Brief Communications

5-HT_{2C} Receptor Agonist Anorectic Efficacy Potentiated by 5-HT_{1B} Receptor Agonist Coapplication: An Effect Mediated via Increased Proportion of Pro-Opiomelanocortin Neurons Activated

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An essential component of the neural network regulating ingestive behavior is the brain 5-hydroxytryptamine2C receptor (5-HT_{2C}R), agonists of which suppress food intake and were recently approved for obesity treatment by the US Food and Drug Administration. 5-HT_{2C}R-regulated appetite is mediated primarily through activation of hypothalamic arcuate nucleus (ARC) pro-opiomelanocortin (POMC) neurons, which are also disinhibited through a 5-HT_{1B}R-mediated suppression of local inhibitory inputs. Here we investigated whether 5-HT_{2C}R agonist anorectic potency could be significantly enhanced by coadministration of a 5-HT_{1B}R agonist and whether this was associated with augmented POMC neuron activation on the population and/or single-cell level. The combined administration of subanorectic concentrations of 5-HT_{2C}R and 5-HT_{1B}R agonists produced a 45% reduction in food intake and significantly greater *in vivo* ARC neuron activation in mice. The chemical phenotype of activated ARC neurons was assessed by monitoring agonist-induced cellular activity via calcium imaging in mouse POMC-EGFP brain slices, which revealed that combined agonists activated significantly more POMC neurons (46%) compared with either drug alone (~25% each). Single-cell electrophysiological analysis demonstrated that 5-HT_{2C}R/5-HT_{1B}R agonist coadministration did not significantly potentiate the firing frequency of individual ARC POMC-EGFP cells compared with agonists alone. These data indicate a functional heterogeneity of ARC POMC neurons by revealing distinct subpopulations of POMC cells activated by 5-HT_{2C}Rs and disinhibited by 5-HT_{1B}Rs. Therefore, coadministration of a 5-HT_{1B}R agonist potentiates the anorectic efficacy of 5-HT_{2C}R compounds by increasing the number, but not the magnitude, of activated ARC POMC neurons and is of therapeutic relevance to obesity treatment.

Introduction

Obesity is a primary global health challenge of the 21st century, with obesity pharmacotherapies representing one of the most profound unmet clinical needs. The dynamic interplay between nutritional intake and the biogenic amine 5-hydroxytryptamine (5-HT), which is derived from the essential dietary amino acid tryptophan, indicates that 5-HT is inextricably linked to energy balance (Fernstrom and Wurtman, 1972; Tecott, 2007). Compounds augmenting the bioavailability of 5-HT by blocking its reuptake and/or stimulating release, such as D-fenfluramine and sibutramine, have been at the forefront of obesity treatment for the past 15 years (Lam et al., 2010). However, such compounds have been withdrawn from clinical use due to off-target effects (Connolly et al., 1997). Therefore, a greater understanding of the cellular networks that underlie the effectiveness of these compounds would aid in maximizing their therapeutic potential and facilitating the generation of more effective 5-HT obesity therapies.

Past efforts revealed that D-fenfluramine requires functional G_q-coupled 5-HT_{2C} receptors (5-HT_{2C}Rs) and G_i-coupled 5-HT_{1B} receptors (5-HT_{1B}Rs) to suppress appetite (Tecott et al., 1995; Lucas et al., 1998). Through these receptors, D-fenfluramine modulates the melanocortin system of the arcuate nucleus of the hypothalamus (ARC), a key energy balance network (Heisler et al., 2002, 2006; Sohn et al., 2011). 5-HT-mediated appetite through the engagement of the ARC melanocortin system occurs via a three-pronged mechanism: (1) direct activation of anorectic pro-opiomelanocortin (POMC) neurons (the endogenous melanocortin receptor agonist) via

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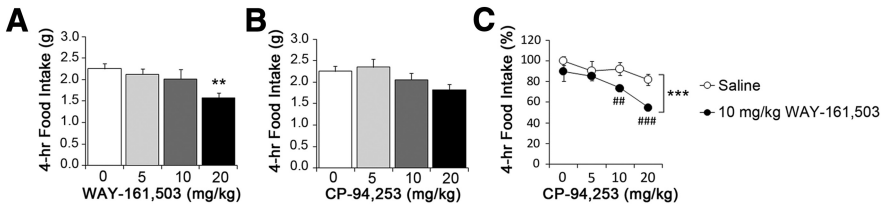

Figure 1. Combined WAY-161503 and CP-94253 significantly reduce feeding at concentrations that alone are not anorectic. ***A***, The 5-HT$_{2C}$R agonist WAY-161503 (20 mg/kg, i.p.) significantly suppresses 4 h feeding compared with vehicle (**$p = 0.010$). ***B***, The 5-HT$_{1B}$R agonist CP-94253 does not significantly influence 4 h feeding. ***C***, Combined subanorectic concentrations of WAY-161503 (10 mg/kg) and CP-94253 (20 mg/kg) significantly suppress 4 h food intake compared with saline and individually administrated drugs (***$p < 0.001$, saline + CP-94253 at 0, 5, 10, 20 mg/kg compared with 10 mg/kg WAY-161503 + CP-94253 at 0, 5, 10, 20 mg/kg; ##$p = 0.008$, 10 mg/kg WAY-161503 + 10 mg/kg CP-94253; and ###$p < 0.001$, 10 mg/kg WAY-161503 + 20 mg/kg CP-94253 compared with saline; $n = 10$ per treatment).

5-HT$_{2C}$Rs, (2) indirect 5-HT$_{1B}$R-mediated disinhibition of anorectic POMC neurons via suppression of local inhibitory inputs, and (3) direct inhibition of orexigenic agouti-related protein (AgRP) neurons (the endogenous melanocortin receptor antagonist/inverse agonist; Heisler et al., 2002, 2006; Sohn et al., 2011). Furthermore, 5-HT$_{2C}$R action exclusively on POMC neurons is required for D-fenfluramine to exert its anorectic effect (Xu et al., 2008, 2010), demonstrating the therapeutic relevance of the 5-HT-melanocortin axis. Indeed, the significance of this system in energy balance regulation was realized in the summer of 2012 when the US Food and Drug Administration approved a 5-HT$_{2C}$R agonist (Lorcaserin; Arena Pharmaceuticals) for obesity treatment.

Here we investigated means with which to improve the anorectic therapeutic profile of this new class of obesity pharmacotherapy. In light of 5-HT's bimodal activation of ARC POMC neurons via its concerted action at 5-HT$_{2C}$Rs and 5-HT$_{1B}$Rs, we examined whether a net gain in 5-HT$_{2C}$R agonist anorectic effect could be obtained by coadministration with a 5-HT$_{1B}$R agonist and, if so, whether this was associated with increased efficacy of POMC neuron activation on either the population or single-cell level.

Materials and Methods

Animals. Transgenic mice on a C57BL/6 background expressing enhanced green fluorescent protein (EGFP) under the control of POMC regulatory elements (POMC-EGFP mice; generous gift from Profs. Richard Simerly and Malcolm Low; Heisler et al., 2002) and wild-type C57BL/6 mice were group housed and maintained on a 12:12 light-dark cycle (lights on at 0700) with *ad libitum* access to chow diet and water unless otherwise stated. All experiments were in accordance with the UK Animals (Scientific Procedures) Act 1986.

Drugs. (4aR)-8,9-Dichloro-2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2-*a*]quinoxalin-5(6*H*)-one (WAY-161503; Tocris Bioscience) and 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-pyrrolo[3,2-*b*]pyridine hydrochloride (CP-94253; Tocris Bioscience) were used as selective 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists, respectively. Drugs were dissolved in distilled water and administered in a volume of 10 ml/kg of body weight.

Effect of 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists on acute food intake. Single housed C57BL/6 mice ($n = 30$) received a habituation intraperitoneal injection of vehicle before a 24 h fast from 0900 h (lights on 0700/off 1900). Mice were then treated intraperitoneally with vehicle, WAY-161503 (5, 10, 20 mg/kg), CP-94253 (5, 10, 20 mg/kg), or a combined dose of WAY-161503 (10 mg/kg) and CP-94253 (0, 5, 10, 20 mg/kg). One hour postinjection, chow pellets were replaced and the 4 h food intake was measured. These studies were performed using a within-subject experimental design with a minimum 3 d treatment-free period.

Immunohistochemistry. To assess *in vivo* ARC neuronal activity, *ad libitum* fed or 24 h fasted C57BL/6 mice were treated intraperitoneally with saline, 10 mg/kg WAY-161503, 20 mg/kg CP-94253, or combined 10 mg/kg WAY-161503 + 20 mg/kg CP-94253 ($n = 10$ per treatment) for 2 h following the onset of the light cycle (0900 h). Two hours later, mice were terminally anesthetized, transcardially perfused with 0.9% saline and then 10% neutral buffered formalin (Sigma), and brains were extracted, prepared, coronally sectioned (25 μm), and processed for cFOS immunoreactivity (FOS-IR) as described previously (Alon et al., 2009; Lam et al., 2009; Marston et al., 2011) using an anti-cFOS rabbit primary antibody (1:8000, catalog #PC38; Calbiochem) and a biotinylated donkey anti-rabbit secondary antibody (1:1000, catalog #711-065-152, Stratech). Images of FOS-IR were generated and analyzed using an Axioskop II microscope (Carl Zeiss) and Adobe Photoshop CS5 software. Quantitative analysis of manually counted FOS-IR cells was conducted at the rostral ARC (−1.58 to −1.94 mm from bregma) and the caudal ARC (−2.18 to −2.46 mm from bregma; Paxinos and Franklin, 2001).

Tissue preparation for calcium imaging and electrophysiology. Coronal brain slices (180 μm thick) containing the ARC (−1.58 to −1.94 mm bregma) were taken from *ad libitum* fed POMC-EGFP mice 2 h after the onset of the light cycle. Slices were prepared as described previously (Heisler et al., 2002, 2006; Blot et al., 2009) and maintained in an artificial cerebrospinal fluid solution containing the following (in mM): 126 NaCl, 2.5 KCl, 21.4 $NaHCO_3$, 1.2 NaH_2PO_4, 10 glucose, 2 Na pyruvate, 1.2 $MgCl_2$, and 2.4 $CaCl_2$, pH 7.3, gassed with 95% O_2 and 5% CO_2.

Calcium imaging. Brain slices from male and female POMC-EGFP (28 ± 1 d, $n = 9$) mice were loaded with fura-2 AM (9.6 μM, 0.04% pluronic acid, 34–36°C for 20 min, followed by a 20 min washout). Imaging was performed at 34–36°C with an Olympus FluoView 1000MPE two-photon laser-scanning microscope, equipped with a MaiTai DeepSee laser (Spectra-Physics) tuned to 790 nm, and via an LUMPlanFI/IR 40× (numerical aperture 0.8) objective. Images were acquired at a frame rate of 5.4 s and spatial averages of somatic fluorescence were monitored as agonists were applied to the bath perfusion. Fluorescence values (F) were corrected for linear bleaching, background subtracted, and expressed as the percentage $\Delta F/F$ baseline.

Electrophysiology. Cell-attached current-clamp recordings were performed on brain slices from male and female (7:14) POMC-EGFP (38 ± 3 d, $n = 21$) mice, at 32–34°C using an EPC-10 amplifier and Patchmaster software (HEKA Elektronik). Action potentials were detected using MiniAnalysis software (Synaptosoft), and interspike intervals assessed before and after bath application of agonists and expressed as percentage change from baseline.

Data analysis. Data were analyzed with Student's t tests, one-way ANOVA, repeated-measures ANOVA, or χ^2 followed by Tukey's or χ^2 *post hoc* tests where appropriate. For all analyses, significance was assigned at $p < 0.05$. Data are presented as mean ± SEM.

Results

Identification of subanorectic concentrations of 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists

5-HT$_{2C}$R agonist WAY-161503 and 5-HT$_{1B}$R agonist CP-94253 suppress appetite (Lee et al., 2002; Heisler et al., 2006; Rosenzweig-Lipson et al., 2006). We first performed a dose–response analysis for 5-HT$_{2C}$R agonist WAY-161503 (5, 10, and 20 mg/kg, i.p.) and 5-HT$_{1B}$R agonist CP-94253 (5, 10 and 20 mg/kg, i.p.) to identify subanorectic concentrations in 24 h fasted mice ($n = 10$ per treatment). The highest dose of WAY-161503 (20 mg/kg, i.p.) suppressed feeding (Fig. 1*A*, $F_{(3,49)} = 4.247$, $p = 0.010$) and none of the concentrations of CP-94253 used significantly influenced 4 h food intake (Fig. 1*B*, $F_{(3,50)} = 1.998$, $p = 0.127$). Nonanorectic concentrations of 10 mg/kg WAY-161503 and 5, 10, and 20 mg/kg CP-94253 were selected for future analyses.

Combined nonanorectic concentrations of 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists significantly suppress food intake

To investigate the effect of a combined nonanorectic dose of a 5-HT$_{2C}$R agonist with increasing nonanorectic concentrations of 5-HT$_{1B}$R agonists on acute feeding behavior, 24 h fasted C57BL/6 mice were treated with either saline + CP-94253 (0, 5, 10, or 20 mg/kg) or 10 mg/kg WAY-161503 + CP-94253 (0, 5, 10, or 20 mg/kg) and 4 h food intake measured ($n = 10$ per treatment). Again, saline, 10 mg/kg WAY-161503, and 5, 10, and 20 mg/kg CP-94253 had no significant effect on feeding behavior alone (Fig. 1*C*). In contrast, combining CP-94253 (5, 10, and 20 mg/kg) with 10 mg/kg WAY-161503 dose-dependently enhanced 5-HT$_{2C}$R agonist anorectic potency (Fig. 1*C*, main effect of treatment, $F_{(1,89)} = 14.686$, $p < 0.001$; main effect of CP-94253 concentrations, $F_{(3,89)} = 7.381$, $p < 0.001$; interaction between treatment and CP-94253 concentration, $F_{(3,89)} = 1.382$, $p = 0.254$). Indeed, 10 mg/kg WAY-161503 combined with CP-94253 suppressed 4 h feeding by 26% at 10 mg/kg (Tukey's *post hoc*, $p = 0.008$) and suppressed feeding by 45% at 20 mg/kg (Tukey's *post hoc*, $p < 0.001$), despite these concentrations of CP-94253 having no significant effect on feeding when combined with saline. These data indicate that the efficiency of a 5-HT$_{2C}$R agonist in inducing anorexia is significantly increased in conjunction with a 5-HT$_{1B}$R agonist.

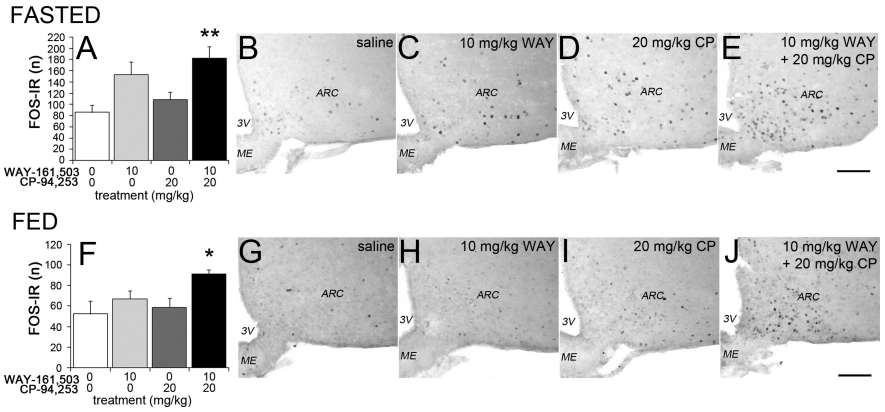

Figure 2. Combined WAY-161503 and CP-94253 significantly increase ARC FOS-IR compared with agonists administrated individually. ***A***, ***F***, Quantitative analysis of FOS-IR neurons in rostral ARC (bregma levels from −1.58 mm to −1.94 mm); combination of 10 mg/kg WAY-161503 and 20 mg/kg CP-94253 increases rostral ARC FOS-IR in *ad libitum* fed (***A***) and 24 h fasted mice (***F***) (*$p = 0.027$, **$p = 0.0049$ compared with saline; $n = 6–7$ per treatment). ***B–E*** and ***G–J***, Representative images of rostral ARC FOS-IR (−1.58 mm to −1.94 mm from bregma) in *ad libitum* fed (***B–E***) and 24 h fasted (***G–J***) mice treated with saline (***B***,***G***), 10 mg/kg WAY-161503 (***C***,***H***), 20 mg/kg CP-94253 (***D***,***I***), or 10 mg/kg WAY-161503 + 20 mg/kg CP-94253 (***E***,***J***). 3V indicates third ventricle; ME, median eminence. Scale bar, 50 μm (***B–E***, ***G–J***).

Combined nonanorectic concentrations of 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists significantly increase ARC FOS-IR

We next sought to investigate the neural mechanism underlying the observed potentiation of anorexia by combining a 5-HT$_{2C}$R agonist with a 5-HT$_{1B}$R agonist. Based on previous reports indicating that both 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists influence the activity of ARC melanocortin neurons (Heisler et al., 2002, 2006; Lam et al., 2008; Sohn et al., 2011), we first examined *in vivo* ARC neuronal activation using FOS-IR. We observed that concentrations of the 5-HT$_{2C}$R (10 mg/kg) and 5-HT$_{1B}$R (20 mg/kg) agonists that did not influence feeding when administered alone also did not alter ARC neuronal activity (Fig. 2). In contrast, combined 5-HT$_{2C}$R agonist (10 mg/kg) with a 5-HT$_{1B}$R agonist (20 mg/kg) substantially increased rostral (−1.58 to −1.82 mm from bregma) ARC FOS-IR compared with saline in both *ad libitum* fed (Fig. 2*A–E*, $F_{(3,22)} = 5.944$, $p = 0.0049$) and 24 h fasted mice (Fig. 2*F–J*, $F_{(3,23)} = 3.774$, $p = 0.027$, $n = 6–7$ per treatment). Neither individual nor combined drug treatment significantly influenced the activity of caudal (−2.18 to −2.46 mm from bregma) ARC neurons in either *ad libitum* fed (saline, 118.4 ± 16.3; WAY-161503, 133.5 ± 13.0; CP-94253, 121.3 ± 25.5; WAY-161503 + CP-94253, 155.0 ± 22.2 FOS-IR neurons, $F_{(3,23)} = 0.808$, $p = 0.504$) or 24 h fasted mice (saline, 84.2 ± 11.1; WAY-161503, 92.3 ± 8.7; CP-94253, 63.0 ± 9.5; WAY-161503 + CP-94253, 91.3 ± 8.4 FOS-IR neurons, $F_{(3,24)} = 2.134$, $p = 0.126$). These data suggest that the population of ARC neurons activated by 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists lie in a particular subregion of the ARC, the rostral portion.

Combined 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists significantly increase the number of responsive ARC POMC neurons

To clarify the chemical phenotype of rostral ARC neurons activated by combined 5-HT$_{2C}$Rs and 5-HT$_{1B}$Rs agonists, we examined changes in intracellular calcium concentrations in acute brain slices from POMC-EGFP mice loaded with fura-2 AM using multiphoton fluorescence imaging (Fig. 3*A*). Approximately 25% of POMC neurons responded to 20 μM WAY-161503 (12 of 45) and 200 nM CP-94253 (19 of 66) alone. In contrast, by combining 20 μM WAY-161503 with 200 nM CP-94253, ~46% of ARC POMC neurons (34 of 74) were responsive, significantly more than individual agonists alone (Fig. 3*B*,*C*, χ^2 (2) = 6.38, $p = 0.041$, $n = 185$). Illustrating that CP-94253's effects on POMC activity are mediated via presynaptic action at 5-HT$_{1B}$Rs, both the synaptic blocker tetrodotoxin (1 μM) and the GABA$_A$ receptor antagonist gabazine (3 μM) prevented CP-94253's effects on POMC activity (0 of 27 and 3 of 42, respectively, χ^2 (2) = 15.4, $p < 0.001$, compared with CP-94253 alone, $n = 135$), whereas gabazine (3 μM) did not influence WAY-161503's effect (χ^2 (1) = 0.971E-02, $p = 0.922$, compared with WAY-161503 alone, $n = 53$; Fig. 3*B*). These findings are consistent with previous reports indicating that a 5-HT$_{2C}$R agonist activates ~25% of POMC neurons (Sohn et al., 2011) and reveal for the first time the proportion of POMC neurons activated by a 5-HT$_{1B}$R agonist. The finding that the 5-HT$_{2C}$R agonist and 5-HT$_{1B}$R agonist activated a similar percentage of POMC neurons illustrates the importance of modulating GABAergic tone on POMC activity in 5-HT's appetitive effects. When combined, the 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists significantly increased the number of ARC POMC neurons activated on a population level.

POMC ARC neurons increase firing rate in response to individual and combined 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists

To explore the nature of ARC POMC neuronal activation in response to WAY-161503 and CP-94253 on a single-cell level, we used cell-attached electrophysiological recording to assess changes in firing frequency in acute brain slices isolated from POMC-EGFP mice. Recordings were made from ventromedial ARC POMC neurons −1.58 to −1.94 mm from bregma where the preponderance of 5-HT responding POMC neurons localize (Sohn et al., 2011). Changes in firing frequency were measured as

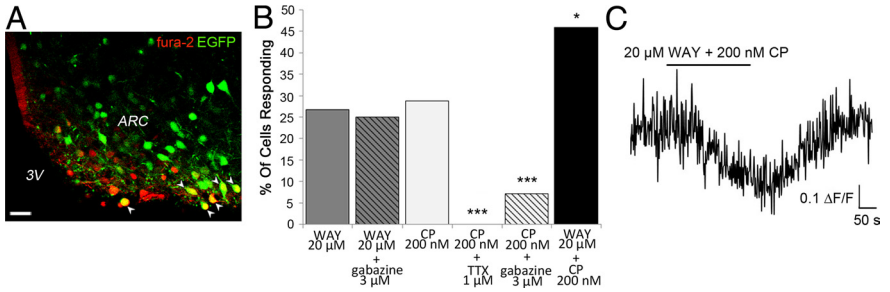

Figure 3. Combined WAY-161503 and CP-94253 increases ARC POMC intracellular calcium concentrations compared with individual agonists. ***A***, Coronal ARC POMC-EGFP brain slice illustrating EGFP neurons (green) loaded with fura-2 (red), dual-labeling (yellow, white arrowheads). 3V indicates third ventricle. Scale bar, 50 μm. ***B***, Combination of 20 μM WAY-161503 and 200 nM CP-94253 increases intracellular calcium concentrations in ARC POMC-EGFP neurons compared with individual treatments (*$p =$ 041, $n = 185$ cells). 1 μM tetrodotoxin (TTX) and 3 μM gabazine block the effect of 200 nM CP-94253 on POMC-EGFP activity (***$p < 0.001$, $n = 135$ cells), whereas 3 μM gabazine has no effect on 20 μM WAY-161503 on POMC-EGFP cells ($p = 0.922$, $n =$ 53 cells). ***C***, Representative trace of POMC-EGFP neuron increasing intracellular calcium concentrations shown by reduced fluorescence (F) expressed as $\Delta F/F$ to the combined dose of 20 μM WAY-161503 and 200 nM CP-94253.

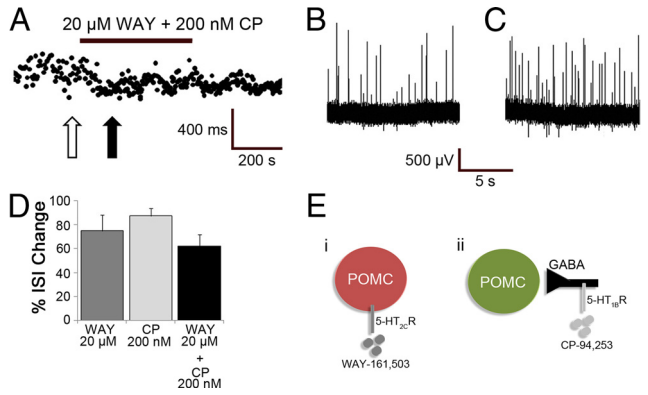

Figure 4. WAY-161503 and CP-94253 increase the firing rate of ARC POMC neurons. ***A***, Representative diagram of POMC-EGFP neuron interspike interval (ISI) in response to coapplication of 20 μM WAY-161503 and 200 nM CP-94253 recorded in a cell-attached electrophysiological configuration (white arrow, baseline trace expanded in ***B*** and black arrow, treatment trace expanded in ***C***). ***D***, Application of 20 μM WAY-161503, 200 nM CP-94253, and 20 μM WAY-161503 + 200 nM CP-94253 achieve a similar degree of reduction in the ISI of ARC POMC-EGFP neurons ($n = 6$–7 per treatment). ***E***, Proposed model of the mechanism through which 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists increase ARC POMC neuron activity. ***Ei***, POMC neurons are directly activated by WAY-161503 via G_q-coupled 5-HT$_{2C}$Rs expressed on POMC neurons. ***Eii***, POMC neurons are disinhibited/indirectly activated by CP-94253 via G_i-coupled 5-HT$_{1B}$R-expressing GABAergic projections onto POMC.

a percentage change of an interspike interval from baseline (Fig. 4*A–C*). ARC POMC neurons treated with 20 μM WAY-161503 and 200 nM CP-94253 administered individually and in combination showed a substantially higher increase in firing frequency (12.8 ± 6.5%, 25.1 ± 13.2%, and 37.7 ± 9.7%, respectively, $n = 19$) and did not have a significantly different effect on the degree of POMC neuron activation (Fig. 4*D*, one-way ANOVA, $F_{(2,18)} = 1.311$, $p = 0.297$, $n = 6$–7 per treatment). These data reveal that whereas combining 5-HT$_{2C}$Rs and 5-HT$_{1B}$Rs agonists recruits a greater number of ARC POMC neurons, on a single-cell level, combined agonist administration fails to potentiate the action of either drug alone.

Discussion

Lorcaserin, a 5-HT$_{2C}$R agonist soon to reach the clinical setting (Smith et al., 2010), was the first new approved obesity treatment by the FDA in 13 years. Here we investigated means with which to improve the anorectic therapeutic profile of 5-HT$_{2C}$R agonists. Utilizing concentrations of compounds that did not influence feeding, we observed that when combining a 5-HT$_{2C}$R agonist with a 5-HT$_{1B}$R agonist, a significant suppression of food intake was obtained. Illustrating a mechanism underlying this effect, we report that 5-HT$_{2C}$R and 5-HT$_{1B}$R agonist coapplication produced a significant increase in the activity of the key appetitive regulator POMC in the ARC on the population level, but not the single-cell level.

POMC is a component of the melanocortin system and acts as a principal gateway through which multiple metabolic cues signal (Broberger, 2005; Zhou et al., 2005; Lam et al., 2010; Pandit et al., 2011). Indeed, both the critical appetitive neurotransmitter 5-HT and the adipocyte-derived hormone leptin reciprocally regulate the activity of POMC and AgRP neurons by directly activating POMC neurons, inhibiting GABAergic inputs onto POMC neurons, and directly inhibiting AgRP neurons (Cowley et al., 2001; Heisler et al., 2002, 2006; Zhou et al., 2005). The important role of GABA in the mediation of POMC tone in the regulation of energy balance has been revealed in a recent study demonstrating that a primary effect of leptin on appetite and body weight is mediated directly through modulation of GABAergic neurotransmission (Vong et al., 2011). We reported previously that half the concentration of the 5-HT$_{1B}$R agonist CP-94253 was sufficient to reduce inhibitory postsynaptic currents onto POMC neurons compared with the concentration required to directly inhibit the activity of AgRP neurons (Heisler et al., 2006). This is consistent with the localization of 5-HT$_{1B}$Rs, which are primarily heteroreceptors influencing the release of other neurotransmitters such as GABA (Stanford and Lacey, 1996). The *in vivo* feeding data together with the calcium imaging and electrophysiological data presented here indicate that the effect of 5-HT$_{1B}$R agonists on appetite is associated with an increase in ARC POMC neuron disinhibition via a suppression of local GABAergic inputs, and that this is as important as direct stimulation of POMC neurons via 5-HT$_{2C}$Rs in mediating 5-HT-induced anorexia.

The identification of functional heterogeneity in ARC POMC neurons was recently revealed in a study showing that POMC neurons responsive to leptin were distinct from those responsive to a 5-HT$_{2C}$R agonist (Sohn et al., 2011). The data presented here further unravel the functional response of POMC neurons to 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists and suggest an additional level of dissociation. Specifically, within the rostral ARC, individual 5-HT$_{2C}$R and 5-HT$_{1B}$R agonists alone activate ~25% of POMC neurons each and, when combined, activate ~46% of POMC neurons. Data obtained on a single-cell level reveal that 5-HT$_{2C}$R and 5-HT$_{1B}$R coadministration is not more effective at activating POMC neurons than individual agonists: they all stimulate POMC neurons to a similar extent. Together, the data indicate that a primary effect of agonists is achieved via activation of distinct subpopulations of POMC neurons, those expressing 5-HT$_{2C}$Rs (Fig. 4*Ei*) and those receiving inhibitory inputs expressing 5-HT$_{1B}$Rs (Fig. 4*Eii*), agonists that when combined thereby significantly increase the proportion of POMC neurons activated. These data provide an additional functional dissociation of ARC POMC

neurons within the rostral ARC and further clarify the heterogeneity of this important population of cells influencing energy balance.

In summary, we report a significant reduction in food intake by combining a 5-HT$_{2C}$R agonist with a 5-HT$_{1B}$R agonist at concentrations that alone do not influence feeding—a combination of agonists that significantly increased POMC neuron activity on a population level. The data obtained therefore provide an effective means of stimulating a greater proportion of these critical appetitive cells, highlight the heterogeneity of ARC POMC neurons, and illustrate the importance of GABAergic modulation of POMC neurons in 5-HT-induced anorexia. These data reveal a powerful means with which it is possible to augment the anorectic potency of a new class of obesity pharmacotherapy, 5-HT$_{2C}$R agonists.

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