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ORIGINAL ARTICLE

Neutral antagonism at the cannabinoid 1 receptor: a safer treatment for obesity

FJ Meye[1], V Trezza[1,2], LJMJ Vanderschuren[1,3], GMJ Ramakers[1] and RAH Adan[1]

Obesity is a global problem with often strong neurobiological underpinnings. The cannabinoid 1 receptor (CB1R) was put forward as a promising drug target for antiobesity medication. However, the first marketed CB1R antagonist/inverse agonist rimonabant was discontinued, as its use was occasionally associated with negative affect and suicidality. In artificial cell systems, CB1Rs can become constitutively active in the absence of ligands. Here, we show that such constitutive CB1R activity also regulates GABAergic and glutamatergic neurotransmission in the ventral tegmental area and basolateral amygdala, regions which regulate motivation and emotions. We show that CB1R inverse agonists like rimonabant suppress the constitutive CB1R activity in such regions, and cause anxiety and reduced motivation for reward. The neutral CB1R antagonist NESS0327 does not suppress constitutive activity and lacks these negative effects. Importantly, however, both rimonabant and NESS0327 equally reduce weight gain and food intake. Together, these findings suggest that neutral CB1R antagonists can treat obesity efficiently and more safely than inverse agonists.

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Keywords: cannabinoid 1 receptor; constitutive activity; inverse agonist; mesolimbic dopamine system; obesity

INTRODUCTION

Obesity is a global problem for both the afflicted individual and society.[1,2] The etiology of obesity often has prominent neurobiological underpinnings that may share many similarities with drug addiction.[3] In order to help treat obesity, adjunctive pharmacological strategies can be used to help facilitate a healthy life style.[4–6] The cannabinoid 1 receptor (CB1R) was a prominent candidate target, because of its key role in energy balance.[7–10] The CB1R antagonists/inverse agonist rimonabant was shown to clinically reduce body weight in humans, but was associated with psychiatric side effects, like anxiety, depression and even suicidality.[7,11–13] Because of these side effects, CB1R antagonists/inverse agonists were abandoned as viable medicine.

Notably, however, the CB1R is a constitutively active receptor in artificial cell systems, as it can adopt active conformations in the absence of any ligand.[14–16] Rimonabant suppresses CB1R constitutive signaling, whereas such inverse agonism is not observed with novel neutral CB1R antagonists like NESS0327.[14–21] CB1R constitutive activity occurs in artificial cell systems, but its physiological occurrence and role in behavior remain unclear. We now show that CB1R constitutive activity regulates neurotransmission in the mesocorticolimbic system, which has an important role in anxiety and depression.[22–26] We also show that the suppression of CB1R constitutive activity has potentially harmful effects on anxiety and motivation for reward. The neutral CB1R antagonist NESS0327 lacks these effects, and is just as capable of reducing weight gain and food intake as rimonabant.

MATERIALS AND METHODS

Animals

All electrophysiological and behavioral experiments were approved by the Animal Ethics Committee of Utrecht University, and were conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and the European regulations (Guideline 86/609/EEC). Electrophysiological experiments were performed in C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) born in our own animal facility. Mice were group housed together with littermates in Makrolon cages (34 × 20 × 13.5 cm). The age of mice ranged from postnatal day 14–21 (ventral tegmental area; VTA) or 22–42 (basolateral amygdala; BLA).

Behavioral experiments were performed in male Wistar rats (Charles River, Sulzfeld, Germany) housed in pairs in Makrolon cages (37.5 × 22.5 × 15.0 cm). At arrival in our animal facility, rats typically weighed between 250–300 g. Rats were handled daily, but otherwise left to recover for at least 1 week before experimentation.

Both mice and rats were kept under controlled conditions (ambient temperature of 20–21 °C, 60–65% relative humidity). Unless otherwise specified, animals had ad libitum access to chow (CRM(E), Special Diet Services, Witham, UK) and water, and were housed on a 12/12 h light cycle with lights on at 07:00 hours. Animals were experimentally naive when tested.

Drugs

The AMPA/Kainate receptor antagonist DNQX, the $GABA_A$ receptor antagonist bicuculline, the voltage-gated Na^+-channel blocker tetrodotoxin (TTX), the direct CB1/2R agonist WIN55,212-2, the FAAH inhibitor URB597 (which inhibits hydrolysis of the endocannabinoid anandamide), the MGL inhibitor URB602 (which inhibits hydrolysis of the endocannabinoid 2-AG), the neutral CB1R antagonist O-2050 and the CB1R inverse agonist AM251 were all from Tocris (Bristol, UK). The neutral CB1R antagonist NESS0327 was from Cayman Chemical (Ann Arbor, MI, USA) and the CB1R inverse agonist SR141716A (rimonabant) was a generous gift from the NIMH Chemical Synthesis and Drug Supply Program. For electrophysiological experiments, these compounds were dissolved in DMSO and bath-applied, with the final bath concentration of DMSO never exceeding 0.1%. For behavioral experiments, we dissolved SR141716A and NESS0327 in a vehicle of 1% DMSO, 4% polyethylene glycol and 5% TWEEN-80. Drugs were injected intraperitoneally (ml kg^{-1} body weight).

[1]Rudolf Magnus Institute, Department of Neuroscience and Pharmacology, University Medical Center Utrecht, Utrecht, The Netherlands; [2]Department of Biology, University 'Roma Tre', Rome, Italy and [3]Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. Correspondence: Professor RAH Adan, Rudolf Magnus Institute, Department of Neuroscience and Pharmacology, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.
E-mail: r.a.h.adan@umcutrecht.nl
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Ex vivo electrophysiology

Experiments were conducted essentially as described earlier[27,28] and are described fully in the Supplementary Information. In short, animals were anesthetized with isoflurane and decapitated. Brain slices of 200 μm were cut on a Vibratome (Leica, Rijswijk, The Netherlands) in a horizontal (VTA) or coronal (BLA) plane and kept in ACSF (in mm): NaCl (124), KCl (3.3), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $MgSO_4$ (1.3), $NaHCO_3$ (20), glucose (10). Whole-cell voltage clamp recordings (HEKA; EPC-10 patch clamp amplifier) were made in VTA dopamine neurons and BLA pyramidal neurons. The pipette solutions for miniature inhibitory postsynaptic current (mIPSC) recordings in the VTA had the following composition (in mM): K-gluconate (78), KCl (77), HEPES (10), EGTA (1), Mg^{2+}-ATP (2), Na^{+}-GTP (0.4), pH = 7.4. Pipette solution for mEPSC recordings in the BLA (in mM) was as follows: Cs-methanesulphonate (120), CsCl (17.5), HEPES (10), BAPTA (5), Mg^{2+}-ATP (2), Na^{+}-GTP (0.4), pH = 7.4. Recordings were performed at −60 mV for mIPSCs and −70 mV for mEPSCs. To isolate mIPSCs, recordings were made in the presence of 1 μM TTX and 20 μM DNQX, whereas mEPSCs were isolated using 1 μM TTX and 20 μM bicuculline. Data were sampled at 20 kHz. Only recordings in which the uncompensated series resistance and cell capacitance were stable over time (<20% change) were included in the final analysis.

Behavioral procedures

Motivated behavior. Sixteen male Wistar rats weighing 350–400 g at the start of the experiment were kept on a reversed light schedule (lights on at 07:00 hours). These rats were tested on a progressive ratio schedule for sucrose pellets (45 mg, formula F, Research Diets, New Brunswick, NJ, USA). These procedures are outlined in the Supplementary Information.

Anxiety-like behavior. Sixty-two male Wistar rats, weighing between 330–400 g, were tested once in the elevated plus maze test, as reported previously.[29] The procedures are described in the Supplementary Information.

Body weight and food intake. Seventy-two male Wistar rats, weighing between 250–350 g at the start of the experiment, were tested. To monitor their food intake, rats were individually housed. Before testing, rats had been

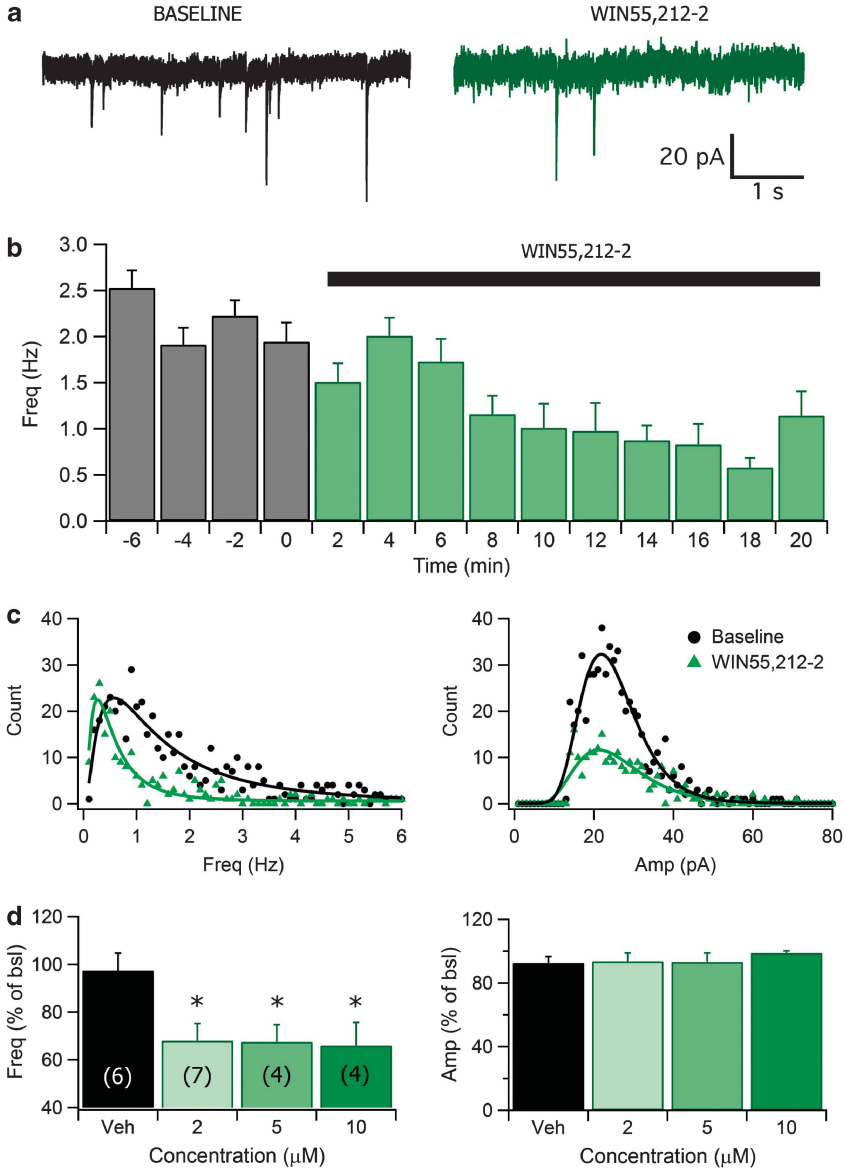

Figure 1. Cannabinoid 1 receptor (CB1R) agonist WIN55,212-2 reduces the GABAergic miniature inhibitory postsynaptic currents (mIPSC) frequency in VTA dopamine neurons. (**a**) Current traces from a representative recording during baseline conditions and in the presence of WIN55,212-2 (2 μM). (**b**) Time line of the effect of WIN55,212-2 (2 μM) from a recording. (**c**) Representative lognormal distributions for baseline (black circles) and WIN55,212-2 (green triangles) with fitted curves for frequency (left) and amplitude (right). (**d**) WIN55,212-2 reduced mIPSC frequency ($F(3,17) = 3.98$, $P < 0.05$), but not the amplitude ($P > 0.10$) compared with vehicle (repeated measures ANOVA). The number of cells is in parentheses. *$P < 0.05$.

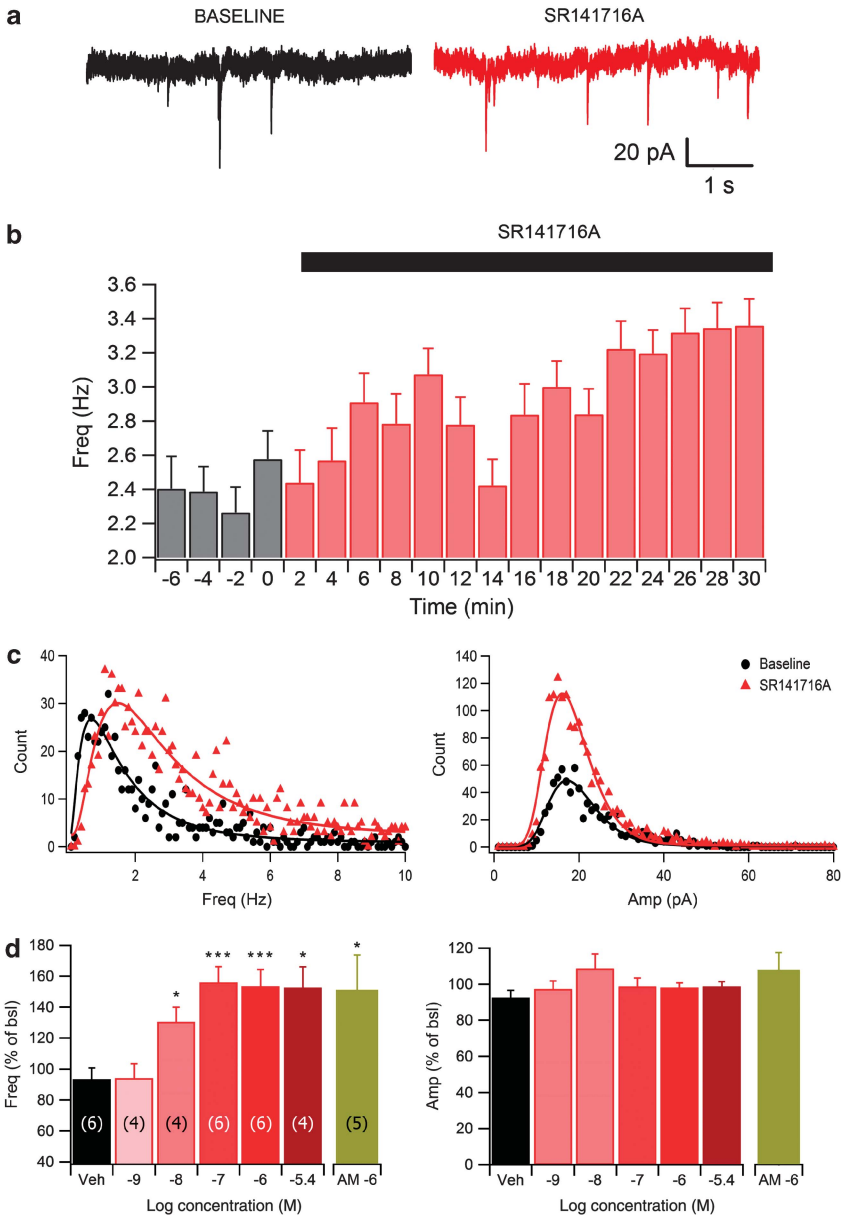

Figure 2. Cannabinoid 1 receptor (CB1R) inverse agonists increase GABAergic miniature inhibitory postsynaptic current (mIPSC) frequency in VTA dopamine neurons. (**a**) Current traces from a representative recording during baseline conditions and in the presence of CB1R inverse agonist SR141716A (1 μM). (**b**) Representative timeline of the effect of SR141716A (1 μM). (**c**) Representative lognormal distributions with fitted curves for baseline (black circles) and SR141716A (red triangles) for mIPSC frequency (left) and amplitude (right). (**d**) Dose–response curve for inverse agonists SR141716A and AM251 (AM) on mIPSC frequency (left) and amplitude (right). The number of experiments is in parentheses. Both SR141716A ($F(5,24) = 9.60$, $P < 0.001$) and AM251 ($F(1,9) = 11.11$, $P < 0.01$; repeated measures ANOVAs) increased the mIPSC frequency compared with baseline. Neither compound affected mIPSC amplitude ($P > 0.1$). $^{*}P < 0.05$; $^{***}P < 0.001$.

habituated to these cages and food intake was established during a 5-day baseline. Then for 8 consecutive days, animals received a cannabinoid antagonist/inverse agonist or vehicle by intraperitoneal injection 30 min before onset of the dark phase. Food intake was recorded by placing the food trays on scales that were continuously digitally monitored using Scales software (Department Biomedical Engineering, UMC Utrecht, The Netherlands).

Analysis. The occurrence and kinetics of synaptic events were analyzed as previously described.[27] Events were scored using MiniAnalysis software (Synaptosoft, Decatur, CA, USA). Event distributions of frequency and amplitude were fitted in Igor Pro (Wavemetrics, Lake Oswego, OR, USA) using a lognormal curve: $f(x) = A^{*}\exp[-0.5^{*}(\ln X - \mu)/\sigma)^2]/(x^{*}\sigma^{*}\sqrt{2\pi})$, as described before.[27,30] In this equation X represents the measured instantaneous frequency or amplitude of an event, A is the relative area under the curve, μ is the mean and σ the standard deviation of the underlying normal distribution. The mean of the lognormal distribution (m) was then calculated using the equation: $m = \exp(\mu + \sigma^2)$. To assess drug effects, two-tailed analysis of variances (ANOVAs) and Bonferroni *post hoc* tests were performed on the means of the normally distributed data. Behavioral data were statistically analyzed using two-tailed repeated measures ANOVA, one-way ANOVAs or two-way ANOVAs, followed by Bonferroni *post hoc* tests. All statistical testing was performed in SPSS 17.0 (Chicago, IL, USA). Bar graphs represent means ± s.e.m. values.

RESULTS

CB1Rs on GABAergic afferents to ventral tegmental area dopamine neurons are constitutively active

Dopamine neurons in the ventral tegmental area (VTA) have a crucial role in appetitive behavior,[31–33] but also in depression and

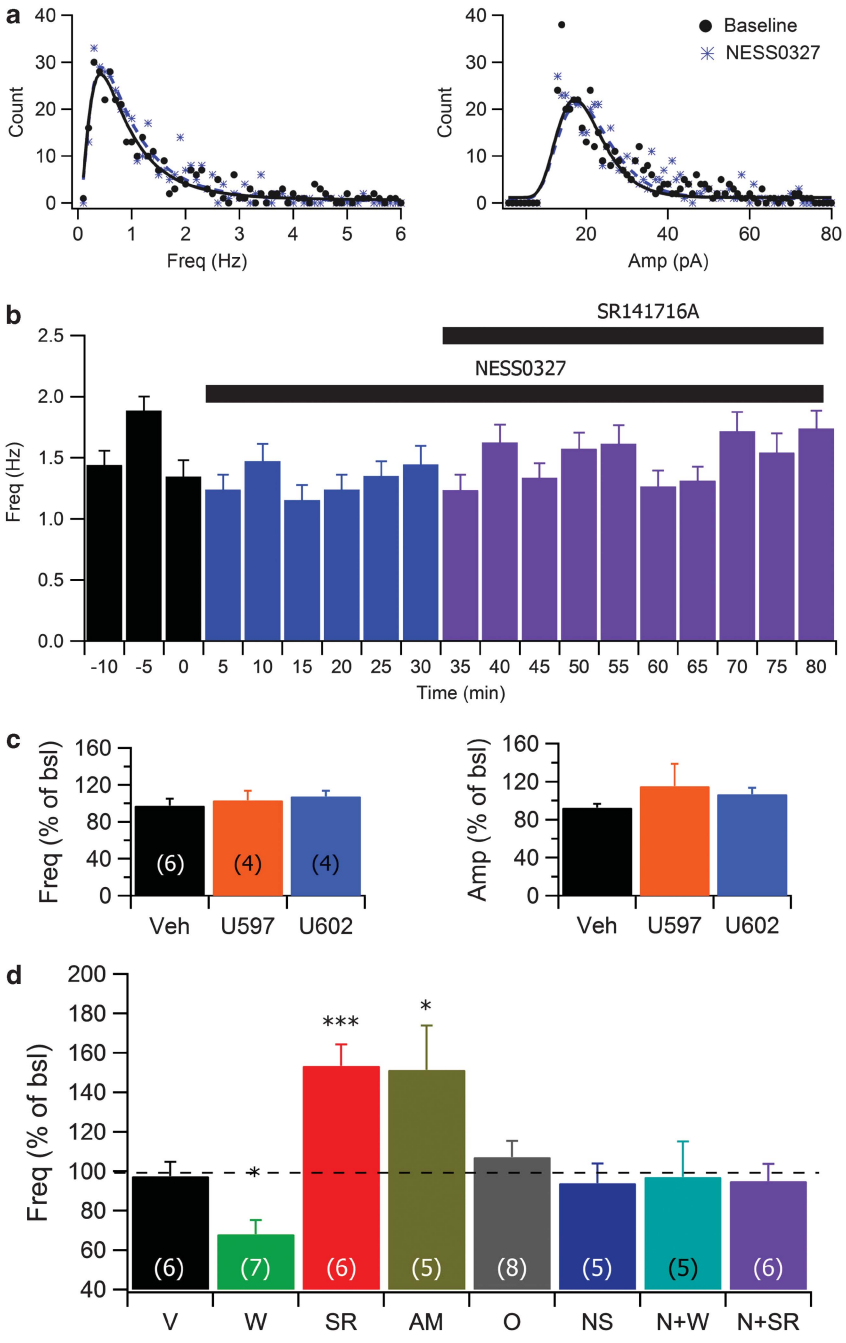

Figure 3. Inverse agonists act by suppressing CB1R constitutive activity on GABAergic projections to VTA dopamine neurons. (**a**) Representative lognormal distributions with fitted curves for baseline (black circles) and CB1R neutral antagonist NESS0327 (0.5 μM; blue stars) for mIPSC frequency (left) and amplitude (right). (**b**) A representative experiment showing that NESS0327 does not affect mIPSC frequency itself, but does block the effect of SR141716A. (**c**) The indirect CB1R agonists URB597 and URB602, which would enhance an anandamide or 2-AG tone if it were present, do not affect mIPSC frequency ($F(2,11) = 1.00$, $P = 0.57$) or amplitude ($P > 0.1$). (**d**) Overall summary of effects of cannabinoid ligands on mIPSC frequency, with the number of experiments in parentheses. Whereas CB1R agonist WIN55,21-2 decreases the frequency, inverse agonists AM251 and SR141716A increase it. Importantly, neutral antagonists O-2050 and NESS0327 do not affect mIPSCs, and NESS0327 blocks the effects of both WIN55,212-2 and SR141716A ($P > 0.1$; repeated measures ANOVA compared with baseline). $^{*}P < 0.05$; $^{***}P < 0.001$.

anxiety.[22–24,34] Cannabinoids disinhibit VTA dopamine neurons by acting on CB1Rs on GABAergic nerve terminals.[35,36] We determined whether these CB1Rs also exhibit constitutive activity in the absence of endocannabinoids. We recorded from VTA dopamine neurons and isolated synaptic GABAergic mIPSCs. In accordance with a presynaptic localization of CB1Rs,[35,36] the CB1/2R agonist WIN55,212-2 reduced mIPSC frequency, but not amplitude (Figure 1).

If some CB1Rs on these GABAergic nerve terminals are constitutively active, suppression of this constitutive activity with an inverse agonist would be expected to produce an effect opposite to that of the agonist. Acute administration of CB1R inverse agonist SR141716A (rimonabant)[15,16] increased mIPSC frequency (Figure 2). This effect was dose-dependent, with an EC_{50} value of 9.25 ± 6.46 nM and a maximal increase in mIPSCs of $55.98 \pm 10.22\%$ at 100 nM (Figure 2d). The effect of SR141716A was

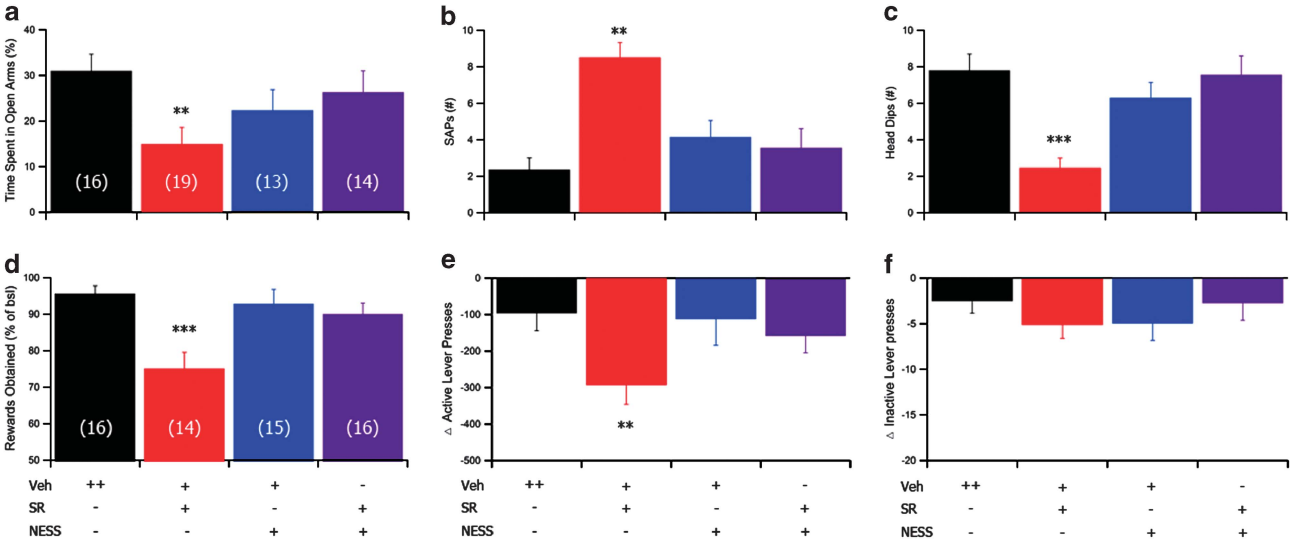

Figure 4. Suppression of cannabinoid 1 receptor (CB1R) constitutive activity causes anxiety and lack of motivation for reward. (**a**) CB1R inverse agonist SR141716A, but not neutral antagonist NESS0327 increases anxiogenic responses in the elevated plus maze on the percentage of time spent in the open arms (%TO; $F(1,58) = 2.21$, $P < 0.05$; two-way ANOVA, Bonferroni *post hoc* testing), (**b**) the number of stretched attend postures (SAPs; $F(1,58) = 15.46$, $P < 0.001$; two-way ANOVA, Bonferroni *post hoc* testing), and (**c**) the number of head dips ($F(1,58) = 16.45$, $P < 0.001$; two-way ANOVA, Bonferroni *post hoc* testing). (**d**) CB1R inverse agonist SR141716A, but not neutral antagonist NESS0327, also lowers the amount of sucrose rewards obtained ($F(3,36) = 5.37$, $P < 0.05$; repeated measures ANOVA, Bonferroni *post hoc* testing) and (**e**) the amount of presses on the active lever $F(3,36) = 2.88$, $P < 0.05$; repeated measures ANOVA, Bonferroni *post hoc* testing), in the progressive ratio task. (**f**) Neither compound had an effect on the amount of presses on the inactive lever, which was not coupled to any reward ($P > 0.1$). $^{**}P < 0.01$; $^{***}P < 0.001$. The number of experiments is in parentheses.

mimicked by AM251 (1 μM), another high-affinity CB1R inverse agonist[37,38] (Figure 2d).

The apparent inverse agonistic effect of SR141716A could also be caused by interference with ongoing endocannabinoid–CB1R signaling. To rule out this possibility, we tested neutral CB1R antagonists, which would also interfere with endocannabinoids acting on CB1Rs, while leaving any constitutive signaling intact. At concentrations sufficient for maximal occupancy of the CB1R,[15,20] the specific neutral antagonists NESS0327 (0.5 μM) and O-2050 (1 μM) did not affect mIPSC frequency (Figures 3a, b and d). Pre-administration of NESS0327 fully blocked the effects of subsequently administered WIN55,212-2 or SR141716A (Figures 3b and d), illustrating the CB1R-dependence of their effects. To corroborate the lack of an endogenous tone in our slice preparations, we also tested the effects of two indirect cannabinoid agonists, URB597 and URB602. These ligands interfere with the degradation of anandamide and 2-AG, respectively, and only exert an effect in the case of ongoing endocannabinoid–CB1R signaling.[39,40] Even at high concentrations and up to 30 min of incubation, neither the FAAH inhibitor URB597 (1 μM) nor the MGL inhibitor URB602 (50 μM) affected mIPSC frequency (Figure 3c).

CB1Rs on glutamatergic afferents to the basolateral amygdala are constitutively active

We determined whether CB1R constitutive activity also occurs at glutamatergic synapses in the basolateral amygdala (BLA). These synapses have an important role in anxiety,[41] and are subject to modulation by cannabinoids in a CB1R-dependent manner.[42] We patch clamped BLA pyramidal neurons and isolated glutamatergic miniature excitatory postsynaptic currents (mEPSCs). The results indicate the occurrence of constitutively active CB1Rs on glutamatergic terminals in the BLA. WIN55,212-2 (2 μM) reduced mEPSC frequency (Supplementary Figures 1a and d), while SR141716A (1 μM) enhanced it (Supplementary Figures 1b and d). NESS0327 (0.5 μM) did not affect mEPSC frequency itself, but prevented the action of WIN55,212-2 and SR141716A (Supplementary Figures 1c and d). None of the ligands affected mEPSC amplitude (Supplementary Figure 1d).

Constitutive CB1R activity regulates anxiety and reward processing

As we established a role of CB1R constitutive activity in neural circuitry involved in processing rewards and emotions, we investigated whether this was indeed associated with behavioral effects on such parameters. We observed that SR141716A (1 mg kg^{-1}) produced strong anxiogenic effects in the elevated plus maze on the amount of time spent in the open arms (Figure 4a), the number of stretched attend postures (SAPs, Figure 4b) and the number of head dips (Figure 4c). The neutral CB1R antagonist NESS0327 (0.1 mg kg^{-1}) was not anxiogenic itself and blocked the effect of SR141716A on all parameters, showing their CB1R-dependence.

In a progressive ratio setup, SR141716A (1 mg kg^{-1}) clearly reduced the motivation for sucrose reward. NESS0327 (0.1 mg kg^{-1}) did not affect such motivation and blocked the effect of SR141716A (Figure 4d). A similar profile of effects was observed for the number of presses on the active lever (Figure 4e). There were no effects of the drugs on presses on the inactive lever (Figure 4f), indicating that the cannabinoids had no general disruptive effect on behavior.

A CB1R neutral antagonist and inverse agonist reduce weight gain equally

The role of the endocannabinoid system in energy balance may particularly involve endocannabinoid-mediated activation of CB1Rs.[8,17,18,43] We therefore investigated next how the CB1R neutral antagonist NESS0327 and the inverse agonist SR141716A compared in their ability to reduce body weight and food intake. In a separate experiment, we did not observe acute effects of the CB1R antagonists on locomotor activity (Supplementary Figure 2).

Rats pretreated with either NESS0327 or SR141716A dose-dependently gained less weight than vehicle controls (Figure 5a). After 8 days of treatment, the dose-dependent reduction in body weight gain was very evident (Figure 5b). The maximal effect of SR141716A (at 1 mg kg^{-1}) and NESS0327 (at 0.1 mg kg^{-1}) was associated with a similar reduction in average food intake during the treatment period (Figure 5c). The CB1R neutral antagonist NESS0327 and the inverse agonist SR141716A equally reduced body weight and food intake (Figures 5b and c). Notably then, the neutral antagonist NESS0327 (0.1 mg kg^{-1}) was maximally effective on food intake and body weight at a dose devoid of effects in the elevated plus maze and the progressive ratio task. Contrarily, the inverse agonist SR141716A (1 mg kg^{-1}), at a dose that exerted similar effects on food intake and body weight as NESS0327, was associated with potentially harmful effects relating to anxiety and hedonic processing.

DISCUSSION

Obesity is a global threat with a diverse etiology that often involves an important neural component.[3] CB1R inverse agonists were originally welcomed as anti-obesity medication, until evidence indicated that they could produce mood disorders and suicidality.[7,11–13,44] We now present evidence that detrimental side effects of CB1R inverse agonists are due to interference with the constitutive activity that CB1Rs exhibit. We show that this constitutive CB1R activity has an important role in regulating GABAergic and glutamatergic neurotransmission in brain regions implicated in anxiety and depression.[22–26,34,45] Most importantly, we demonstrate that a neutral CB1R antagonist, which leaves the constitutive CB1R activity intact, is just as efficient in reducing weight gain as a CB1R inverse agonist, but lacks potentially harmful effects on anxiety and motivation.

Our findings indicate that constitutive CB1R activity does not just occur in artificial systems, but also regulates neurotransmission in native brain tissue. The effect of SR141716A cannot readily be attributed to a mechanism of action other than suppression of constitutive CB1R activity.[16] We determined that the inverse agonistic effect of SR141716A was CB1R mediated, as it was fully blocked by the specific neutral antagonist NESS0327.[20] The dose–response curve of SR141716A on mIPSC frequency in the VTA suggested a low nanomolar affinity, comparable to a previously reported established CB1R-mediated effect of SR141716A.[14] Moreover, the effect of SR141716A on mIPSC frequency was mimicked by AM251, another CB1R inverse agonist.[37,38] We also determined, using both neutral antagonists and indirect agonists, that the inverse agonistic effect of SR141716A in our slice preparation was not because of interference with ongoing endocannabinoid signaling. These findings highlight a role for CB1R constitutive activity in regulation of neurotransmission in the mesocorticolimbic regions under close to physiological conditions.

The behavioral differences between SR141716A and NESS0327 suggest that CB1R constitutive activity has important functions *in vivo* as well. Our findings indicate that while CB1R constitutive activity does not have a large role in food intake and body weight regulation, it is significantly involved in anxiety and motivation for reward. This is particularly relevant in light of the adverse effects of the discontinued weight-loss drug rimonabant (SR141716A), which include anxiety and depression.[11,12] The CB1R neutral antagonist NESS0327 was not anxiogenic in our study, a finding supported by a previous report that found no anxiogenic effect of another CB1R neutral antagonist: AM4113.[19] We now provide evidence that such findings can be attributed specifically to the lack of interference with CB1R constitutive activity. Interestingly, endocannabinoid–CB1R signaling, likely in the BLA, is known to be involved in both anxiety and fear.[39,46] This suggests that, despite endocannabinoid involvement in emotional processing, prominent anxiety mainly occurs when CB1R constitutive activity is suppressed on top of interference with endocannabinoid signaling. Similarly, endocannabinoids may well contribute to motivated behaviors,[47] but our findings suggest that reduced motivation for sucrose reward may not be observed until CB1R constitutive activity is suppressed. It is clear that decreased motivation for (food-related) rewards could be a useful quality of a weight-loss drug, but it is conceivable that it also encompasses a great risk. Lack of motivation and drive (avolition) is an important

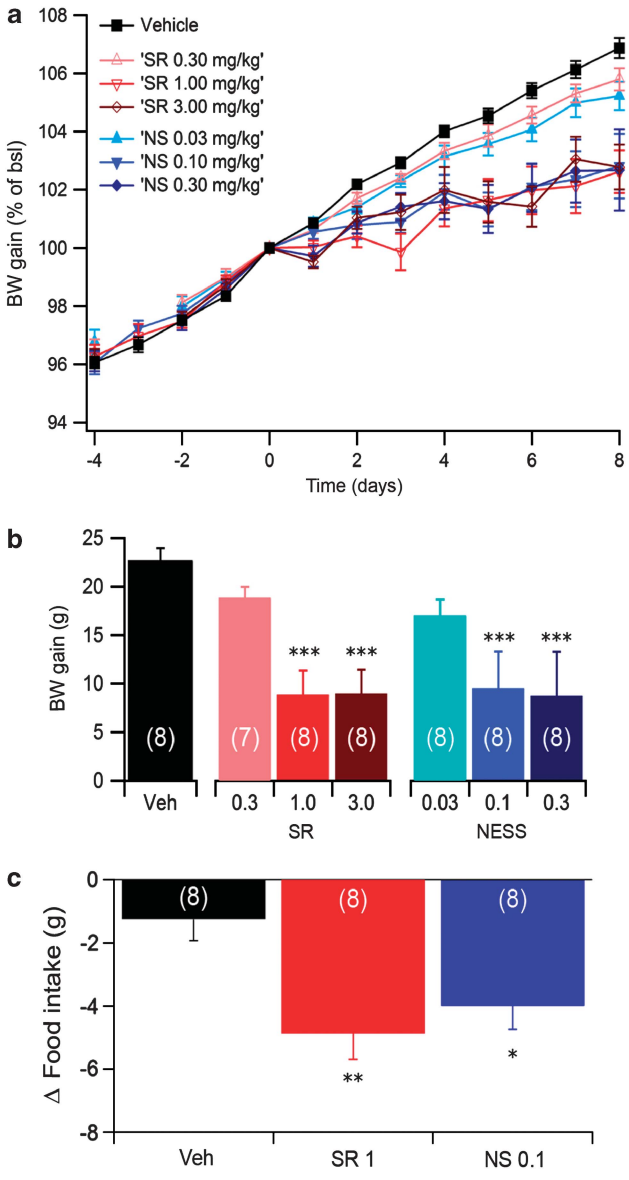

Figure 5. Both types of cannabinoid 1 receptor (CB1R) antagonist reduce body weight gain and suppress food intake. (**a**) The inverse CB1R agonist SR141716A and the neutral CB1R antagonist NESS0327 both dose-dependently reduce weight gain over time ($F(48\,512) = 5.10$, $P < 0.001$; repeated measures ANOVA, treatment × time interaction). (**b**) Dose-dependent changes in weight gain after 8 days of cannabinoid antagonist administration ($F(6,64) = 8.17$, $P < 0.001$; one-way ANOVA followed by Bonferroni *post hoc* testing). NESS0327 (0.1 mg kg^{-1}) and SR141716A (1.0 mg kg^{-1}) produced a comparable ($P > 0.1$) maximal reduction in body weight gain. (**c**) Associated changes in food intake after cannabinoid antagonist administration ($F(2,21) = 6.41$, $P < 0.01$; one-way ANOVA followed by Bonferroni *post hoc* testing. NESS0327 (0.1 mg kg^{-1}) and SR141716A (1.0 mg kg^{-1}) produced the same reduction in food intake ($P > 0.1$). *$P < 0.05$; **$P < 0.01$; ***$P \leqslant 0.001$. The number of experiments is in parentheses.

component of major depressive disorder,[34,48] and there is evidence to suggest that an impaired mesocorticolimbic dopamine system is at the root of this.[23,34] In this light, our findings offer a potential mechanism by which rimonabant could produce such symptoms, and suggest that neutral antagonists like NESS0327 may be less likely to produce them.

Whereas NESS0327 and SR141716A differed on potentially harmful effects, they showed great similarity in their therapeutically relevant effects on body weight and food intake. This corresponds well with previous reports that interference with endocannabinoid signaling at the CB1R, likely within hypothalamic, forebrain and midbrain regions, is sufficient for effects on energy balance.[8,17,18,43,49–51] Therefore, CB1R neutral antagonists, which only interfere with endocannabinoid–CB1R signaling, may prove to be drugs that are efficient in treating obesity, while lacking many of the deleterious side effects associated with CB1R inverse agonists.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)