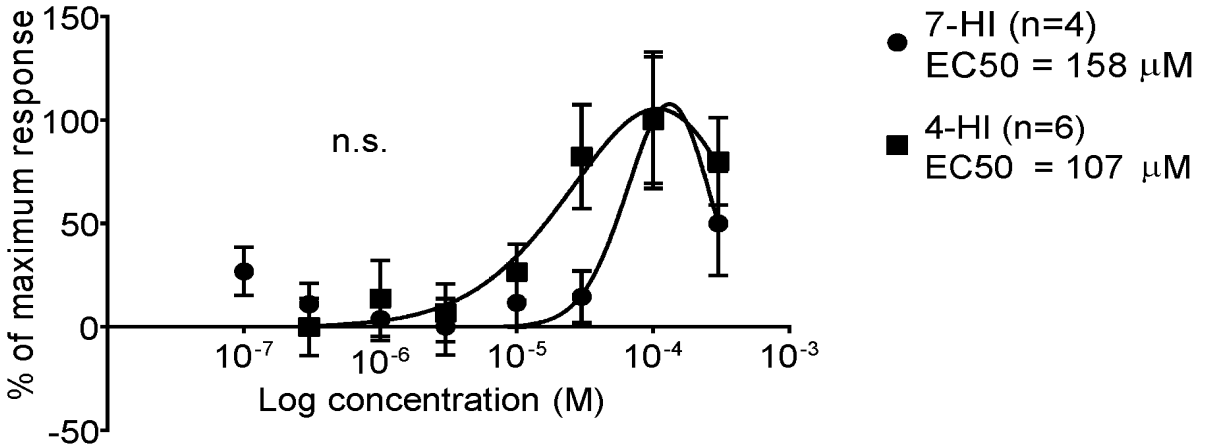


Fig. 12E

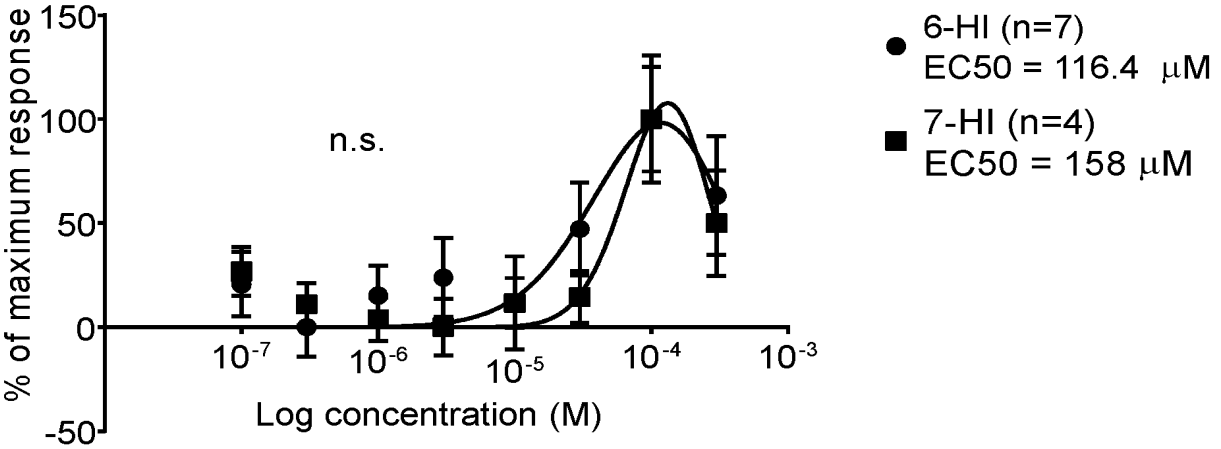
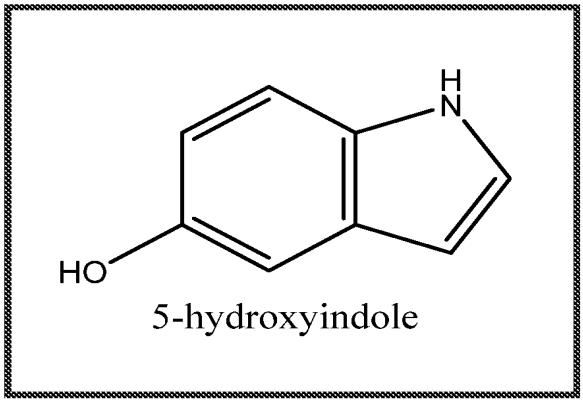
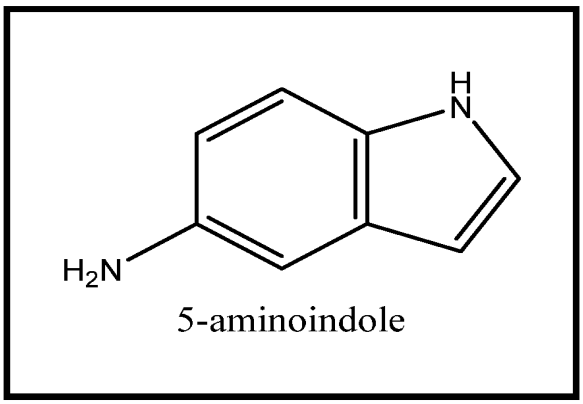


Fig. 12F


HO
H
N
5-hydroxyindole


H2N
H
N
5-aminoindole

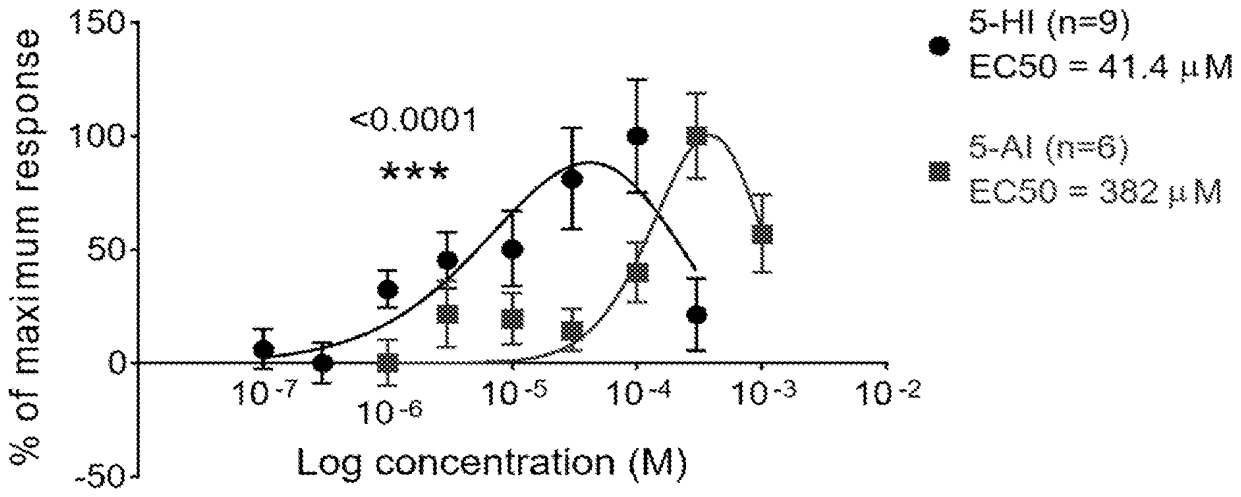
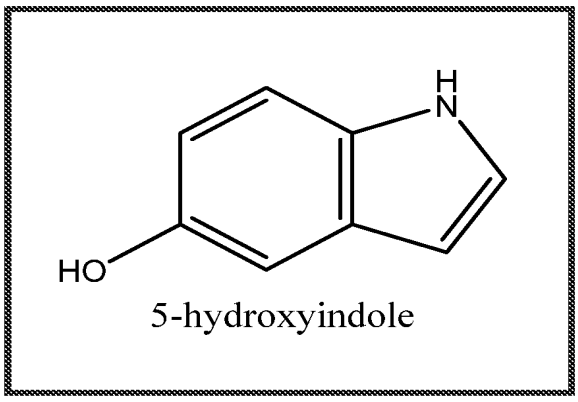
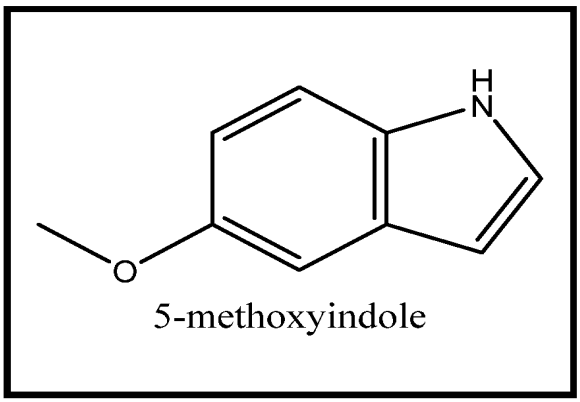

% of maximum response
150
100
50
0
-50
<0.0001
***
10-7
10-6
10-5
10-4
10-3
10-2
Log concentration (M)
5-HI (n=9)
EC50 = 41.4 µM
5-AI (n=6)
EC50 = 382 µM

Fig. 13A



5-hydroxyindole



5-methoxyindole

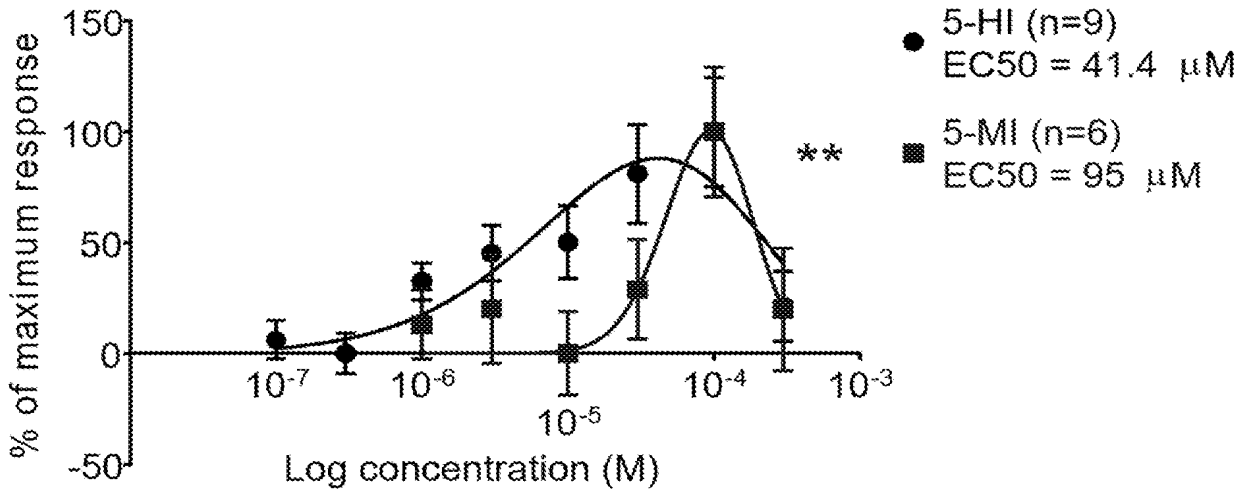
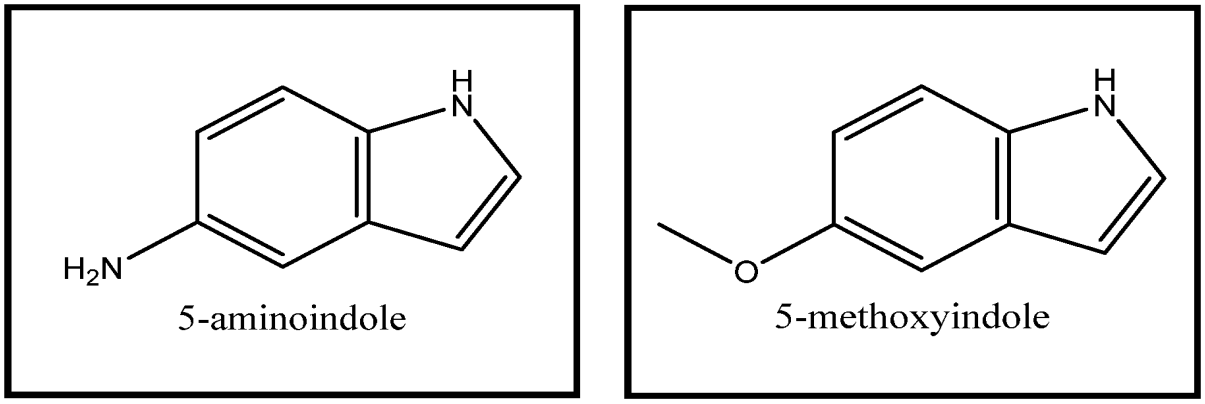


Fig. 13B


H
N
H_2N
5-aminoindole
H
N
O
5-methoxyindole

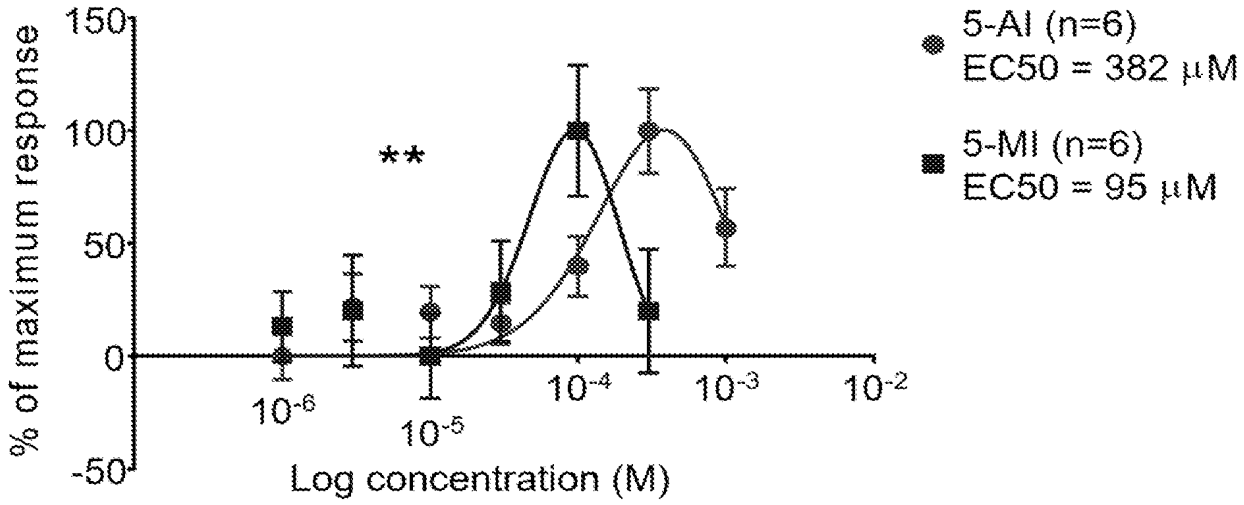

150
100
50
0
-50
% of maximum response
**
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Log concentration (M)
5-AI (n=6)
EC50 = 382 μM
5-MI (n=6)
EC50 = 95 μM

Fig. 13C

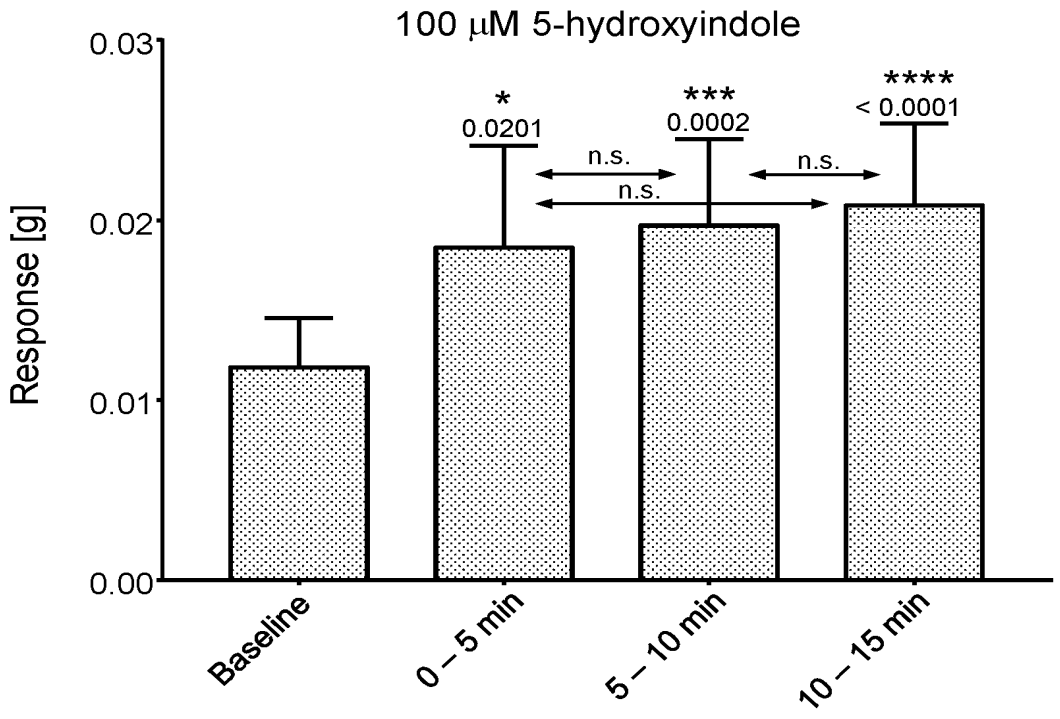


Fig. 14A

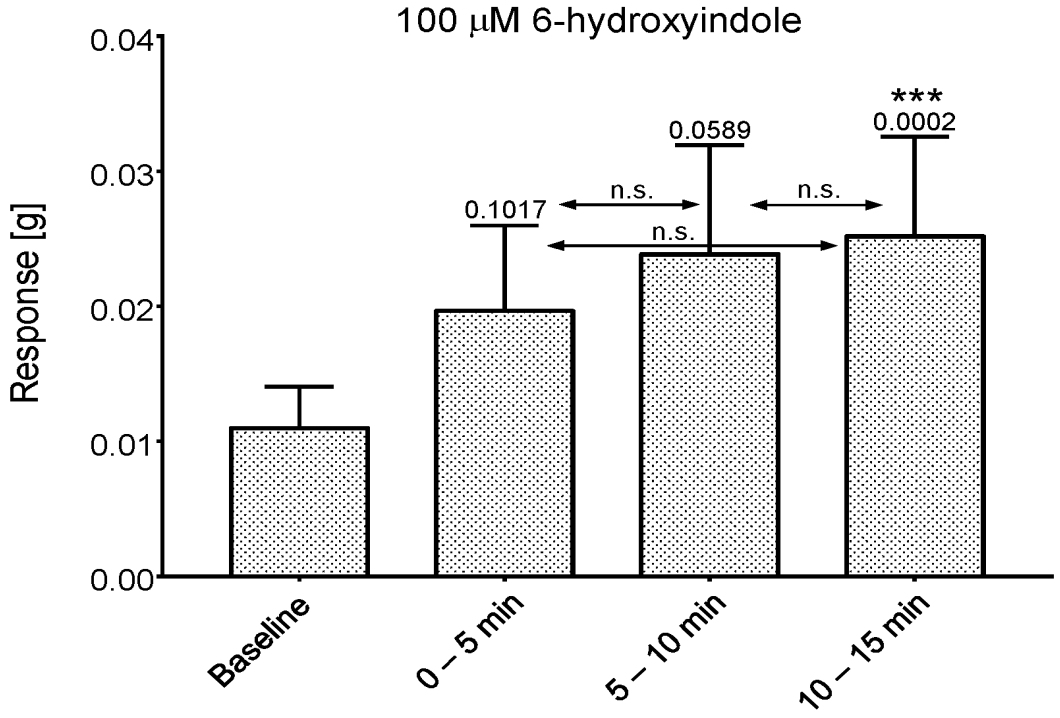


Fig. 14B

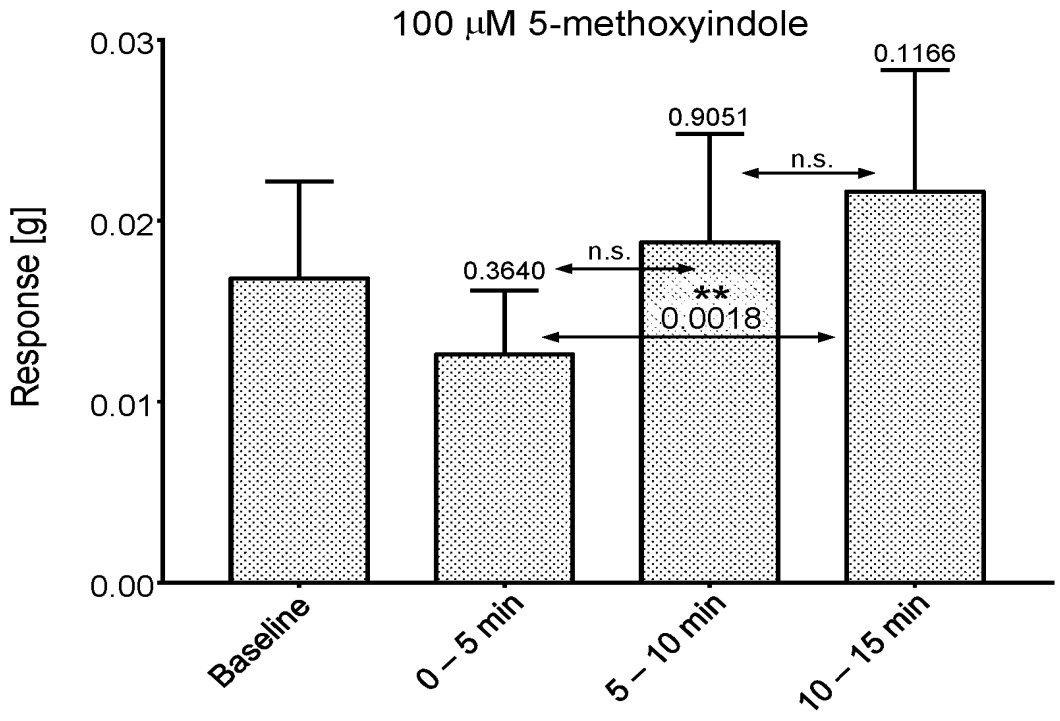


Fig. 14C

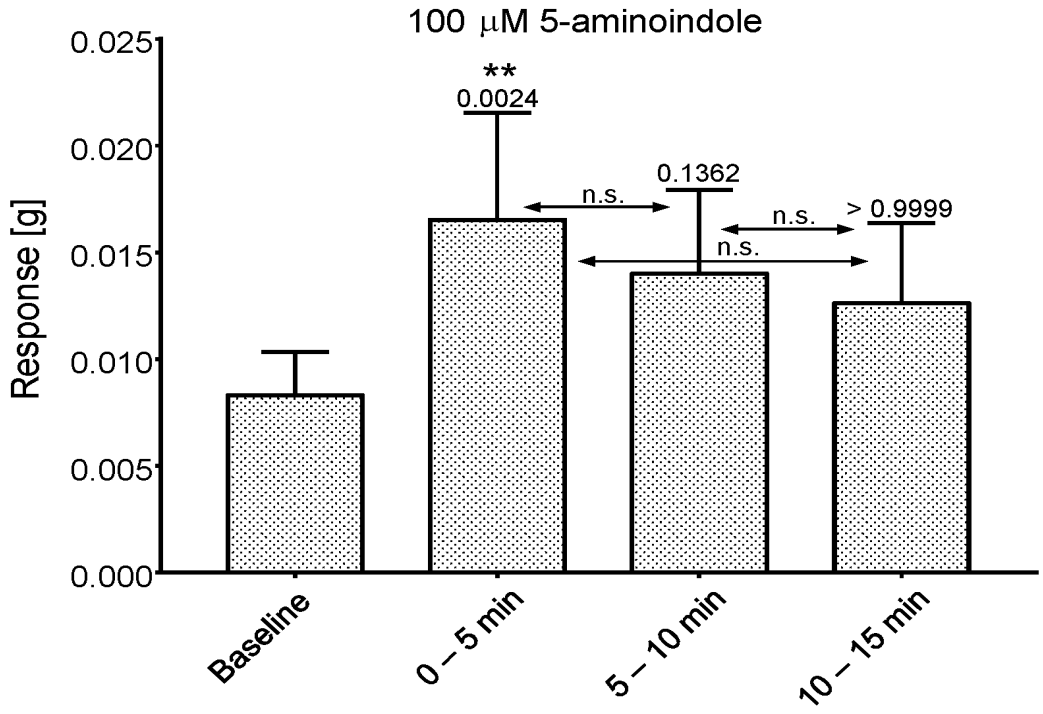


Fig. 14D

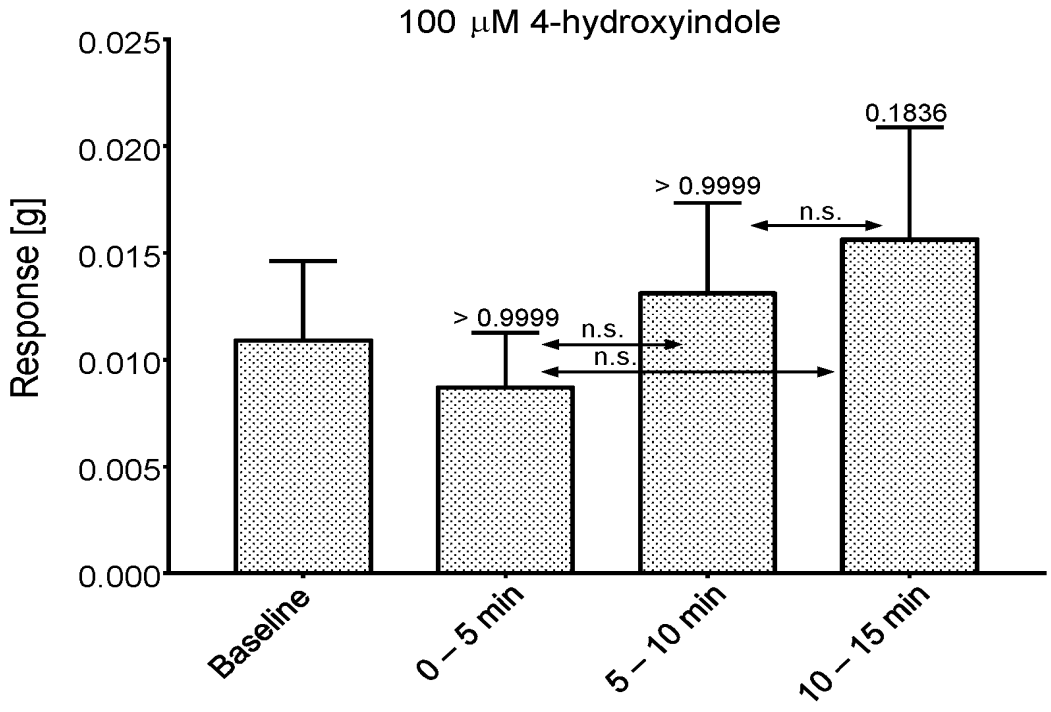


Fig. 14E

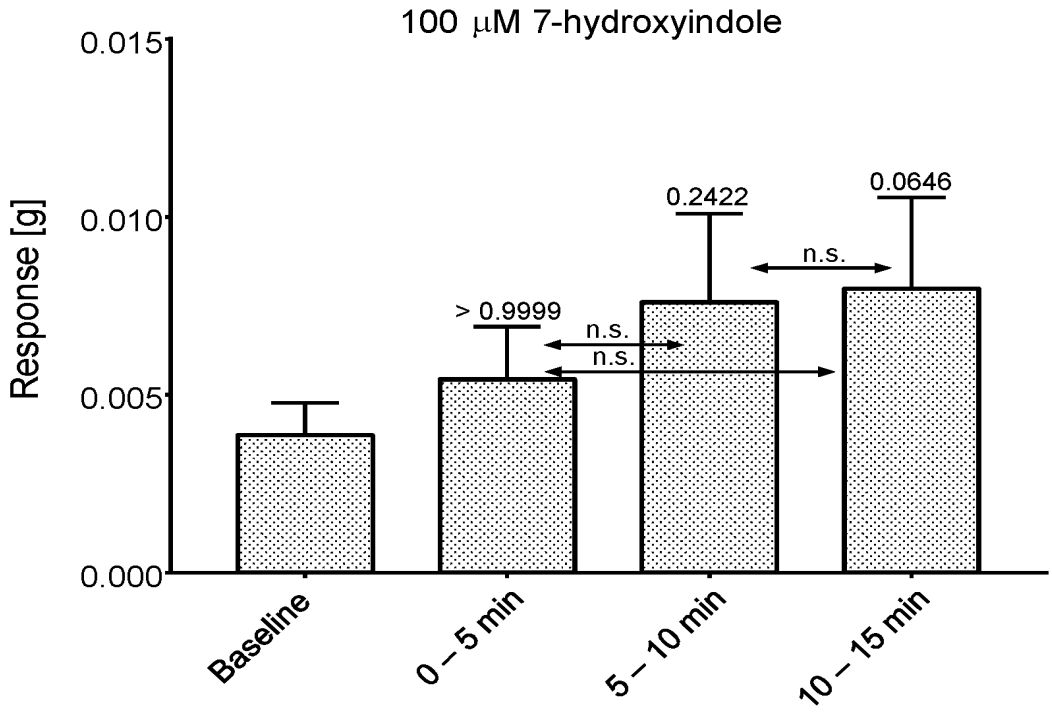


Fig. 14F

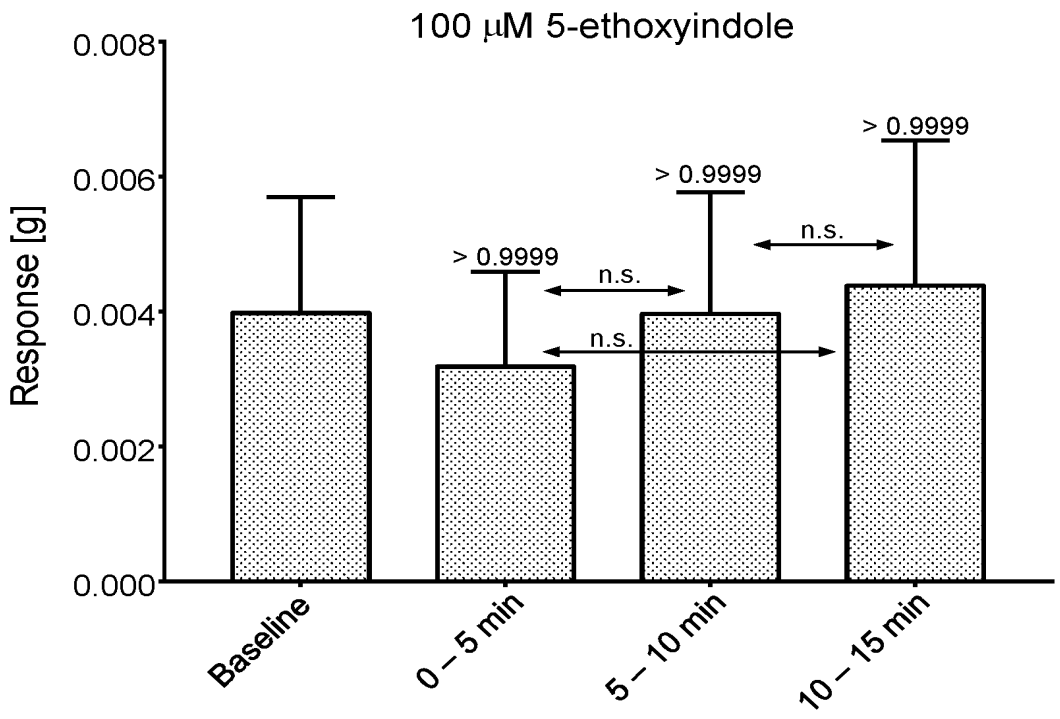

100 μM 5-ethoxyindole
Response [g]
0.008
0.006
0.004
0.002
0.000
> 0.9999
> 0.9999
> 0.9999
n.s.
n.s.
n.s.
Baseline
0 – 5 min
5 – 10 min
10 – 15 min

Fig. 14G

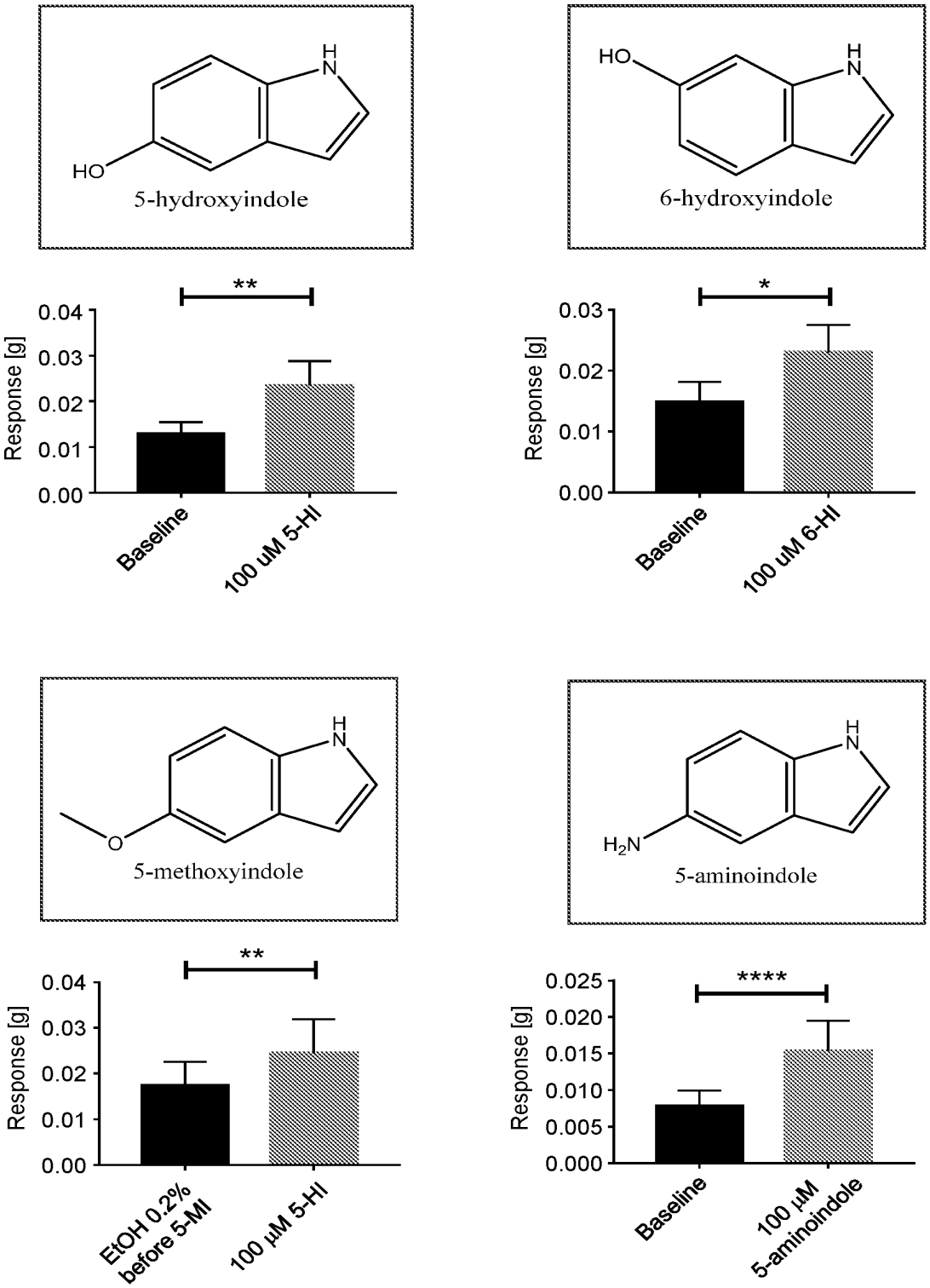

HO
H
N
5-hydroxyindole
HO
H
N
6-hydroxyindole
**
Response [g]
0.04
0.03
0.02
0.01
0.00
Baseline
100 uM 5-HI
*
Response [g]
0.03
0.02
0.01
0.00
Baseline
100 uM 6-HI
O
H
N
5-methoxyindole
H2N
H
N
5-aminoindole
**
Response [g]
0.04
0.03
0.02
0.01
0.00
EtOH 0.2%
before 5-MI
100 µM 5-HI
****
Response [g]
0.025
0.020
0.015
0.010
0.005
0.000
Baseline
100 µM
5-aminoindole

Fig. 15

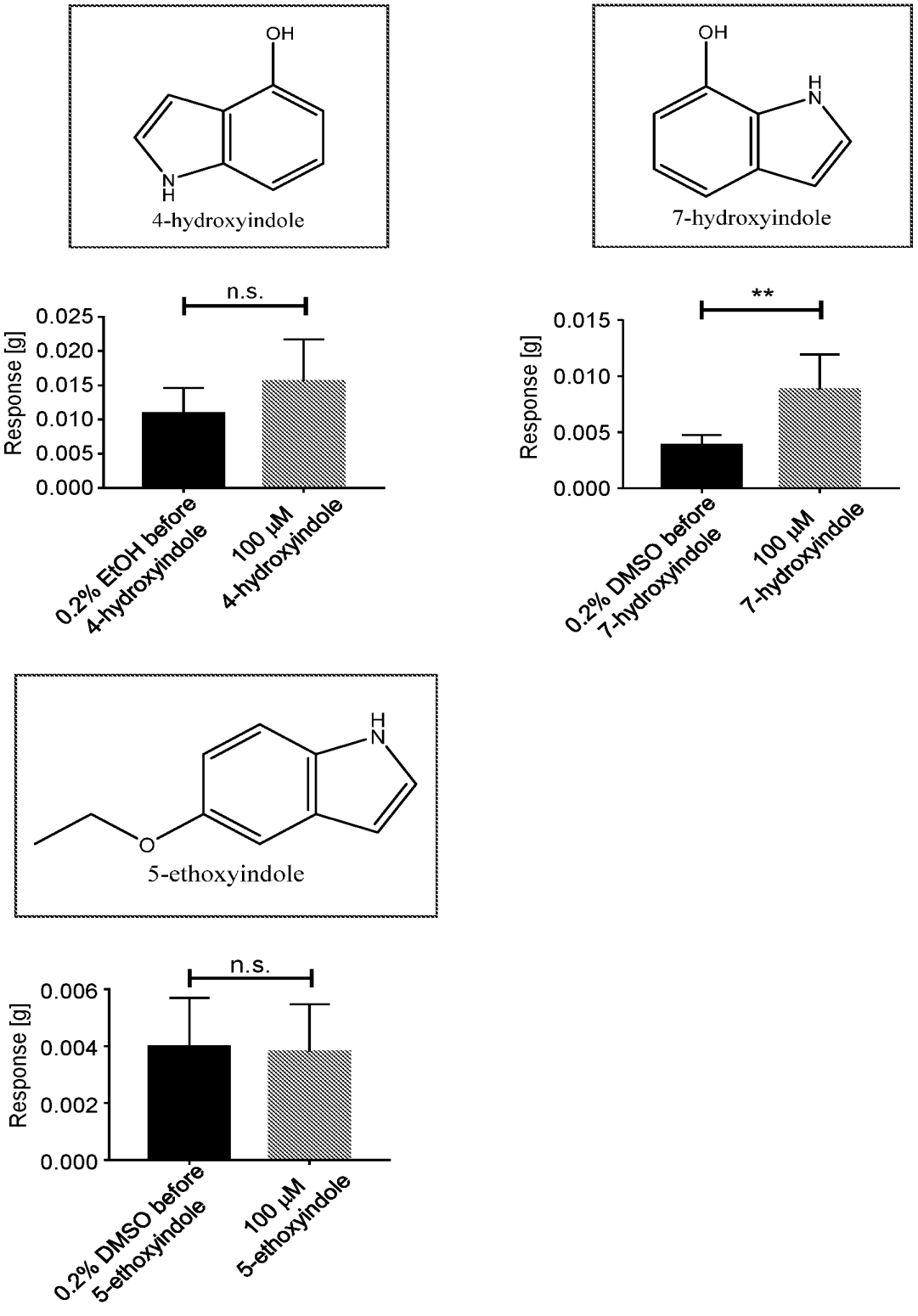

OH
N
H
4-hydroxyindole
OH
H
N
7-hydroxyindole
n.s.
Response [g]
0.025
0.020
0.015
0.010
0.005
0.000
0.2% EtOH before 4-hydroxyindole
100 µM 4-hydroxyindole
**
Response [g]
0.015
0.010
0.005
0.000
0.2% DMSO before 7-hydroxyindole
100 µM 7-hydroxyindole
H
N
O
5-ethoxyindole
n.s.
Response [g]
0.006
0.004
0.002
0.000
0.2% DMSO before 5-ethoxyindole
100 µM 5-ethoxyindole

Fig. 15, Cont'd

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2021/050434

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/404 A61P1/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/161268 A1 (TEXAS A & M UNIV SYS [US]) 21 September 2017 (2017-09-21)	1-3, 5-15, 17-19
A	claim 23	16,20,21

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 November 2021	18/11/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Trifilieff, Sylvie

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2021/050434

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Waclawiková Barbora ET AL: "Gut bacteria-derived 5-hydroxyindole is a potent stimulant of intestinal motility via its action on L-type calcium channels", PLoS biology, 1 January 2021 (2021-01-01), page e3001070, XP55853046, United States DOI: 10.1371/journal.pbio.3001070 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7857600/pdf/pbio.3001070.pdf [retrieved on 2021-10-20] the whole document	1-21
A	WO 2014/125084 A1 (TECH UNIVERSITÄT MÜNCHEN [DE]) 21 August 2014 (2014-08-21) claims 12,16	1-21
A	US 7 732 479 B2 (UNIV TEL AVIV FUTURE TECH DEV [IL]) 8 June 2010 (2010-06-08) column 14, line 65; claim 1; figure 4	1-21

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2021/050434

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2021/050434

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017161268 A1	21-09-2017	EP 3429361 A1	23-01-2019
		EP 3430128 A1	23-01-2019
		EP 3430395 A1	23-01-2019
		US 2019032014 A1	31-01-2019
		US 2019083462 A1	21-03-2019
		US 2020123498 A1	23-04-2020
		WO 2017161268 A1	21-09-2017
		WO 2017161301 A1	21-09-2017
		WO 2017161307 A1	21-09-2017
WO 2014125084 A1	21-08-2014	EP 2956450 A1	23-12-2015
		US 2015376164 A1	31-12-2015
		WO 2014125084 A1	21-08-2014
US 7732479 B2	08-06-2010	AT 539745 T	15-01-2012
		EP 1793816 A2	13-06-2007
		US 2008194667 A1	14-08-2008
		US 2010240726 A1	23-09-2010
		WO 2006018850 A2	23-02-2006