SUPPLEMENTAL INFORMATION

The nucleus accumbens 5-HTR_4-CART pathway ties anorexia to hyperactivity

Alexandra Jean, PhD[1,2,3,4]*, Laetitia Laurent, PhD[1,2,3]*, Joel Bockaert, PhD[1,2,3], Yves Charnay, PhD[5], Nicole Dusticier[6], André Nieoullon, PhD[6], Michel Barrot, PhD[7], Rachael Neve, PhD[8] and Valérie Compan, PhD[1,2,3,4]

*Authors equally contribute to this study

[1] CNRS, UMR-5203, Institut de Génomique Fonctionnelle, Montpellier, F-34094, France
[2] INSERM, U661, Montpellier, F-34094, France
[3] Universités de Montpellier 1 & 2, UMR-5203, Montpellier, F-34094, France
[4] Université de Nîmes, Nîmes, F-30000, France

[5]Hôpitaux Universitaires de Genève, Division de Neuropsychiatrie,
CH-1225 Chêne-Bourg, Switzerland.

[6]CNRS UMR7288, Université d'Aix-Marseille,
F-13 288 Marseille, France.

[7]CNRS UPR3212, Institut des Neurosciences Cellulaires et Intégratives,
F-67084 Strasbourg, France.

[8]Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology,
Cambridge, MA 02139-4307, USA.

Correspondence: Valerie.Compan@igf.cnrs.fr, Tel: (33) 4 34 35 93 10, Fax: (33) 4 67 54 24 32

List of Supplemental Information:

Supplemental Experimental Procedures

Animals

All experiments were performed using male 129/SvTer mice[1, 2], housed in groups of 5 per cage of the same genetic background and sex with food (pellet form, 16.5% crude proteins, 3.6% crude fat, 4.6% crude fibers, 5.2% ash) and tap water available *ad libitum* in a temperature - controlled environment with a 12-h light/dark cycle (light onset at 07:00) at our animal facility (Institut de Génomique Fonctionnelle, France). Male 129/SvPas mice were obtained from Charles River Laboratories (L'Arbresle, France). Mice were habituated to the testing room 8 days until experiments, performed in accordance with the *Guide for Care and Use of Laboratory Animals* (CNRS and the Regional Ethical Committee's guidelines, project agreement n°C34-172-13; authorization n° 21CAE011).

Surgery

As described[3], mice were anesthetized by i.p. injection of ketamine (60 mg/kg) and xylasine (15 mg/kg) and placed in a David Kopf stereotaxic frame. Each compound used (see below) was dissolved in NaCl (9‰) and stored at 4°C until use. A sterile 26-gauge stainless steel guide was unilaterally implanted in the left shell NAc at the following coordinates from the bregma: A + 1.6 mm, H - 4.3 mm, L + 0.07 mm according to the brain atlas.[4] Each compound was infused in the NAc (1 µl/min) using a microsyringe nanopump (CE, Myneurolab, St Louis, MO) in freely moving animals. The localization of the injection site was systematically assessed for each mouse.

Pharmacological and nucleic acid treatments in freely moving mice

As previously established[3], the sequences of doubled-stranded were (mouse mRNA) for si5-HTR_4: sense, 5'-UAAGUCUUUCAGACGUGCC99-3'; antisense, 5'-GGCACGUCUGAAAGACUUA99-3', and siCART: sense, 5'-ACGCAUUCCGAUCUACGAG99-3'; antisens, 5'-CUCGUAGAUCGGAAUGCGU99-3') or of control (siCt: 0.05 µg/µl) siRNA (siCt for 5-HTR_4: sens, 5'-AUGAUGGCAACUGAUCGAC99-3'; antisens, 5'-GUCGAUCAGUUGCCAUCAU99-3'; siCt for CART: sens, 5'-CCUGAAUAGACCAUUCGCG99-3'; antisens, 5'-CGCGAAUGGUCUAUUCAGG99-3'). Generation of HSV-5-HTR_4 constructs was previously described.[5] Briefly, a cDNA fragment comprising the open reading frame of the *mHtr4* gene encoding 5-HTR_4 (obtained from the full-length 5-HT_{4a} splice variant) was inserted in the HSVPrpUC amplicon vector. The amplicon construct was packaged with the 5dl1.2 helper virus, purified on a sucrose gradient and suspended in 10% sucrose. The HSV immediate-early gene promoter IE4/5 regulates the transgene expression. The HSV plasmids were stored at -80°C. The HSV-LacZ construct (control) and HSV-5-HTR_4 were injected in the NAc at the concentration of 10^7 infectious units/ml.

Biochemical analyses

WT_4 and KO_4 mice were sacrificed by decapitation 5 min following the end of the open-field session and the brains were immediately removed and kept on ice in order to evaluate the levels of tissue 5-HT and 5-HIAA. Tissue samples containing NAc, striatum, dorsal hippocampus and amygdala were microdissected from 1 mm thick sections at -20°C using micropunches following the landmarks of the stereotaxic atlas (NAc +1.6 mm, striatum +1.0 mm, dorsal hippocampus -2.2 mm, amygdala -3.2 mm from the bregma).[4] All samples were stored at -80°C and treated as we described to determine 5-HT and 5-HIAA levels by high performance liquid chromatography with electrochemical detection.[6]

The receptor autoradiography was performed using frozen serial coronal brain 12 µm-thick sections from treated and control mice were processed to measure binding of 5-HTR_4 to specific antagonists [^{3}H]GR113808, and then [^{125}I]SB207710 because [^{3}H]GR113808 was not anymore available on the market.[2, 3] Briefly, sections were incubated in the appropriate buffer supplemented with 10 µM pargyline, 0.01% ascorbic acid and [^{125}I]SB207710 (specific activity: 2000 Ci/mmol, final concentration: 0.02 nM; Amersham, Piscataway, NJ, USA), at 37°C for 30 min. Non-specific binding was determined on consecutive sections incubated in the presence of 1 µM GR113808 [Sigma-Aldrich, St. Louis, MO, USA]. All sections were exposed to KODAK X-Omat Blue (PerkinElmer, Waltham, MA, USA) for 5 days at 4°C. Sections from both groups were processed together in order to compare signals on the same film.

Place conditioning paradigm

Mice were tested in two zones (43.2 × 13.5 × 30.5 cm) separated by a rectangular central area for 30-min on the preconditioning day (day 1), conditioning days (days 2–7) and the testing phase (day 8). With the exception of the preconditioning and testing phases, when mice were treated with NaCl injection and had free access to any zone, mice received i.p. administration of MDMA or NaCl combined with i.p. administration of NaCl or RS39604 in the NAc, 30-min before being confined to a single conditioning zone on alternate days. Mice were exposed to one zone/treatment pairing per day. Treatments were counterbalanced between the two zones and control mice were NaCl treated each day. A preference score is the difference between times spent by each mouse in the MDMA-, NaCl-, RS39604-, or MDMA plus RS39604-paired zone during the preconditioning and testing phases.

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Supplemental Table S1

Table S1. Binding site density for [^{3}H]GR113808 (0.1 nM) in adult WT_{1B} and KO_{1B} mice

Regions	Genotypes	Bound 5-HTR_4 (fmol/mg protein)
Prefrontal cortex	WT_{1B}	118.5 ± 11.7
	KO_{1B}	104.0 ± 19.2
Striatum	WT_{1B}	136.8 ± 5.5
	KO_{1B}	140.7 ± 18.2
Hippocampe (dorsal)	WT_{1B}	183.8 ± 4.3
	KO_{1B}	168.6 ± 16.5
Amygdala	WT_{1B}	157.9 ± 26.7
	KO_{1B}	180.5 ± 27.2

Values are expressed as means ± SEM from 3-6 frontal brain sections per region and per mouse (5 mice per group). Assuming an even concentration of 1 mg of protein per 10 mg of brain tissue.

Supplemental Table S2

Table S2. Tissue 5-HT and 5-HIAA levels in KO_4 and WT_4 mice

Regions Genotypes	**Environment**	**5-HT** µg/g wet weight	**5-HIAA** µg/g wet weight	**5-HIAA/5-HT** 5-HT turnover	**Difference** %
NAc					
WT_4	Home cage	1.23 ± 0.14	1.01 ± 0.09	0.82 ± 0.05	
	Open-field	0.65 ± 0.08 &	1.03 ± 0.11	1.60 ± 0.09 &&	+95
KO_4	Home cage	1.00 ± 0.09	1.12 ± 0.18	1.12 ± 0.13 *	
	Open-field	0.96 ± 0.11	0.94 ± 0.05	1.02 ± 0.09	
Striatum					
WT_4	Home cage	0.94 ± 0.06	1.10 ± 0.06	1.16 ± 0.04	
	Open-field	0.93 ± 0.15	1.30 ± 0.09	1.54 ± 0.20 &	+32
KO_4	Home cage	1.06 ± 0.09	1.28 ± 0.12	1.21 ± 0.07	
	Open-field	1.20 ± 0.11	1.18 ± 0.06	1.01 ± 0.07	
Hippocampus (dorsal)					
WT_4	Home cage	0.69 ± 0.07	0.97 ± 0.13	1.40 ± 0.14	
	Open-field	0.46 ± 0.06	0.84 ± 0.09	1.97 ± 0.37	
KO_4	Home cage	0.59 ± 0.06	0.98 ± 0.09	1.66 ± 0.14	
	Open-field	0.44 ± 0.04	0.83 ± 0.06	1.91 ± 0.14	
Amygdala					
WT_4	Home cage	0.98 ± 0.08	0.73 ± 0.10	0.73 ± 0.05	
	Open-field	0.56 ± 0.04	0.60 ± 0.08	1.10 ± 0.16	
KO_4	Home cage	0.88 ± 0.11	0.78 ± 0.12	0.88 ± 0.07	
	Open-field	0.76 ± 0.05	0.53 ± 0.05	0.73 ± 0.12	

Values are expressed as means ± SEM of 5-HT and 5-HIAA (n = 4-6) and 5-HIAA/5-HT ratio for WT_4 and KO_4 mice. & $p<0.05$, && $p<0.001$ compared to basal condition (home cage); * $p<0.05$ to WT_4

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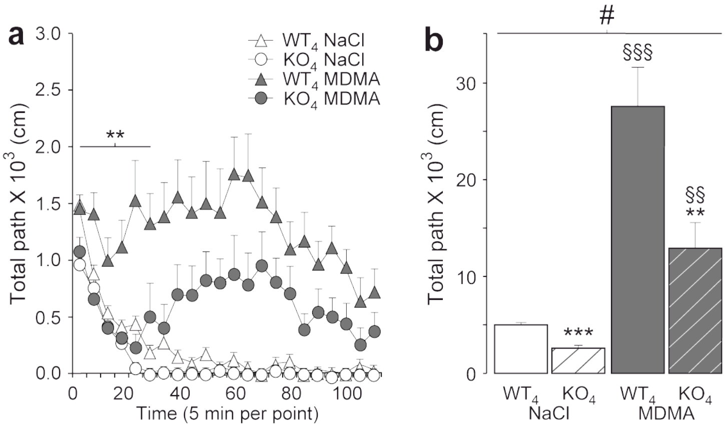

Figure S1. Novelty- and MDMA-induced hyperactivity is reduced in KO_4
(**a**, **b**) Total distance traveled (**a**) every 5-min over (**b**) 110-min following i.p. administration of MDMA (n = 14 WT_4, 12 KO_4) compared to saline (n=9 WT_4, 10 KO_4). Data are expressed as means ± SEM; ** $p<0.01$, *** $p<0.001$ compared to WT_4; §§ $p<0.01$, §§§ $p<0.001$ compared to NaCl; # $p<0.05$ genotype x treatment interaction.

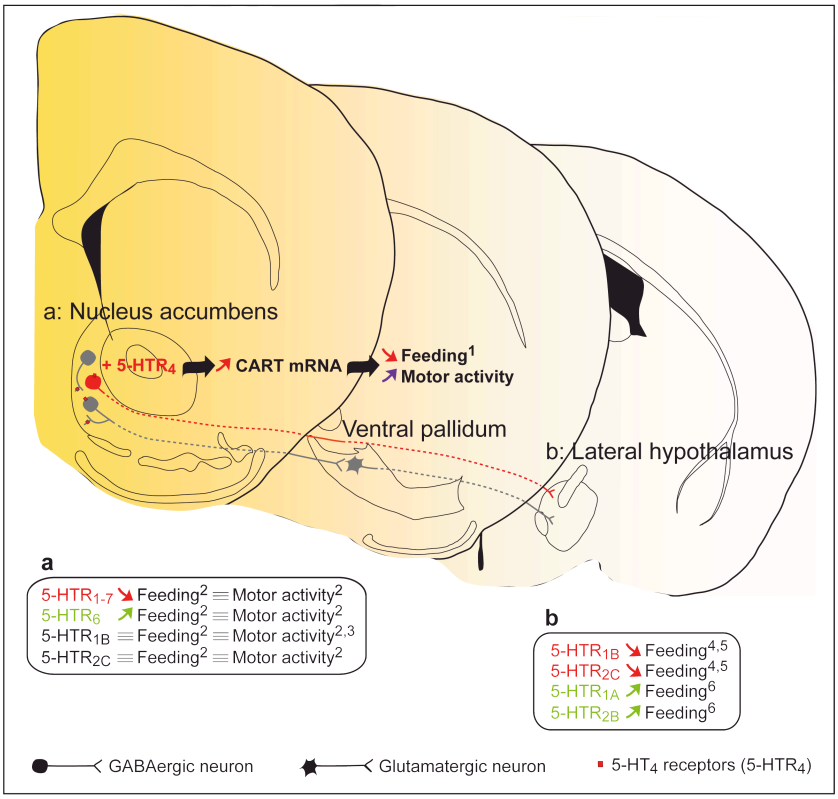

Figure S2. Opposite changes in feeding and locomotion under the control of 5-HTR_4
The regulation of feeding behavior may preferentially depend on (**a**) 5-HTR_4, 5-HTR_6 and 5-HTR_7 of the NAc and (**b**) on 5-HTR_{1A}, 5-HTR_{1B}, 5-HTR_{2B} and 5-HTR_{2C} of the hypothalamus. Only 5-HTR_4 in mice ties anorexia to hyperactivity (present study), consistent with the KO_4 mice's phenotype[2]. Efferent neurons of the NAc to the lateral hypothalamus might mediate anorexia and hyperactivity. 1.[3], 2.[8], 3.[9], 4.[10], 5.[11], 6.[12].

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References

1. Saudou F, Amara DA, Dierich A, LeMeur M, Ramboz S, Segu L *et al.* Enhanced aggressive behavior in mice lacking 5-HT1B receptor. *Science* 1994; **265**(5180)**:** 1875-1878.

2. Compan V, Zhou M, Grailhe R, Gazzara RA, Martin R, Gingrich J *et al.* Attenuated response to stress and novelty and hypersensitivity to seizures in 5-HT4 receptor knock-out mice. *J Neurosci* 2004; **24**(2)**:** 412-419.

3. Jean A, Conductier G, Manrique C, Bouras C, Berta P, Hen R *et al.* Anorexia induced by activation of serotonin 5-HT4 receptors is mediated by increases in CART in the nucleus accumbens. *Proc Natl Acad Sci U S A* 2007; **104**(41)**:** 16335-16340.

4. Franklin KBJ, and Paxinos, G. *The mouse brain in stereotaxic coodinates.* . Academic press: San Diego, 1997.

5. Lucas G, Compan V, Charnay Y, Neve RL, Nestler EJ, Bockaert J *et al.* Frontocortical 5-HT4 receptors exert positive feedback on serotonergic activity: viral transfections, subacute and chronic treatments with 5-HT4 agonists. *Biol Psychiatry* 2005; **57**(8)**:** 918-925.

6. Dusticier N, Nieoullon A. Comparative analysis of the effects of in vivo electrical stimulation of the frontal cortex and g-butyrolactone administration on dopamine and dihydroxyphenylacetic acid (DOPAC) striatal contents in the rat. *Neurochem Int* 1987; **10:** 275-280.

7. Robledo P, Balerio G, Berrendero F, Maldonado R. Study of the behavioural responses related to the potential addictive properties of MDMA in mice. *Naunyn Schmiedebergs Arch Pharmacol* 2004; **369**(3)**:** 338-349.

8. Pratt WE, Blackstone K, Connolly ME, Skelly MJ. Selective serotonin receptor stimulation of the medial nucleus accumbens causes differential effects on food intake and locomotion. *Behav Neurosci* 2009; **123**(5)**:** 1046-1057.

9. Przegalinski E, Filip M, Papla I, Czepiel K. Effects of 5-HT1B receptor ligands microinjected into the accumbal shell or core on the cocaine-induced locomotor hyperactivity in rats. *J Physiol Pharmacol* 2002; **53**(3)**:** 383-394.

10. Heisler LK, Pronchuk N, Nonogaki K, Zhou L, Raber J, Tung L *et al.* Serotonin activates the hypothalamic-pituitary-adrenal axis via serotonin 2C receptor stimulation. *J Neurosci* 2007; **27**(26)**:** 6956-6964.

11. Heisler LK, Jobst EE, Sutton GM, Zhou L, Borok E, Thornton-Jones Z *et al.* Serotonin reciprocally regulates melanocortin neurons to modulate food intake. *Neuron* 2006; **51**(2)**:** 239-249.

12. Yadav VK, Oury F, Suda N, Liu ZW, Gao XB, Confavreux C *et al.* A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell* 2009; **138**(5)**:** 976-989.