Supplementary material

1 Materials and Methods

1.1 Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured as recommended and grown to a relative density of 40–70%. For radioligand-binding assays, cells were transfected with 35 µg DNA using TransIT®-293 transfection reagent (Mobitec, Goettingen, Germany). The cDNA mixture comprised 20% 5-HT3A cDNA and 80% 5-HT3C cDNA. For aequorin assays, 15 µg plasmid DNA were transfected. The cDNA mixture comprised 67% apo-aequorin cDNA, 8.25% 5-HT3A cDNA, and 24.75% 5-HT3C cDNA. Cells were analyzed 48 h post transfection. During cultivation, cells were maintained in a humidified atmosphere at 37 ℃ and 5% CO_2.

1.2 Expression constructs

The human 5-HT3 subunit encoding cDNAs from *HTR3A* and *HTR3C* (GenBank accession numbers: D49394 and AF459285) were cloned into a pcDNA3 expression vector (ThermoFisher Scientific, Waltham, Massachusetts, the US). A mutant of the 5-HT3C subunit was constructed by site-directed mutagenesis in which the Asn (N) residue 163 was replaced with Lys (K) using the "Quick change" site-directed mutagenesis system (Stratagene, La Jolla, California, the US). After cloning, the fidelity of the cDNA sequences was verified by sequence analysis using the MEGABACE system (GE Healthcare, Munich, Germany) as indicated by the manufacturer. Aequorin cDNA (GenBank accession number L29571) encoding a jellyfish photoprotein was originally derived from cytAEQ/pcDNA1 (ThermoFisher Scientific, Waltham, Massachusetts, the US) and subcloned into *Hin*dIII/*Xba*I-digested pcDNA 3.1/Zeo (+) (ThermoFisher Scientific, Waltham, Massachusetts, the US).

1.3 Drugs

5-HT creatinine sulfate from Sigma-Aldrich (St. Louis, Missouri, the US) was used. [^{3}H]-3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone ([^{3}H]GR65630, specific activity 77.2 Ci/mmol resp. 86 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Boston, Massachusetts, the US).

1.4 Solutions

Drug solutions were prepared daily from aqueous stocks (stored at –20 ℃).

1.5 Membrane preparation and radioligand-binding assay

To prepare crude membranes, transiently transfected cells from a 175 cm^2 cell culture flask were harvested 48 h post transfection and washed once with binding assay buffer (NaCl 150 mM; $CaCl_2$ 1.8 mM; $MgCl_2$ 1 mM; HEPES 10 mM; KCl 5.4 mM; pH 7.4). All steps were carried out on ice. After cells were resuspended in 2 ml buffer, they were homogenized with a glass potter and pelleted by centrifugation (1000 x g, 4 min, 4 ℃). Supernatant was re-centrifuged in an ultracentrifuge (40,000 x g, 25 min, 4 ℃). After the pellet was washed once with buffer, an additional centrifugation step followed (40,000 x g, 25 min, 4 ℃). The final pellet was resuspended in 0.5 ml buffer and homogenized by pipetting through a 0.4 x 20 mm-gauge needle. Protein measurement of 10 µl membrane suspension was done by the method of Lowry et al. [1] using bovine serum albumin as standard. Membranes were diluted to a protein concentration of 0.33 µg/µl and stored at -80 ℃ until use.

For saturation experiments, 4 µg of membranes were incubated in triplicates with six increasing concentrations of [^{3}H]GR65630 (0.02–1.5 nM) in a final reaction volume of 0.5 ml. After an incubation time of 60 min at room temperature, incubation mixes were filtered through GF/B-filters (Whatman, Kent, the UK) using a Brandel cell harvester and washed

three times with 2 ml of ice-cold buffer. Radioactivity was measured in a liquid scintillation counter (Beckman, Fullerton, California, the US). Non-specific binding was determined using mock-transfected cells.

1.6 Aequorin luminescence assay

1.6.1 Cell preparation

HEK 293 cells were harvested by centrifugation (180 x g, 4 min) 48 h post transfection and resuspended in 1.5 ml of DMEM/Ham's F12 + 10% FCS. From this point, all steps were performed under light protection. Cell suspension was supplemented with coelenterazine (Nanolight, Pinetop, Arizona, the US) at a final concentration of 5 µM and incubated for 3 h at room temperature. Following coelenterazine incubation, cells were harvested by centrifugation (45 x g, 3 min) and resuspended in aequorin assay buffer (NaCl 150 mM; $CaCl_2$ 1.8 mM; KCl 5.4 mM; HEPES 10 mM; glucose 20 mM; pH 7.4) to obtain an approximate cell density of 2.5–3.5 x 10^{-6} cells/ml. An incubation time of 10–20 min at room temperature followed.

1.6.2 Aequorin assay

For agonist concentration response curves, a white 96-well Teflon plate with 80 µl of cell suspension per well was placed into a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). Prior to injection of the agonist, baseline luminescence was measured for 5 s at a sampling rate of 2 Hz. After injection of 20 µl agonist solution to the cells, light emission was recorded for 15–60 s at the same sampling rate.

Each drug concentration was measured in quadruplicate per transfection. After measurement of the agonist-induced light signal, the remaining aequorin luminescence was determined by injecting 100 µl of cell lysis solution (Triton X-100 0.1% [vol/vol]; $CaCl_2$ 50 mM) and recording luminescence at 0.5-s intervals for 15 s in the case of 5-HT maximal responses.

2 Data analysis

Peak values for the concentration response curves were obtained by subtracting baseline luminescence from the agonist-induced peak maximum luminescence. In the case of 5-HT maximal responses, peak luminescence (RLU_{peak}) was normalized against total aequorin luminescence (RLU_{max}) after cell lysis to control for differences in transfection efficiency and cell number ($RLU_{peak}/RLU_{peak} + RLU_{max}$). Concentration-response curves, saturation binding curves, EC_{50} constants, and the binding constants K_d and B_{max} were calculated using GraphPad Prism® 8.0 (San Diego, California, US). Data are represented as mean±SEM. Data were analyzed using ANOVA followed by Dunnett's post-test. The unpaired Student's t-test was used to compare results between two groups. Differences were considered significant with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3 Results of the UK and Ireland cohorts

These results should be interpreted with caution because the sample sizes were limited and because the UK cohort only included participants with IBS-D.

3.1 *HTR3* SNP analysis using the dominant and the recessive model

As shown in Supplementary Table 4, depressive symptoms became more severe with increasing numbers of minor *HTR3A* c.-42C>T alleles in the UK cohort according to the recessive model ($F = 4.056$, $p = 0.048$). Irish participants who were carriers of the homozygous minor allele *AA* genotype of *HTR3C* c.489C>A all had significantly more severe depressive and anxiety symptoms according to the recessive model ($F_{depressive} = 9.251$, $p_{depressive} = 0.005$; $F_{anxiety} = 8.584$, $p_{anxiety} = 0.007$).

3.2 Effect of SNP scores on depressive and anxiety symptoms

SNP scores ranged from 0 to 5 in the UK cohort and from 0 to 4 in the Irish cohort. No

significant differences in SNP scores were observed between sexes (F_{UK} = 0.274, p_{UK} = 0.602; $F_{Ireland}$ = 1.954, $p_{Ireland}$ = 0.173) or IBS subtypes ($F_{Ireland}$ = 0.412, $p_{Ireland}$ = 0.666). As shown in Supplementary Table 5, there was no significant trend between depressive or anxiety symptoms and increasing SNP scores in the two cohorts.

References

1. Lowry OH, Rosebrough NJ. Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J biol Chem.* 1951;193:265-275.

Supplementary Table 1 Genotype frequency of SNPs in total and separated for sex and IBS subtypes

		HTR3A c.-42C>T (rs1062613)			*HTR3B c.386A>C* (rs1176744)			*HTR3C c.489C>A* (rs6766410)			*HTR3E c.*76G>A* (rs62625044)		
		CC	*CT*	*TT*	*AA*	*AC*	*CC*	*CC*	*CA*	*AA*	*GG*	*GA*	*AA*
Total		61.5(374)	34.1 (207)	4.4 (27)	49.7 (302)	41.4 (252)	8.9 (54)	34.2 (208)	48.4 (294)	17.4 (106)	88.3 (537)	11.2 (68)	0.5 (3)
Sex	Male	64.1 (118)	32.1 (59)	3.8 (7)	55.4 (102)	35.9 (66)	8.7 (16)	38.6 (71)	45.7 (84)	15.8 (29)	85.9 (158)	13.6 (25)	0.5 (1)
	Female	60.4 (256)	34.9 (148)	4.7 (20)	47.2 (200)	43.9 (186)	9.0 (38)	32.3 (137)	49.5 (210)	18.2 (77)	89.4 (379)	10.1 (43)	0.5 (2)
χ^2 value			0.839			3.755			2.300			1.555	
p value			0.658			0.153			0.317			0.460	
IBS subtypes	IBS-C	54.0 (47)	39.1 (34)	6.9 6)	47.1 (41)	37.9 (33)	14.9 (13)	34.5 (30)	54.0 (47)	11.5 (10)	82.8 (72)	16.1 (14)	1.1 (1)
	IBS-D	66.7 (172)	28.3 (73)	5.0 (13)	50.0 (129)	40.7 (105)	9.3 (24)	34.9 (90)	45.0 (116)	20.2 (52)	86.8 (224)	12.4 (32)	0.8 (2)
	IBS-M	58.9 (155)	38.0 (100)	3.0 (8)	50.2 (132)	43.3(144)	6.5(17)	33.5 (88)	49.8 (131)	16.7 (44)	91.6 (241)	8.4 (22)	0 (0)
χ2 value			9.116			6.037			4.318			7.254	
p value			0.058			0.196			0.365			0.123	

Notes: Values are % (n). **Abbreviations:** IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; IBS-M, IBS with mixed bowel habits.

Supplementary Table 2 Allele frequency of SNPs in total and separated for sex and IBS subtypes

		HTR3A c.-42C>T (rs1062613)		*HTR3B c.386A>C (rs1176744)*		*HTR3C c.489C>A (rs6766410)*		*HTR3E c.*76G>A (rs62625044)*	
		C	*T*	*A*	*C*	*C*	*A*	*G*	*A*
Total		78.5 (955)	21.5(261)	70.4 (856)	29.6 (360)	58.4 (710)	41.6 (506)	93.9 (1142)	6.1 (74)
Sex	Male	80.2 (295)	19.8 (73)	73.4 (270)	26.6 (98)	61.4 (226)	29.6 (142)	92.7 (341)	7.3(27)
	Female	77.8 (660)	22.2 (188)	69.1 (586)	30.9 (262)	57.1 (484)	42.9 (364)	94.5 (801)	5.5 (47)
χ^2 value		0.829		2.241		1.987		1.446	
p value		0.363		0.134		0.159		0.229	
IBS	IBS-C	73.6 (128)	26.4 (46)	66.1 (115)	33.9(59)	61.5 (107)	38.5 (67)	90.8 (158)	9.2 (16)
subtypes	IBS-D	80.8 (417)	19.2 (99)	70.3 (363)	29.7 (153)	57.4 (296)	42.6 (220)	93.0 (480)	7.0 (36)
	IBS-M	77.9 (410)	22.1 (116)	469.6 (408)	30.4 (178)	58.4 (307)	42.6 (220)	95.8 (504)	4.2 (22)
χ^2 value		4.249		1.129		0.914		6.995	
p value		0.119		0.568		0.633		0.030	

Notes: Values are % (n). **Abbreviations:** IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; IBS-M, IBS with mixed bowel habits.

Supplementary Table 3 Hardy–Weinberg equilibrium evaluation

		Observed value	Expected value	χ^2 value	*p* value
HTR3A c.-42C>T (rs1062613)	*CC*	61.5 (374)	61.7 (375)	0.059	0.808
	CT	34.1 (207)	33.7 (205)		
	TT	4.4 (27)	4.6 (28)		
HTR3B c.386A>C (rs1176744)	*AA*	49.7 (302)	49.6 (301)	0.019	0.890
	AC	41.4 (252)	41.7 (253)		
	CC	8.9 (54)	8.7 (53)		
HTR3C c.489C>A (rs6766410)	*CC*	34.2 (208)	34.1 (207)	0.015	0.904
	CA	48.4 (294)	48.6 (295)		
	AA	17.4 (106)	17.3 (105)		
*HTR3E c.*76G>A* (rs62625044)	*GG*	88.3 (537)	88.2 (536)	0.282	0.595
	GA	11.2 (68)	11.5 (70)		
	AA	0.5 (3)	0.3 (2)		

Notes: Values are % (n).

Supplementary Table 4 Effect of *HTR3* SNPs on depressive and anxiety symptoms according to the dominant model and the recessive model in the UK and Ireland cohorts

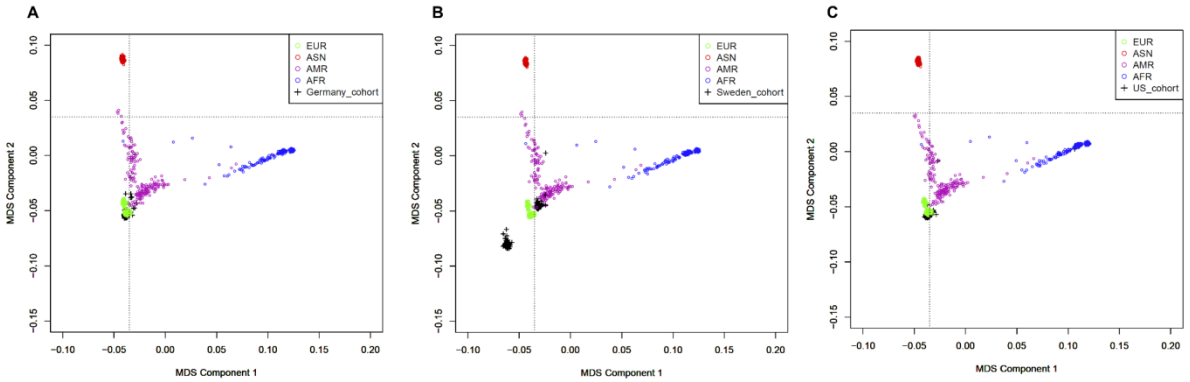
	Models	Mental symptoms	SNPs	Total	Sex		IBS subtypes		
					Male	Female	IBS-C	IBS-D	IBS-M
UK	**Dominant model**	***Depressive symptoms***	*HTR3A c.-42C>T (rs1062613)*	1.031	0.012	1.572		1.031	
			HTR3B c.386A>C(rs1176744)	0.728	3.425	0.003		0.728	
			HTR3C c.489C>A(rs6766410)	1.710	**5.605** ↑	0.022		1.710	
			*HTR3Ec.*76G>A(rs62625044)*	0.673	0.300	0.450		0.673	
		Anxiety symptoms	*HTR3A c.-42C>T (rs1062613)*	0.945	0.030	0.844		0.945	
			HTR3B c.386A>C(rs1176744)	0.520	2.457	0.001		0.520	
			HTR3C c.489C>A(rs6766410)	2.155	2.240	0.381		2.155	
			*HTR3Ec.*76G>A(rs62625044)*	0.380	0.041	1.165		0.380	
	Recessive model	***Depressive symptoms***	*HTR3A c.-42C>T (rs1062613)*	**4.056** ↑	0.300	**9.992** ↑		**4.056** ↑	
			HTR3B c.386A>C(rs1176744)	1.943	2.545	0.245		1.943	
			HTR3C c.489C>A(rs6766410)	0.016	0.253	0.072		0.016	
			*HTR3Ec.*76G>A(rs62625044)*	3.305	0.253	**4.188** ↑		3.305	
		Anxiety symptoms	*HTR3A c.-42C>T (rs1062613)*	0.378	0.041	0.655		0.378	
			HTR3B c.386A>C(rs1176744)	0.689	2.317	0.253		0.689	
			HTR3C c.489C>A(rs6766410)	0.883	0.082	1.025		0.883	
			*HTR3Ec.*76G>A(rs62625044)*	0.830	0.072	1.561		0.830	
Ireland	**Dominant model**	***Depressive symptoms***	*HTR3A c.-42C>T (rs1062613)*	1.492	0.219	1.088	0.154	0.333	0.694
			HTR3B c.386A>C(rs1176744)	0.001	0.571	0.153	3.600	0.333	0.002
			HTR3C c.489C>A(rs6766410)	**4.483** ↑	0.160	**4.356** ↑	0.800		3.875
			*HTR3Ec.*76G>A(rs62625044)*	0.000	0.219	0.164			0.084
		Anxiety symptoms	*HTR3A c.-42C>T (rs1062613)*	2.605	0.159	2.022	0.000	0.000	3.643
			HTR3B c.386A>C(rs1176744)	0.015	0.628	0.033	**32.000**↓		0.215
			HTR3C c.489C>A(rs6766410)	0.976	0.173	0.506	0.125		1.436
			*HTR3Ec.*76G>A(rs62625044)*	0.100	0.159	0.451			0.064
	Recessive model	***Depressive symptoms***	*HTR3A c.-42C>T (rs1062613)*	0.098		0.090			0.177
			HTR3B c.386A>C(rs1176744)	0.047		0.043			
			HTR3C c.489C>A(rs6766410)	**9.251** ↑	3.226	**5.690** ↑			**12.128**↑
			*HTR3Ec.*76G>A(rs62625044)*						
		Anxiety symptoms	*HTR3A c.-42C>T (rs1062613)*	2.447		2.482			1.763
			HTR3B c.386A>C(rs1176744)	0.158		0.214	0.429		
			HTR3C c.489C>A(rs6766410)	**8.584** ↑	3.947	**5.345** ↑			**6.622** ↑
			*HTR3Ec.*76G>A(rs62625044)*						

Notes: F values are shown in the table. Arrows represent the direction of the associations. ↑, dependent variable increases from major to minor alleles; ↓, dependent variable decreases from major to minor alleles. **Abbreviations:** IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; IBS-M, IBS with mixed bowel habits.

Supplementary Table 5 Association between depressive and anxiety symptoms and SNP score of the four tested polymorphisms in the UK and Ireland cohorts

			Total	Sex		IBS subtypes		
				Male	Female	IBS-C	IBS-D	IBS-M
UK	***Depressive symptoms***	*F* value	2.050	1.818	0.416	-	2.050	-
		p-trend value	0.157	0.175	0.522	-	0.157	-
	Anxiety symptoms	*F* value	0.064	4.225	2.235	-	0.064	-
		p-trend value	0.801	0.057	0.141	-	0.801	-
Ireland	***Depressive symptoms***	*F* value	4.118	1.306	3.250	0.375	0.333	2.784
		p-trend value	0.054	0.371	0.087	0.756	0.667	0.114
	Anxiety symptoms	*F* value	3.099	1.110	2.124	0.563	-	4.423
		p-trend value	0.091	0.403	0.161	0.686	-	0.051

Abbreviations: IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; IBS-M, IBS with mixed bowel habits.



Supplementary Figure 1 Results of the population stratification test. A. Population stratification test of German participants; B. Population stratification test of Swedish participants; C. Population stratification test of the US participants. Multidimensional scaling plot of the Germany, Sweden, and US cohorts against 1000 Genomes Project data. The 10 main components were used as covariates in the association tests to correct for any remaining population stratification.