Loss of cholecystokinin and glucagon-like peptide-1-induced satiation in mice lacking serotonin 2C receptors

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Submitted 4 August 2008; accepted in final form 7 November 2008

Asarian L. Loss of cholecystokinin and glucagon-like peptide-1-induced satiation in mice lacking serotonin 2C receptors. Am J Physiol Regul Integr Comp Physiol 296: R51–R56, 2009. First published November 12, 2008; doi:10.1152/ajpregu.90655.2008.—To investigate the role of serotonin 2C receptors (2CR), which are expressed only in the central nervous system, in the satiating actions of the gut peptides CCK and glucagon-like peptide 1 (GLP-1), we examined 1) the effect of null mutations of serotonin 2CR (2CR KO) on the eating-inhibitory potencies of dark-onset intraperitoneal injections of 0.9, 1.7, or 3.5 nmol/kg (1, 2, or 4 μg/kg) CCK and 100, 200, and 400 nmol/kg (33, 66, or 132 μg/kg) GLP-1, and 2) the effects of intraperitoneal injections of 1.7 nmol/kg CCK and 100 nmol/kg GLP-1 on neuronal activation in the brain, as measured by c-Fos expression. All CCK and GLP-1 doses decreased 30-min food intake in wild-type (WT) mice, but none of them did in 2CR KO mice. CCK increased the number of cells expressing c-Fos in the nucleus tractus solitarii (NTS) of WT, but not 2CR KO mice. CCK induced similar degrees of c-Fos expression in the paraventricular (PVN) and arcuate (Arc) nuclei of the hypothalamus of both genotypes. GLP-1, on the other hand, increased c-Fos expression similarly in the NTS of both genotypes and increased c-Fos expression more in the PVN and Arc of 2CR KO mice, but not WT mice. These results indicate that serotonin signaling via serotonin 2CR is necessary for the full satiating effects of CCK and GLP-1. In addition, they suggest that the satiating effects of the two peptides are mediated by different neural mechanisms.

ON THE BASIS OF FULFILLMENT of criteria for endocrine satiation signals initially proposed by Gibbs et al. (20), intestinal CCK and GLP-1 are now considered to be physiological controls of eating (4, 10, 19, 32). Both hormones are released during meals, and premeal administration of doses producing plasma hormone levels that are similar to prandial levels is sufficient to inhibit meal size in the absence of adverse effects in humans. Furthermore, administration of CCK1 receptor antagonists in humans or rats, or GLP-1 receptor antagonists in rats, increases food intake (Williams D, personal communication); CCK1 receptor antagonists also block the satiating effects of CCK secretagogues in humans and rats. Although the understanding of the roles of these peptides in physiology of eating has increased tremendously in the last two decades (19, 39, 41, 54), the brain mechanisms underlying their effects are still unclear.

Serotonin appears to be a key neurotransmitter in the central mediation of eating, including the control of meal size. Extracellular concentrations of serotonin and its metabolite 5-HIAA increase in rats’ brains during spontaneous meals (44). The serotonin agonist fenfluramine, which both releases synaptic serotonin and blocks serotonin reuptake (14, 21), inhibits eating in rats by reducing meal size without affecting meal frequency or causing adverse behavioral effects (8, 9, 16, 29, 52). The most extensively studied serotonin receptor in relation to the control of eating is the serotonin 2C receptor (2CR) (51, 56). 2CR are widely expressed in the CNS, but not in the periphery (2, 34, 57). Several pharmacological studies suggest that peripheral CCK activates brain serotonergic pathways to elicit satiation and that 2CR are involved in these pathways (22, 46, 53). The pharmacological tools used, however, have not always been highly selective to 2CR and have not been administered locally, which is important because several populations of serotonin receptors in the brain and in the periphery have been implicated in CCK satiation (25, 49). Whether serotonin or 2CR are also part of the mechanism of GLP-1 satiation has not been tested yet.

To determine whether 2CR are necessary for the full satiating effects of CCK and GLP-1, the satiating actions of IP injections of these peptides in wild-type (WT) mice and mice with a genetic deletion of 2CR (2CR KO) were compared. Furthermore, using the expression of c-Fos protein as an indication of neuronal activation, we also investigated whether intraperitoneal CCK and GLP-1 activate the paraventricular (PVN) and arcuate (Arc) nuclei of the hypothalamus and the nucleus tractus solitarii (NTS) of the hindbrain similarly in WT and 2CR KO mice.

MATERIALS AND METHODS

Subjects and housing. Homozygote male mice bearing a null mutation of the X-linked htr2c gene (2CR KO: congenic on a C57BL/6J background) and age- and body weight-matched wild-type (WT) mice were obtained by breeding heterozygote female mice (kindly provided by Dr. Lawrence Tecott, University of California, San Francisco, CA, USA) with C57BL/6J male mice (Jackson Laboratories, Sulzfeld, Germany) in our animal facility. The phenotype of the 2CR KO mice was originally described by Tecott and his colleagues (56). Before entering the experimental protocol, the mice were adapted to individual housing in solid bottom acrylic cages with wood chip bedding in a room maintained at 22 ± 1.0°C on a 12:12-h LD cycle (lights off at 1200). Mice were maintained on standard pelleted laboratory chow (#3436, Nafag, Gossau, Switzerland) and tap water. All procedures were approved by the Veterinary Office of the Canton of Zurich.

PCR verification of genotype. The genotypes of all mice were assessed with standard PCR techniques. Genomic DNA was extracted from tail biopsies using proteinase K digestion followed by isopropanol precipitation. DNA was then amplified by PCR using a primer pair of 5′-GCTGCTGATGCGTTGGGCATG-3′ and 5′-CACCACACACCAAGCTGCTG-3′, which specifically amplified a fragment of 193 base pairs of the htr2c gene. Each PCR reaction was carried out in a 50-μl volume with 100 ng of genomic DNA, 1× PCR buffer, 10 mM MgCl2, 0.2 mM of each dNTP, 0.2 μM of each primer, and 2.5 U of AmpliTaq Gold DNA polymerase (ABI). The PCR conditions were: 95°C for 10 min, 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were separated using 2% agarose gels containing 0.5 μg/ml ethidium bromide. DNA gel images were obtained with the Alpha Innotech FluorChem 2 Imager (Alpha Innotech Corporation, San Leandro, CA), and PCR products were visualized using the Alpha Innotech FluorChem 2 software. The identity of the PCR products was confirmed by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

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set that selectively amplifies a 114-bp fragment on the WT htr2c DNA. PCR was performed in a 25-μl reaction mixture containing the following components: 11.875 μl H2O, 0.5 μl Flexi-PCR buffer (5×), 2 μl MgCl2 (25 mM), 1 μl dNTP (10 mM each), 1 μl htr2c forward (10 μM, 5′ gcTc AG Tc TgA AAT gTg T 3′), 1 μl htr2c reverse (10 μM, 5′ cgg AcT gCT AAA TgF ggT c 3′), 2 μl Neo D forward (10 μM, 5′ cAc cTT gCT ccT gcc gag AAA 3′), and 0.125 μM Go-Taq Flexi Polymerase. The PCR amplification conditions were 95°C (4 min), 95°C (30 s), 57°C (45 s), 72°C (30 s) for 40 cycles, 72°C (1 min), 4°C (hold). All reagents were purchased from Catalys AG (Wallisellen, Switzerland). The primers were synthesized by Microsynth (Balgach, Switzerland).

**Experiment 1: effect of 2CR KO on CCK- and GLP-1-induced satiation.** Twelve WT and 12 2CR KO mice (average body weight for both genotypes, 26 ± 1 g) were used. Thirty minutes prior to dark onset, the food from the previous day was removed and weighed, and the body weights of the mice were recorded. Just before dark onset, saline (0.9% NaCl, B. Braun Medical AG, Emmenbrücke, Switzerland) was intraperitoneally injected and food intake was measured 0.5, 1, and 2 h later. Because of the short effect of the peptides, 24-h data were not collected. Mice were adapted to a 12-h light/dark cycle condition for 1 wk. The experiment was designed in two cross-over test trials for each dose and peptide tested. Three doses of CCK-8 (0.9, 1.7, and 3.5 nmol/kg, or 1, 2, and 4 μg/kg; Bachem, Bubendorf, Switzerland) and GLP-1 (100, 200, or 400 nmol/kg, or 33, 66, and 132 μg/kg; Bachem) were tested using separate crossover designs. Intraperitoneal injections were 0.01 ml/g body wt. The two trials of each crossover were done on consecutive days, and the different peptide doses were tested 4 days apart.

**Experiment 2: effect of 2CR KO on CCK- and GLP-1-induced c-Fos expression.** A different group of 16 2CR KO and 17 WT mice (body weight, 27 ± 1 g) underwent the same adaptation and test procedure as in experiment 1. One week later, they were perfused for c-Fos immunocytochemistry. On the day of the perfusion, saline (n = 5/genotype), CCK (1.7 nmol/kg, or 1, 2, and 4 nmol/kg; Bachem) and GLP-1 (100 nmol/kg, or 33, 66, and 132 μg/kg; Bachem) were injected intraperitoneally and food intake was measured 0.5, 1, and 2 h later. Because of the short effect of the peptides, 24-h data were not collected. Mice were adapted to a 12-h light/dark cycle condition for 1 wk. The experiment was designed in two cross-over test trials for each dose and peptide tested. Three doses of CCK-8 (0.9, 1.7, and 3.5 nmol/kg, or 1, 2, and 4 μg/kg; Bachem, Bubendorf, Switzerland) and GLP-1 (100, 200, or 400 nmol/kg, or 33, 66, and 132 μg/kg; Bachem) were tested using separate crossover designs. Intraperitoneal injections were 0.01 ml/g body wt. The two trials of each crossover were done on consecutive days, and the different peptide doses were tested 4 days apart.

**Statistical analysis.** Data were analyzed with two-way ANOVA (genotype × treatment) followed by Bonferroni-Holm post hoc tests (30). The treatment factor was within subjects for the food intake ANOVA and between subjects for the c-Fos ANOVA. Three post hoc comparisons were of interest: 1) CCK or GLP-1 vs. saline in WT mice 2) CCK or GLP-1 vs. saline in 2CR KO mice 3) the effect of genotype on CCK or CCK satiation (WT saline-WT CCK or GLP-1 vs. 2CR KO saline −2CR KO CCK or GLP-1). Data for each dose and peptide were analyzed separately. A robust statistical approach was used to increase statistical power (12, 42). Food intake and c-Fos data were converted to standard scores using the median absolute deviation method, and standard scores with absolute values >2.57 (i.e., P < 0.01) were excluded. The standard error of the difference (SED) for each comparison is reported as an index of experiment-wise residual variability.

**RESULTS**

**Experiment 1: effect of 2CR KO on CCK- and GLP-1-induced satiation.** In WT mice, IP injections of CCK produced a significant decrease in 30-min food intake so that mice ate 79% (after 0.9 nmol/kg), 66% (after 1.7 nmol/kg), and 76% (after 3.5 nmol/kg) less than the corresponding saline intake [F(1,19) = 10.52, F(1,21) = 4.63, and F(1,21) = 9.14, P < 0.004, 0.044, and 0.006, SEDs = 0.027, 0.026 and 0.029 g, for 0.9, 1.7, and 3.5 nmol/kg, respectively, Fig. 1]. Lack of functional serotonin 2CR reversed the CCK-induced inhibition of eating. Thirty-minute food intake after CCK in 2CR KO mice were not significantly different from those of corresponding saline controls. Finally, the difference between saline and CCK was greater in WT vs. 2CR KO mice after 0.9 and 3.5, but not after 1.7 nmol/kg CCK. Figure 2 shows similar findings for GLP-1 treatment. The three doses of GLP-1 decreased food intake by 60% (100 nmol), 54% (200 nmol), and 63% (400 nmol) in WT mice [F(1, 15) = 6.19, F(1, 14) = 0.36, and F(1, 14) = 0.04, P < 0.02, >0.05, and 0.05, SEDs = 0.020, 0.028

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**Fig. 1. Food intake (g) in the first 30 min following intraperitoneal CCK injection in wild-type (WT) and 2CR receptor (2CR) knockout (KO) mice. J** Food intake is shown in response to saline (open bars) or the indicated doses of CCK (hatched bars) intraperitoneally injected at dark onset in nondeprived mice. *P < 0.05, saline vs. CCK, within genotype; +P < 0.05, (WT saline-WT CCK) vs. (KO saline-KO CCK).
and 0.034 g, for 100, 200 and 400 nmol/kg, respectively]. There was no significant effect of GLP-1 on food intake in 2CR KO mice. Following 100 nmol/kg and 200 nmol/kg, but not 400 nmol/kg, the difference between saline and GLP-1 was greater in WT than in 2CR KO mice (Fig. 2).

In the 1- and 2-h food intake data, there were similar trends as in the 30-min data, but there were no significant effects of gene or treatment for either CCK or GLP-1 (data not shown). Experiment 2: effect of 2CR KO on CCK- and GLP-1-induced c-Fos expression. Intraperitoneal injections of CCK induced large increases in c-Fos expression in the cNTS of WT mice but not in the cNTS of 2CR KO mice [Fig. 3, left; F (1, 9) = 10.46, P < 0.05, SED = 0.2 cells]. CCK increased c-Fos expression in the PVN and Arc of WT and 2CR KO mice similarly. A different pattern of c-Fos expression was observed after IP GLP-1 injection: GLP-1 increased c-Fos expression in the cNTS of WT and 2CR KO mice similarly, whereas in the PVN and Arc, GLP-1 induced a much larger increase in c-Fos expression in the 2CR KO mice than in the WT mice [Fig. 3, right; F (1, 11) = 7.43, P < 0.05, SED = 0.5 cells]. A photomicrograph is shown in Fig. 4.

DISCUSSION

The eating data reported here clearly indicate that serotonin signaling via 2CR is necessary for the full satiating effects of intraperitoneal CCK and GLP-1 in mice. These data are the first evidence that serotonin contributes to GLP-1 satiation and extend previous pharmacological investigations of serotonin’s role in CCK satiation in rats. We assume that the critical serotonin 2CR lesion occurred in the brain because there is little or no serotonin 2CR expression outside the brain (2, 34, 57). We also measured the hormones’ effects on c-Fos expression in the cNTS, the PVN, and the Arc because these are areas that have been implicated in the control of eating by CCK, GLP-1, and serotonin. These data suggest that the satiating effects of CCK and GLP-1 are mediated by partially independent neural mechanisms, in that a 2CR-serotonergic input to the NTS may be necessary for CCK, but not GLP-1, satiation. 2CR-serotonergic input also appeared to reduce neuronal activity in the Arc in response to GLP-1, but not to CCK, although this change appears unlikely to be causally related to satiation. We discuss the CCK and GLP-1 findings, in turn, below.

CCK. Although 2CR KO mice ate relatively normally in our test situation, they showed no sign of a reduction in eating across a range of intraperitoneal CCK doses that transiently reduced eating by about 60–80% in WT mice. These data indicate that serotonin 2CR are necessary for CCK satiation in mice and extend a number of pharmacological investigations of the role of serotonin in CCK satiation in rats. Stallone et al.’s (53) report that the serotonin 1/2 receptor antagonist metergoline reversed the satiating action of CCK first implicated serotonin neurotransmission in CCK satiation. Poeschla et al. (46) demonstrated that CCK’s eating-inhibitory effect was blocked by the serotonin 2A/2C receptor antagonist mianserin, but not by the serotonin 2A receptor antagonist ketanserin or by three serotonin 3 receptor antagonists. Because mianserin has nanomolar affinity for the 2CR and only micromolar affinity for the 2A receptor, these data suggested that peripheral CCK interacted with endogenous serotonin signals via 2CR to elicit satiety (note that at the time of their report, serotonin 2CR were called 1C receptors). This pharmacological evidence supports the interpretation that the lack of CCK satiation that we observed in 2CR KO mice was due to disruption of acute serotonin signaling, rather than to early developmental changes or other secondary effects that can arise from the genetic deletion and may mask the intended cell- or tissue-specific molecular effects of the knockout in adult-
Nevertheless, because pharmacological manipulations of serotonin signaling have sometimes produced different results in mice and rats, it will be important to extend the pharmacological analysis (e.g., treatment with the selective serotonin 2CR antagonist SB 242089) to mice (6).

Our second major finding was that CCK does not increase c-Fos expression in the cNTS of 2CR KO mice as it does in the WT mice or in rats. Because increases in c-Fos expression reflect increases in neuronal activity and because the cNTS is the site of projections of the abdominal vagal afferents mediating CCK satiation, these data suggest that the lack of functional serotonin 2CR in the NTS blocks CCK satiation by decreasing the initial central processing of the vagal afferent CCK signal. The interpretation that NTS serotonin 2C may be necessary for CCK satiation parallels earlier reports implicating two other central signaling molecules in CCK satiation. First, Rinaman (47) reported that the satiating action of intraperitoneal CCK was reduced in rats that received injections of a saporin-dopamine beta-hydroxylase antibody conjugate into the NTS to lesion noradrenergic neurons. Unlike our results, however, CCK induced normal amounts of c-Fos expression in the NTS in lesioned rats. More recently, Hayes and Covasa (25) reported that direct injections of the serotonin 3 receptor (3R) antagonist ondansetron into the NTS blocked the satiating action of IP CCK in rats; c-Fos responses were not measured. Taken together, and assuming there are no important species differences, these data suggest that NTS serotonin 2C, 3R, and noradrenaline are each necessary and that no two of them alone are sufficient for CCK satiation. Finally, it is relevant to note that results from rats with chronic supracollicular decerebrations indicate that hindbrain neural networks are also sufficient for the satiating action of exogenous CCK (24).

In contrast to the absence of normal CCK-induced c-Fos in the cNTS in 2CR KO mice, CCK-induced c-Fos responses in the PVN and Arc were normal in 2CR KO mice, indicating that activation of the neurons in these nuclei by CCK is not sufficient to produce satiation. This is surprising because 2CR are expressed in both the Arc and PVN (27, 28), because pharmacological data implicate both the PVN and Arc in the serotonergic control of eating (50, 59), because 2CR-serotonergic inputs to the Arc-PVN melanocortinergic neurons appear to be part of the physiological control of eating in mice (3, 36, 38), and because both the PVN and the Arc have been implicated in the satiating action of CCK (13, 17, 35, 37, 38, 42). Furthermore, the absence of CCK-induced c-Fos activation in the NTS of 2CR KO mice suggests that NTS-to-hypothalamus signaling would be reduced. We speculate that the continued CCK-induced activation of neurons in the Arc and PVN of 2CR KO mice arises from a population of NTS neurons that receives CCK vagal inputs and that is independent of 2CR signaling. These neurons, however, are apparently not sufficient to produce CCK satiation.

GLP-1. Intraperitoneal injections of doses of GLP-1 that reduced eating in WT mice 50–60% had no reliable effect in 2CR KO mice. These are the first data that we know implicating serotonin in GLP-1 satiation.

GLP-1 is expressed both by L-cells in the ileum and colon and by neurons in the NTS. Intestinal GLP-1 is released during meals and appears to reach GLP-1 receptors (GLP-1R) in a variety of peripheral and central loci (32, 45, 55). Which
populations of GLP-1R mediate the satiating actions of peripheral GLP-1 remains unclear. Some data suggest that the satiating action of IP GLP-1 may depend on abdominal vagal afferents in rats (1, 26, 48), and the GLP-1 agonist exendin-4 still inhibited eating in chronic supracollicular decerebrate rats, indicating that caudal hindbrain neural processing is sufficient to mediate GLP-1 satiation. In light of these data, the lack of effect of intraperitoneal GLP-1 on eating in 2CR KO mice coupled with normal cNTS c-Fos expression was unexpected. Analogous to the interpretation above, however, it may be that intraperitoneal GLP-1 affects more than one population of NTS neurons, one of which has a 2CR input and is necessary for satiation and the other of which expresses c-Fos but is not sufficient for satiation. One possibility is that this latter population contributes to GLP-1’s incretin response, which also appears to involve vagal afferent signalling (7, 32).

The stimulatory effect of IP GLP-1 on c-Fos in the Arc and PVN of WT mice was increased in 2CR KO mice. This suggests that these hypothalamic neurons normally receive an inhibitory 2CR input whose absence in 2CR KO mice increases GLP-1-induced c-Fos expression. The source of this 2CR input and the function of these neurons are unclear, but their activation cannot be sufficient for GLP-1 satiation. It also seems unlikely that they reflect a stress response because Heisler et al. (28) have recently reported that 2CR stimulate rather than inhibit PVT CRH-expressing neurons that are involved in the hypothalamic-pituitary-adrenal axis (HPA). An apparently more likely alternative is that the increase in GLP-1-induced c-Fos in the Arc and PVN of 2CR KO mice is related to the loss of 2CR input to inhibitory GABAergic interneurons in Arc and PVN (11, 30). Whether intraperitoneal GLP-1 truly affects Arc or PVN GABA neurons and the functional implications of such an effect remains unknown.


discusses contributions to the control of ingestion across phyla from nematodes and mollusks to mammals. This situation both underscores the importance of understanding serotonin’s roles in eating and the complications facing analysis of this unusually pleiotropic molecule.

**ACKNOWLEDGMENTS**

The author thanks Angelika Weber, Michael Koss, and Susanne Günnel for technical assistance, Dr. Lawrence Tecott for the serotonin 2CR knockout mice, Dr. Lora Heisler for helpful discussions, and Drs. Wolfgang Langhans and Nori Geary for comments on the penultimate draft.

**GRANTS**

This work was supported by ETH-Zürich.

**REFERENCES**


*Perspectives and Significance*

Progress in unraveling the myriad functions of serotonin neurotransmission has accelerated rapidly in recent years. This progress, catalyzed by linked advances in pharmacology and molecular genetics, includes several aspects relevant to the physiology of eating. Among these are the identification of serotonin 1BR and 2CR inputs in Arc proopiomelanocortin and neuropeptide Y/agouti-related peptide neurons that are putative controllers of energy homeostasis (27), the identification of serotonin 2CR inputs in CRH neurons of the PVN that control HPA axis function (28), the identification of serotonergic controls of eating within the caudal brain stem (23), including identification of serotonin 3R as a mediators of CCK satiation (25), the identification of serotonin as a crucial mediator in illness anorexia (57), and the identification of serotonergic dysfunction in neural pathways mediating mood, reward, and hedonics as potential causes of anorexia and bulimia nervosa (33). The present results extend earlier pharmacological evidence that serotonin 2CR are crucial in the mediation of CCK satiation and begin a new chapter in the story, namely, that serotonin 2CR are also crucial in the mediation of GLP-1 satiation. Taken together, these diverse data suggest that serotonin is extensively, if not ubiquitously, involved in the neural control of eating in its many manifestations. This situation is perhaps unsurprising given that, as Tecott (57) has pointed out, serotonin is a phylogenetically ancient neurotransmitter that contributes to the control of ingestion across phyla from nematodes and mollusks to mammals.
SEROTONIN 2C RECEPTORS AND CCK AND GLP-1 SATIATION