Behavioral/Systems/Cognitive

Cannabinoid Receptor 1 in the Vagus Nerve Is Dispensable for Body Weight Homeostasis But Required for Normal Gastrointestinal Motility

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The cannabinoid receptor 1 (CB_1R) is required for body weight homeostasis and normal gastrointestinal motility. However, the specific cell types expressing CB_1R that regulate these physiological functions are unknown. CB_1R is widely expressed, including in neurons of the parasympathetic branches of the autonomic nervous system. The vagus nerve has been implicated in the regulation of several aspects of metabolism and energy balance (e.g., food intake and glucose balance), and gastrointestinal functions including motility. To directly test the relevance of CB_1R in neurons of the vagus nerve on metabolic homeostasis and gastrointestinal motility, we generated and characterized mice lacking CB_1R in afferent and efferent branches of the vagus nerve (*Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice). On a chow or on a high-fat diet, *Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice have similar body weight, food intake, energy expenditure, and glycemia compared with *Cnr1*$^{flox/flox}$ control mice. Also, fasting-induced hyperphagia and after acute or chronic pharmacological treatment with SR141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide] (CB_1R inverse agonist) paradigms, mutants display normal body weight and food intake. Interestingly, *Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice have increased gastrointestinal motility compared with controls. These results unveil CB_1R in the vagus nerve as a key component underlying normal gastrointestinal motility.

Introduction

The cannabinoid receptor 1 (CB_1R) belongs to the endocannabinoid system (Matsuda et al., 1990; Piomelli, 2003) and is widely expressed in the mammalian body. In central and peripheral neurons, CB_1R modulates neurotransmitter release (Marsicano and Lutz, 1999; Piomelli, 2003). Pharmacological blockade of CB_1R reduces food intake and exerts anti-obesity effects in mice and humans and also improves lipid and glucose profiles of overweight and diabetic subjects (Ravinet Trillou et al., 2003; Després et al., 2005, 2006; Van Gaal et al., 2005; Addy et al., 2008). Deletion of CB_1R in mice leads to reduced food intake, body adiposity, and increased insulin sensitivity (Cota et al., 2003; Ravinet Trillou et al., 2004). Interestingly, CB_1R null mice are hypophagic after fasting and are insensitive to the anorectic actions of SR141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide] (CB_1R inverse agonist), suggesting that CB_1R mediates the inhibitory effect of this drug on food intake (Di Marzo et al., 2001). In summary, CB_1R exerts important functions on the control of body energy, glucose, and lipid balance.

The use of cell-specific CB_1R genetic manipulation has indicated some of the critical sites in which CB_1R regulates metabolic homeostasis. For example, CB_1R in glutamatergic neurons has been reported to be required for the orexigenic effect of cannabinoids (Bellocchio et al., 2010). Also, CB_1R in forebrain and sympathetic neurons has been shown to be required for normal energy expenditure (Quarta et al., 2010). Nevertheless, the role of CB_1R in other neuronal sites, for example, the parasympathetic branch of the autonomic nervous system, is yet to be known.

The CB_1R also regulates gastrointestinal functions, for instance, motility. Of note, diarrhea is a frequent untoward side effect observed in patients treated with CB_1R inverse agonist (Despres et al., 2005; Addy et al., 2008), and hypermotility of food through the intestines may reduce absorption of water and nutrients by the intestine and be an underlying cause of diarrhea. In rodents, inhibition of CB_1R increases gastrointestinal motility, whereas activation of CB_1R inhibits it (Colombo et al., 1998; Izzo et al., 1999; Landi et al., 2002; Pinto et al., 2002; Capasso et al., 2005). Also, CB_1R null mice have increased gastrointestinal motility (Yuece et al., 2007). Moreover, it has been suggested that CB_1R modulates acetylcholine release from myenteric neurons (Coutts and Pertwee, 1997; Coutts and Izzo, 2004).

Neurons of the vagus nerve have been shown to control body energy/glucose metabolism (Williams et al., 2000; Rossi et al.,

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2011) and upper gastrointestinal functions. CB_1R is abundantly expressed in both vagal afferent and efferent neurons (Burdyga et al., 2004). Capsaicin deafferentation ablates the orexigenic effect of CB_1R agonist (Gómez et al., 2002), and vagotomy impairs CB_1R regulation of gastrointestinal motility (Krowicki et al., 1999). Thus, it has been suggested that CB_1R in these neurons regulates feeding/body energy balance and gastrointestinal motility. To directly test these hypotheses, we generated and characterized mice lacking CB_1R in vagal afferent and efferent neurons located in the nodose ganglia and dorsal motor nucleus of the vagus (DMV).

Materials and Methods

Animal care. Care of animals and all procedures were approved by University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Mice were housed in groups of four to five mice on a 12 h dark/light cycle with *ad libitum* access to water and food, unless otherwise specified. Mice were fed on a standard chow diet or, if mentioned, on a high-fat diet (TD88137; Harlan Teklad). All studies were performed using male mice.

Generation of Cnr1$^{flox/flox}$; Phox2b–Cre *mice.* Mice containing a Cre-conditional *Cnr1* null allele (*Cnr1*$^{flox/wt}$) were generated in the laboratory of Pierre Chambon for Sanofi-Aventis and then imported by University of Texas Southwestern Medical Center. The targeting plasmid was constructed using genomic DNA of mouse strain 129/Sv. The single encoding exon of *Cnr1* was flanked by loxP sites. The first loxP site was cloned upstream of *Cnr1* start codon, and the loxP–FRT–Neomycin–FRT cassette was cloned downstream of *Cnr1* stop codon. The targeting vector contained 2.1 kb of genomic DNA between loxP sites and 3.8 and 3 kb of genomic DNA as 5′ and 3′ homologous arms, respectively. The targeting plasmid was electroporated into 129 embryonic stem (ES) cells, and Neomycin-resistant clones were screened for homologous recombination as described below. Screening of 3′ end homologous recombination was performed by PCR using ES cell genomic DNA as template and the following primers: Neomycin forward (Neo F), AGGGGCTCGCGCCAGCCGAAGTGTT; and 3′ end reverse (3′ end R), ACAGCAGTCTCAATGATGCTACCAG. If ES cells contain a targeted allele, the expected PCR amplicon is ~4 kb. Screening of 5′ end homologous recombination was performed by Southern blot using NheI as restriction enzyme and a probe between the 5′ end NheI site and the 5′ end edge of the construct. Expected bands are 12 kb (*Cnr1* targeted) and 10 kb (*Cnr1*^{wt}). Targeting was further confirmed by Southern blot in ES cell genomic DNA digested with restriction enzymes NheI or HindIII and a probe against the Neomycin cassette. Expected bands are 12 kb (NheI DNA fragment) and 7.2 kb (HindIII DNA fragment). Chimeric mice (F0) were bred to wild-type mice to generate mice bearing the targeted *Cnr1* allele (F1). These F1 mutants were bred to a ubiquitously expressing FLPe recombinase (Flp) transgenic line. Successful removal of the flippase recognition target (FRT)-flanked phosphoglycerate kinase (PGK)–Neomycin cassette was confirmed by PCR in *Cnr1*$^{flox/wt}$ mice bearing a ubiquitously expressing Flp transgene (F2). These F2 mice were bred to wild-type mice, and offspring mice containing the FLP-recombined FRT-flanked PGK–Neomycin (F3) were selected by PCR genotyping. These F3 mutant mice were used to establish the Cre-conditional *Cnr1* null line. *Cnr1*$^{flox/flox}$ mice were mated to *Phox2b–Cre* transgenic mice (line 1; Scott et al., 2011) to obtain the study groups that were in a mixed C57BL/6 and 129 genetic background. Mice were genotyped by PCR using primers CB_1R forward (ACCACCTTCCTCATGTTAACCT) and CB_1R reverse (GACCAGAGACAGCTCCAGA) for amplification of the *Cnr1*^{wt} (197 bp) or *Cnr1*^{flox} allele (247 bp) and CB_1R forward and CB_1R-D (GGGTAGTTAGGCTTCAGATTTGGA) for amplification of the Cre-recombined *Cnr1* null allele (*Cnr1*$^{\Delta}$) (419 bp). Mice that underwent the "delta event," which has been described previously (Balthasar et al., 2004), were excluded from the studies. To genotype for *Phox2b–Cre* allele, a PCR reaction was performed, as described previously (Scott et al., 2011), using the following primers: Phox2b forward, CCGTCT

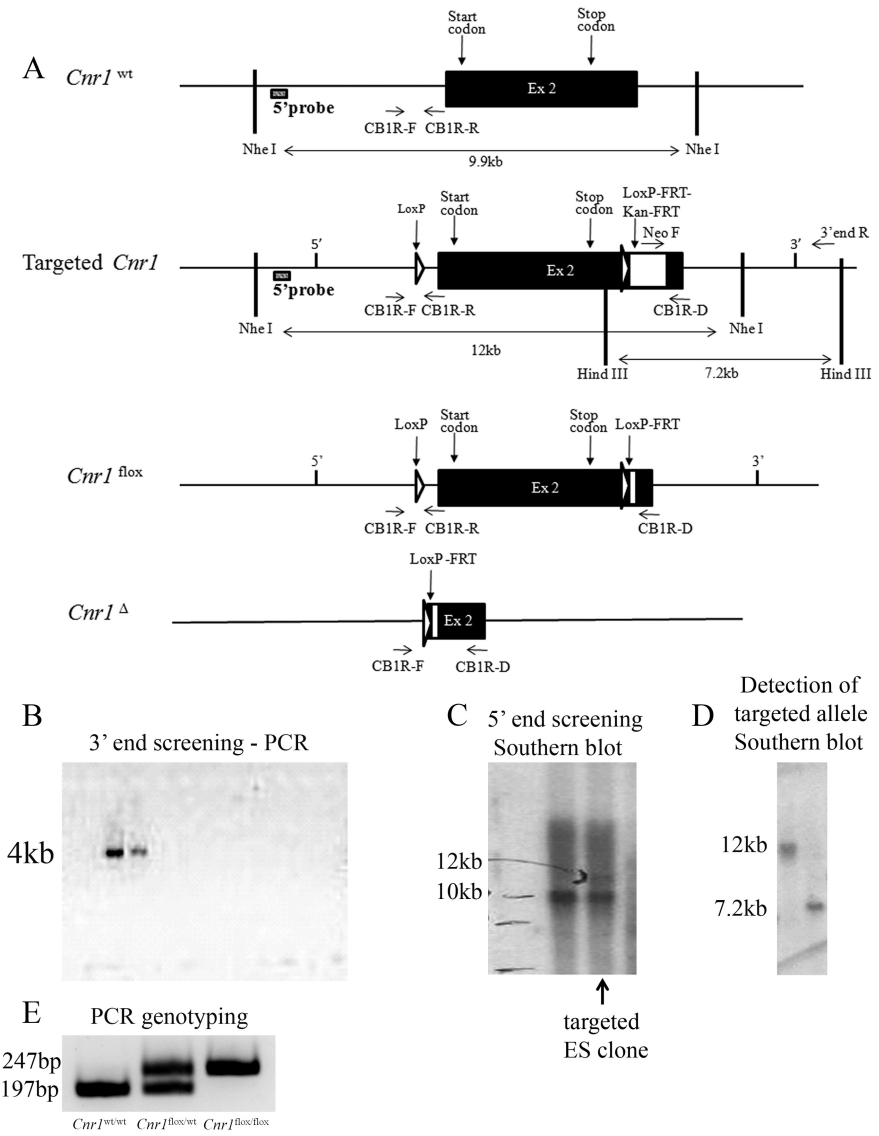

Figure 1. Generation of mice bearing a Cre-conditional *Cnr1* null allele (*Cnr1*$^{flox/wt}$). Schematic representation of the targeting strategy. Represented are *Cnr1*^{wt}, targeted *Cnr1*, *Cnr1*^{flox}, and *Cnr1*$^{\Delta}$ alleles (***A***). Screening of 3′ end homologous recombination by PCR. A 4 kb PCR amplicon expected in ES cells bearing *Cnr1* targeted allele (***B***). Screening of 5′ end homologous recombination by Southern blot using NheI as the restriction enzyme and a probe upstream of 5′ edge of the construct. Expected 12 kb (*Cnr1* targeted) and 10 kb (*Cnr1* wt) bands are indicated (***C***). Detection of the *Cnr1* targeted sequences in the single clone deemed targeted according to ***B*** and ***C*** (***D***). NheI and HindIII were the restriction enzymes used on left and right lanes, respectively, and the probe was against the Neomycin cassette (***D***). Expected PCR amplicons from tail genomic DNA of *Cnr1*$^{w/w}$, *Cnr1*$^{flox/wt}$, and *Cnr1*$^{flox/flox}$ mice (***E***).

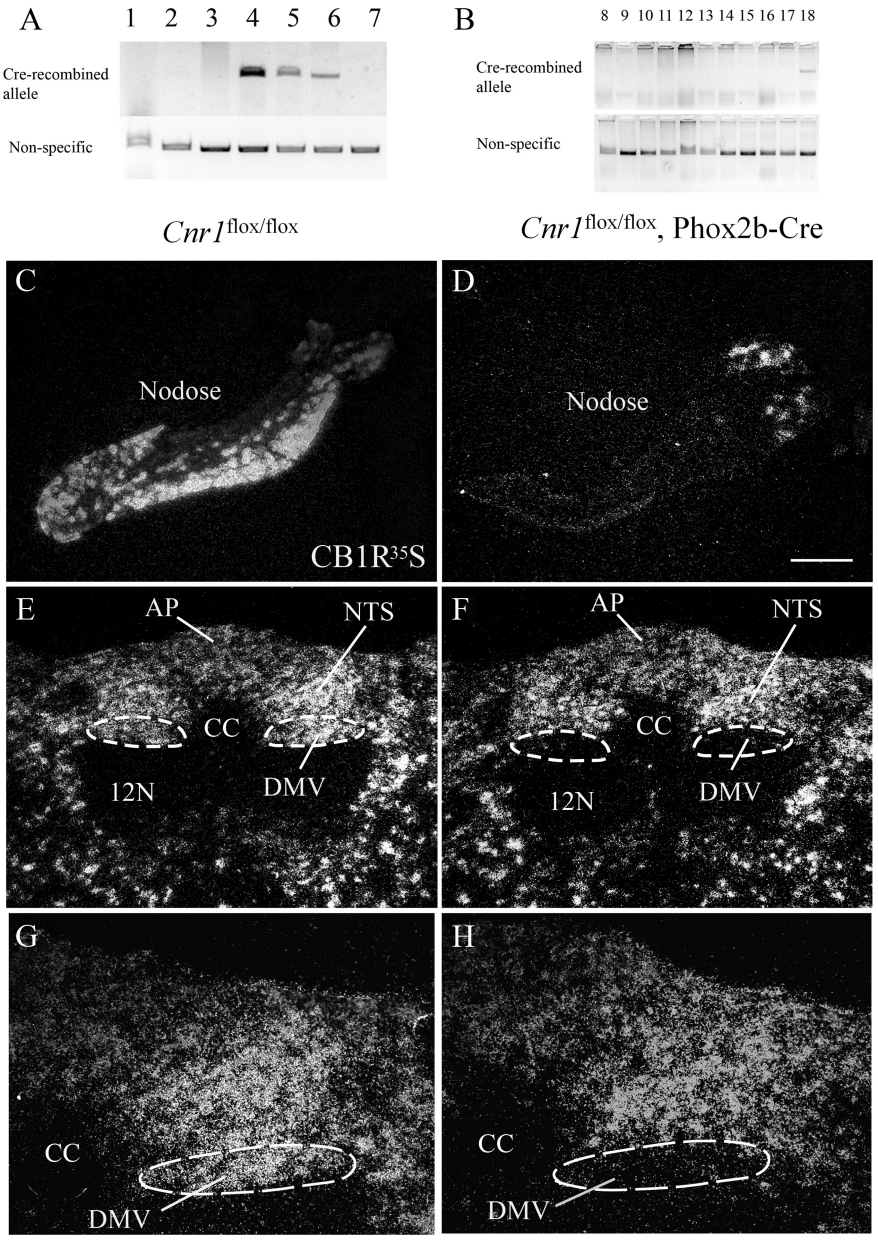

Figure 2. Deletion of CB_1R in nodose and DMV neurons. Detection of Cre-recombined *Cnr1* null allele by PCR using genomic DNA of a $Cnr1^{flox/flox}$; *Phox2b–Cre* mouse [***A***: ***1***, forebrain; ***2***, hypothalamus; ***3***, pituitary; ***4***, midbrain; ***5***, hindbrain; ***6***, nodose; ***7***, tail as negative control; ***B***: ***8***, heart; ***9***, kidney; ***10***, stomach; ***11***, duodenum; ***12***, jejunum; ***13***, ileum; ***14***, colon; ***15***, pancreas; ***16***, liver; ***17***, perigonadal white adipose tissue; ***18***, positive control, hindbrain]. *In situ* hybridization histochemistry for *Cnr1* mRNA (*in situ* probe complementary to *Cnr1* exon 2) in nodose and hindbrain sections of $Cnr1^{flox/flox}$ (***C***, ***E***, ***G***) and $Cnr1^{flox/flox}$; *Phox2b–Cre* (***D***, ***F***, ***H***) male mouse. Scale bar: ***C–F***, 200 μm; ***G***, ***H***, 100 μm. AP, Area postrema; 12N, 12 nerve; CC, central canal; NTS, nucleus of the solitary tract.

CCACATCCATCTTT; Phox2b reverse, GTACGGACTGCTCTGGTGGT; and Cre reverse, ATTCTCCCACCGTCACTACG. Male mice littermates were used for all the experiments performed.

In situ *hybridization histochemistry.* Mice were anesthetized with chloral hydrate (500 mg/kg, i.p.) and perfused transcardially with diethylpyrocarbonate (DEPC)-treated water 0.9% PBS, followed by 10% neutral buffered Formalin. Brains and nodose ganglion were dissected and placed in the same fixative for 4–6 h at 4°C, immersed in 20% sucrose in DEPC-treated PBS, pH 7.0, at 4°C for 24 h. Tissue was sliced into 25 μm sections on a freezing microtome. Sections from brain and nodose ganglion were mounted onto SuperFrost plus slides (Thermo Fisher Scientific) and stored at −20°C. Before hybridization, sections were fixed in 4% formaldehyde for 20 min, dehydrated in ascending concentrations of ethanol, cleared in xylene for 15 min, rehydrated in descending concentrations of ethanol, and placed in prewarmed 0.01 M sodium citrate buffer, pH 6.0. Sections were pretreated for 10 min in a microwave, dehydrated in ethanol, and air dried. The CB_1R riboprobe was generated by *in vitro* transcription with [^{35}S]UTP. The ^{35}S-labeled probe was diluted (10^6 dpm/ml) in hybridization solution containing 50% formamide, 10% dextran sulfate, and 1× Denhardt's solution (Sigma). The hybridization solution (120 μl) was applied to each slide, and they were incubated overnight at 57°C. Sections were then treated with 0.002% RNAase A solution and submitted to stringency washes in decreasing concentrations of sodium chloride/SSC. Sections were dehydrated and enclosed in x-ray film cassettes with BMR-2 film (Eastman Kodak) for 48 h. Slides were dipped in NTB2 autoradiographic emulsion (Eastman Kodak), dried, and stored at 4°C for 14 d. Slides were developed with a D-19 developer (Eastman Kodak).

The CB_1R probe (antisense) was transcribed from PCR fragments amplified using the following primers: forward, CTG CAA GAA GCT GCA ATC TG; and reverse, TGG CGA TCT TAA CAG TGC TC. This sequence is complementary to part of exon 2, which is the single encoding exon in *Cnr1*. Hybridization with the sense probe was performed as negative control.

Gastrointestinal motility. Gastrointestinal motility was measured using the charcoal method as described previously (Rossi et al., 2003). Male mice at 10–12 weeks of age were fasted overnight with water *ad libitum* and received a single injection of vehicle or SR141716 (10 mg/kg, i.p.) at time 0. At 30 min, mice received 100 μl of a solution of 10% charcoal–5% Arabic gum in saline (Sigma-Aldrich) by oral gavage, and, at 50 min, mice were killed by cervical dislocation and the intestine were quickly dissected. Immediately after dissection, the intestine was placed in cold 10% Formalin solution until the tissue was straightened, and the distance traveled by the solution was measured.

mRNA content. Four-hour-fasted mice were killed, and stomach and small intestine were quickly dissected, snap frozen in liquid nitrogen, and stored at −80°C until additional processing. Total RNA was isolated using Trizol (Invitrogen) following the protocol of the manufacturer. RNA samples were treated with DNase I (Roche Applied Science) and retro-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen). qPCR analysis was performed using TaqMan assays (Applied Biosystems).

Stool analysis. Mice were individually housed, and stool samples were collected from the cages. Calorimetric and fat content analysis was performed by Central Analytical Lab, University of Arkansas Poultry Science, using ANSI/ASTM D2015-77 and AOAC 920.39C methods, respectively.

Body weight, metabolic rate, and food intake. Body weight measurement was performed weekly starting at 5 weeks of age. Metabolic rate parameters (oxygen consumption, carbon dioxide production, and respiratory exchange ratio) and food intake were measured by indirect calorimetry using the TSE labmaster system (TSE Systems). Approximately 8-week-old mice were transferred to the TSE labmaster system and allowed to acclimatize for 4 d, and data were collected for the following 3–4 d.

Pharmacological SR141716 treatment. Single-housed 7- to 8-week-old mice were fasted for 24 h, and, right before the dark cycle, mice received a single intraperitoneal injection of vehicle or SR141716 (3 mg/kg). Food intake was measured for the following 2 h.

For chronic treatment with SR141716, mice were fed on high-fat diet for ~8 weeks. Mice were single housed, and blood glucose, serum metabolites, and body composition were assessed before and after the pharmacological treatment. Blood glucose was measured using a standard glucometer (One Touch Ultra; Lifescan). Blood was centrifuged to collect serum for analysis of insulin (Crystal Chem), fatty acids (Wako Diagnostics), and triglycerides levels (Wako Diagnostics). Body composition was analyzed using the Echo MRI-100 system (Echo Medical Systems).

Data analyses. All values are reported as mean ± SEM. Analyses of the data were performed using GraphPad Prism software (GraphPad Software). Statistical significance was determined by two-tailed unpaired Student's *t* test or two-way ANOVA, followed by Bonferroni's *post hoc* test (** $p < 0.05$ and *** $p < 0.01$).

Results

Generation and validation of *Phox2b–Cre; Cnr1$^{flox/flox}$* mice

A Cre-conditional *Cnr1* null allele (*Cnr1^{flox}*) was generated by flanking exon 2 of *Cnr1* allele with loxP sites (Fig. 1*A*). A 4 kb PCR amplicon was observed in two ES clones screened for homologous recombination at the 3′ end (Fig. 1*B*). In one of these clones, the two expected bands at 12 and 10 kb, from *Cnr1* targeted and *Cnr1*^{wt} allele, were detected by Southern blot (Fig. 1*C*). Additional analysis of this single clone by Southern blot allowed the detection of a 12 and 7.2 kb band in the DNA digested with the restriction enzymes NheI and HindIII, respectively (Fig. 1*D*). *Cnr1$^{flox/wt}$* mice were mated, and *Cnr1$^{w/w}$*, *Cnr1$^{flox/wt}$*, and *Cnr1$^{flox/flox}$* offspring were obtained at the expected ratio according to the Mendelian distribution of alleles (Fig. 1*E*). *Cnr1$^{flox/wt}$* mice were crossed to *Phox2b–Cre* transgenic mice (Scott et al., 2011) to generate the study groups.

To determine whether *Cnr1$^{flox/flox}$; Phox2b–Cre* mice have Cre-recombined *Cnr1* allele in Phox2b neurons, we first performed PCR assays using genomic DNA from different brain areas and different organs/tissues. The Cre-recombined *Cnr1* allele is present in midbrain, hindbrain, and nodose ganglia (Fig. 2*A*), all sites in which Phox2b neurons are located. Cre-recombined *Cnr1* allele was not detected in all other tissues tested, including the stomach, different parts of the small intestine, and colon (Fig. 2*B*). Second, we performed *in situ* hybridization histochemistry to detect *Cnr1* mRNA in brain tissue. In *Cnr1$^{flox/flox}$; Phox2b–Cre* mice, distribution of *Cnr1* mRNA was similar to the pattern observed in *Cnr1$^{flox/flox}$* mice, including parabrachial nucleus (data not shown) and nucleus of the solitary tract (Fig. 2*E–H*). However, *Cnr1* mRNA was not detected in great part of nodose ganglia and DMV (Fig. 2*C–H*). Thus, these results show that *Cnr1$^{flox/flox}$; Phox2b–Cre* mice lack CB_1R in nodose ganglia and DMV neurons.

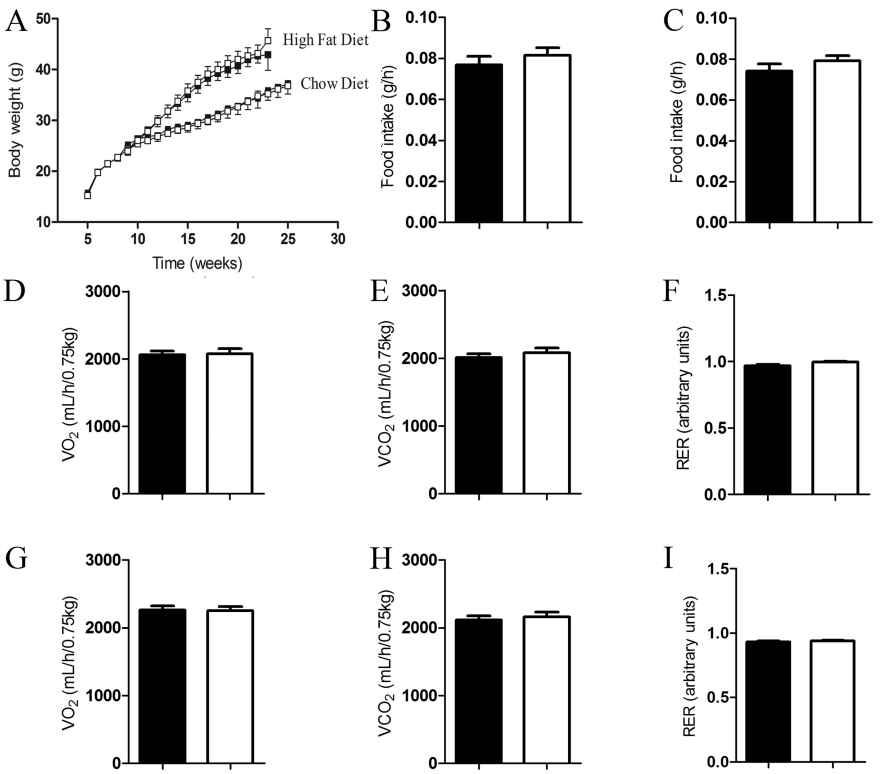

Figure 3. CB_1R in nodose and DVM neurons does not regulate body energy balance. Body weight curve of standard chow or high-fat fed mice (***A***) (chow diet: *Cnr1$^{flox/flox}$*, $n = 7$ and *Cnr1$^{flox/flox}$; Phox2b–Cre*, $n = 7$; high-fat diet: *Cnr1$^{flox/flox}$*, $n = 13$ and *Cnr1$^{flox/flox}$; Phox2b–Cre*, $n = 16$). Food intake of mice fed on standard chow (***B***) or high-fat diet (***C***). Oxygen consumption, carbon dioxide production, and respiratory exchange ratio of mice fed on chow (***D–F***) or high-fat diet (***G–I***) (chow diet: *Cnr1$^{flox/flox}$*, $n = 13$ and *Cnr1$^{flox/flox}$; Phox2b–Cre*, $n = 13$; high-fat diet: *Cnr1$^{flox/flox}$*, $n = 12$ and *Cnr1$^{flox/flox}$; Phox2b–Cre*, $n = 12$). Filled black symbols/bars and open symbols/bars represent *Cnr1$^{flox/flox}$* and *Cnr1$^{flox/flox}$; Phox2b–Cre* mice, respectively. Results are expressed as means ± SEM. Statistical analyses were performed using two-tailed unpaired Student's *t* test.

Body weight homeostasis in *Phox2b–Cre; Cnr1$^{flox/flox}$* mice

CB_1R controls food intake, energy expenditure, and thus body weight homeostasis (Cota, 2007; Quarta et al., 2010). To test whether CB_1R in the nodose/DMV is required for body weight homeostasis, we measured body weight, food intake, and energy expenditure in mice lacking CB_1R in the nodose/DMV neurons. On chow or high-fat diet feeding regimens, *Cnr1$^{flox/flox}$; Phox2b–Cre* mice have similar body weight compared with *Cnr1$^{flox/flox}$* controls (Fig. 3*A*). Food intake, oxygen consumption, carbon dioxide production, and respiratory exchange ratio were also similar between *Cnr1$^{flox/flox}$; Phox2b–Cre* and *Cnr1$^{flox/flox}$* mice (Fig. 3*B–I*).

CB_1R also regulates fasting-induced hyperphagia and mediates the anorexigenic effect of SR141716 (Di Marzo et al., 2001). Thus, we tested whether CB_1R in the nodose/DMV neurons is required for normal fasting-induced hyperphagia. Food intake after 24 h fasting was also similar between *Cnr1$^{flox/flox}$* and *Cnr1$^{flox/flox}$; Phox2b–Cre* mice (Fig. 4*A*). SR141716-treated *Cnr1$^{flox/flox}$* mice significantly reduce food intake compared with vehicle-treated *Cnr1$^{flox/flox}$* mice, similar to previously reported results (Fig. 4*A*) (Di Marzo et al., 2001). The anorexigenic effect of SR141716 in *Cnr1$^{flox/flox}$; Phox2b–Cre* mice was similar to the effect observed in *Cnr1$^{flox/flox}$* mice (Fig. 4*A*). Interestingly, SR141716-treated *Cnr1$^{flox/flox}$; Phox2b–Cre* mice tended to have longer meals and reduced rate of food intake compared with *Cnr1$^{flox/flox}$* mice, but the differences between the groups were not statistically significant (data not shown). In the chronic SR141716 treatment study, *Cnr1$^{flox/flox}$* mice treated with

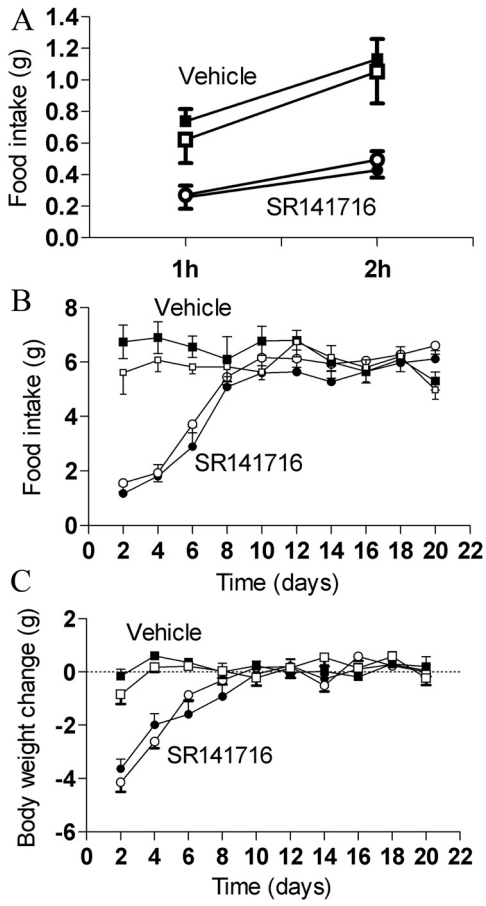

Figure 4. CB_1R in nodose and DVM neurons is not required for the anorexigenic effect and anti-obesity effect of SR141716. Fasting-induced hyperphagia (***A***) of mice fed on chow diet. Food intake (***B***) and body weight (***C***) curves of mice fed on high-fat diet and treated with 10 mg/kg SR141716 or vehicle (vehicle treated: *Cnr1*$^{flox/flox}$, $n = 6$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 7$; SR141716 treated: *Cnr1*$^{flox/flox}$, $n = 8$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 10$). Mice received daily intraperitoneal injections right before the beginning of dark cycle. Symbols represent *Cnr1*$^{flox/flox}$ (filled) and *Cnr1*$^{flox/flox}$*; Phox2b–Cre* (open). Results are expressed as means ± SEM.

SR141716 had reduced food intake during approximately the first week compared with vehicle-treated *Cnr1*$^{flox/flox}$ mice (Fig. 4*B*). The anorexigenic effect of SR141716 was transient as reported previously (Ravinet Trillou et al., 2003; Cota et al., 2009). Also, body weight reduction was observed in SR141716-treated compared with vehicle-treated *Cnr1*$^{flox/flox}$ mice (Fig. 4*C*). In *Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice, the anorexigenic and body weight reducing effects of SR141716 were similar to those observed in *Cnr1*$^{flox/flox}$ mice (Fig. 4*B*,*C*). Fed and fasted blood glucose, fatty acids, and triglycerides were similar between *Cnr1*$^{flox/flox}$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice either before treatment or after treatment (data not shown). As for body composition analysis, fat mass was reduced in SR141716-treated compared with vehicle-treated *Cnr1*$^{flox/flox}$ mice, but similar fat mass was observed in *Cnr1*$^{flox/flox}$*; Phox2b–Cre* compared with *Cnr1*$^{flox/flox}$ mice, either before or after treatment (data not shown). Altogether, these results suggest that CB_1R in the nodose/DMV neurons is not required to control body weight, food intake, energy expenditure, blood glucose, fatty acids, triglycerides, and fat mass and to mediate the effects of SR141716 on these parameters.

Gastrointestinal motility in *Phox2b–Cre; Cnr1*$^{flox/flox}$ mice

Pharmacological administration of CB_1R antagonist (SR141716) or genetic deletion of CB_1R in mice increases gastrointestinal motility (Coutts and Izzo, 2004; Yuece et al., 2007). *In vitro* data suggest that CB_1R in cholinergic fibers of the parasympathetic branch neurons mediate this effect (Coutts and Pertwee, 1997; Coutts and Izzo, 2004). However, the CB_1R-expressing sites that mediate this effect are unknown. Here we tested whether CB_1R in nodose/DMV neurons is required to regulate gastrointestinal motility. On chow diet, *Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice had increased gastrointestinal motility compared with *Cnr1*$^{flox/flox}$ mice (Fig. 5*A*). Also, SR141716-treated mice had increased gastrointestinal motility compared with vehicle-treated mice (Fig. 5*A*). These results suggest that CB_1R in nodose/DMV neurons is required for normal gastrointestinal motility in chow diet feeding conditions.

High-fat diet increases gastrointestinal motility (Izzo et al., 2009). Thus, we investigated the relevance of CB_1R in nodose/DMV neurons on regulation of gastrointestinal motility in the context of high-fat diet. On high-fat diet, *Cnr1*$^{flox/flox}$*; Phox2b–Cre* mice had increased gastrointestinal motility compared with *Cnr1*$^{flox/flox}$ mice (Fig. 5*B*), but this parameter was not affected by SR141716 treatment in both genotypes (Fig. 5*B*). These results suggest that CB_1R in nodose/DMV neurons is required for normal gastrointestinal motility also in the high-fat diet feeding condition.

To exclude the possibility that the increase in gastrointestinal motility observed in *Cnr1*$^{flox/flox}$*; Phox2b–Cre* was a result of the transgene per se, we performed experiments using *Cnr1*$^{w/w}$*; Phox2b–Cre* and *Cnr1*$^{w/w}$ mice fed on chow diet. We observed similar gastrointestinal motility in both groups (data not shown), indicating that the increase in gastrointestinal motility in *Cnr1*$^{flox/flox}$*; Phox2b–Cre* results from deletion of CB_1R in Phox2b neurons and not by an effect attributable to the *Phox2b–Cre* transgene itself.

CB_1R is expressed in several neurons of the small intestine, and the majority of those are cholinergic (Coutts et al., 2002).

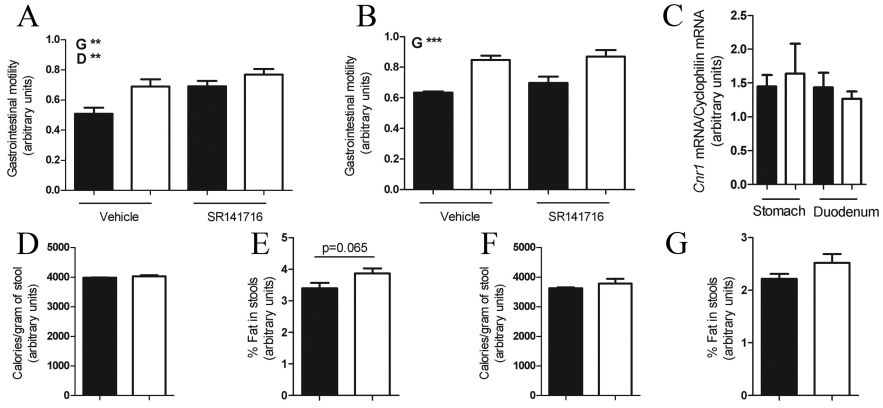

Figure 5. CB_1R in nodose and DMV neurons is required for gastrointestinal motility. Gastrointestinal motility in vehicle- or SR141716-treated mice fed on standard chow (***A***) (vehicle treated: *Cnr1*$^{flox/flox}$, $n = 10$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 10$; SR141716 treated: *Cnr1*$^{flox/flox}$, $n = 9$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 9$) or high fat (***B***) (vehicle treated: *Cnr1*$^{flox/flox}$, $n = 6$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 6$; SR141716 treated: *Cnr1*$^{flox/flox}$, $n = 7$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 7$). qPCR analysis of stomach and duodenum total mRNA (*Cnr1*$^{flox/flox}$, $n = 5$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 5$) (***C***). Calorimetric analyses or percentage of fat content of stools of mice fed on standard chow (***D***, ***E***) or high fat (***F***, ***G***) (*Cnr1*$^{flox/flox}$, $n = 6$ and *Cnr1*$^{flox/flox}$*; Phox2b–Cre*, $n = 6$). Bars represent *Cnr1*$^{flox/flox}$ (filled) and *Cnr1*$^{flox/flox}$*; Phox2b–Cre* (open). Results are expressed as means ± SEM. Statistical analyses were performed using two-way ANOVA (***A***, ***B***) or Student's *t* test (***C–G***). For ***A***, genotype (G), $F_{(1,31)} = 8.6$, $p < 0.05$; drug (D), $F_{(1,31)} = 8.9$, $p < 0.05$; and interaction, $F_{(1,31)} = 1.3$, $p = 0.25$. For ***B***, genotype (G), $F_{(1,17)} = 23.3$, $p < 0.05$; drug, $F_{(1,17)} = 1.16$, $p = 0.29$; and interaction, $F_{(1,17)} = 0.3$, $p = 0.61$.

To investigate whether the phenotype on gastrointestinal motility could be a result of reduced *Cnr1* mRNA expression in the stomach or small intestine, we measured *Cnr1* mRNA levels in those tissues. In either the stomach or duodenum, similar levels of *Cnr1* mRNA was detected in samples from $Cnr1^{flox/flox}$ and $Cnr1^{flox/flox}$*; Phox2b–Cre* mice (Fig. 5*C*). Thus, these results indicate that the phenotype on gastrointestinal motility is not the result of reduced *Cnr1* mRNA expression in the stomach or small intestine.

To further investigate whether increased gastrointestinal motility would result in reduced absorption of nutrients, we performed calorimetric and fat content analysis in the stools. Similar calories per gram or fat content was observed in stools of $Cnr1^{flox/flox}$ and $Cnr1^{flox/flox}$*; Phox2b–Cre* mice fed on chow diet (Fig. 5*D*,*E*). Of note, fat content tended to be higher in stools of $Cnr1^{flox/flox}$*; Phox2b–Cre* mice, but differences were not statistically significant. Also, similar calories per gram or fat content was observed in $Cnr1^{flox/flox}$ and $Cnr1^{flox/flox}$*; Phox2b–Cre* mice fed on a high-fat diet (Fig. 5*F*,*G*). Therefore, these data suggest that increased gastrointestinal motility does not lead to reduced absorption of nutrients, a result that is in agreement with the unchanged energy balance of $Cnr1^{flox/flox}$*; Phox2b–Cre* mice.

Discussion

CB_1R is widely expressed and regulates several physiological processes. Genetic deletion studies have demonstrated that CB_1R regulates body weight and gastrointestinal motility; nevertheless, the sites mediating these actions remain to be identified. Several results show that the vagus nerve controls aspects of energy metabolism, including food intake and blood glucose homeostasis (Williams et al., 2000; Fan et al., 2004; Rossi et al., 2011). Moreover, CB_1R in the vagus nerve has been suggested as an important molecule underlying normal feeding and consequentially body weight homeostasis. By using the Cre/loxP system, we generated mice lacking CB_1R in afferent (sensory) and efferent (motor) vagal neurons. Notably, deletion of CB_1R expression in vagal neurons did not significantly alter energy balance regulation or glucose homeostasis. In contrast, we found that CB_1R expressed by Phox2b neurons is required for the regulation by CB_1R of gastrointestinal motility.

SR141716 was considered a promising pharmacological drug for the treatment of obesity and diabetes. However, because of its psychotropic effect (increased depression), the process of additional development of this drug was halted (Di Marzo, 2008). However, the concurrent effects of CB_1R inverse agonist on mood and body weight may be separated if the CB_1R sites governing mood and body weight were to be identified. Thus, it is of interest to identify the sites expressing CB_1R that regulate energy balance in an attempt to dissociate the beneficial effects of SR141716 on body weight reduction from its psychiatric side effect. Notably, if CB_1R expressed by the nodose/DMV neurons is relevant for control of body energy metabolism, it would represent a possible target for brain-impermeable CB_1R inverse agonist anti-obesity drugs. However, our data support the view that CB_1R in those neurons are not required for regulation of body energy balance, and, as such, these sites should be ruled out as potential targets for development of anti-obesity CB_1R inverse agonist drugs.

Diarrhea is a frequent side effect reported by patients treated with SR141716 (Van Gaal et al., 2005; Addy et al., 2008). Indeed, this is a common side effect of anti-obesity drugs (Cahoon, 2010), and increase in gastrointestinal motility is one underlying cause of diarrhea. Importantly, despite the discomfort that it may generate, alteration in gastrointestinal motility is often observed in gastrointestinal diseases, such as irritable bowel syndrome (prevalence of 9–23% worldwide according to the International Foundation for Functional Gastrointestinal Disorders) and may lead to severe consequences, such as inflammation of the gastrointestinal tract. Several studies indicate that CB_1R controls gastrointestinal motility; nevertheless, the neurons that express CB_1R that mediate it are unclear. It has been suggested that CB_1R acts to control acetylcholine release from neurons of the myenteric neurons (Coutts and Pertwee, 1997). CB_1R colocalizes with several cholinergic neurons of the enteric nervous system (Coutts et al., 2002), but we do not observe deletion of *Cnr1* mRNA in the duodenum and stomach of $Cnr1^{flox/flox}$*; Phox2b–Cre* mice. Indeed, it has been reported previously that the *Phox2b–Cre* transgenic line used in this study does not express the transgene in the enteric nervous system (Ferreira-Gomes et al., 2011). The transgenic *Phox2b–Cre* mouse line used in this study has been reported to express Cre in a few other sites in addition to the nodose/DMV (Rossi et al., 2011), but we do not believe that these sites contribute to the phenotype observed because they have not been suggested previously to regulate gastrointestinal motility. Our results suggest that CB_1R in the nodose/DMV is required for control of gastrointestinal motility.

Anandamide levels increase during fasting in small intestine, and it has been suggested that it is a metabolic cue signaling through the vagal circuitry to stimulate feeding (Gómez et al., 2002). Conversely, given the fact that during fasting there is no major need of motility to have the food traveling through the digestive tract, it is plausible that anandamide may serve as a cue to suppress gastrointestinal motility.

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