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Effects of acute low-dose combined treatment with naloxone and AM 251 on food intake, feeding behaviour and weight gain in rats

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ABSTRACT

Low-dose combinations of naloxone and rimonabant produce additive effects on food intake and feeding behaviour, yet abolish the scratching syndrome typically induced by rimonabant per se. To assess the generality of these findings, we have examined the acute effects of low-dose combinations of naloxone (0.1 mg/kg) and the rimonabant derivative AM 251 (0.5 and 1.0 mg/kg) on food intake, feeding behaviour and weight gain in non-deprived male rats. Although ineffective when given alone, combined treatment with naloxone and 0.5 mg/kg AM 251 significantly and selectively suppressed mash intake and time spent feeding. By itself, 1.0 mg/kg AM 251 failed to alter any measure of feeding behaviour but did reduce food consumption and induce scratching behaviour. Co-administration of naloxone with 1.0 mg/kg AM 251 not only significantly suppressed both food intake and feeding behaviour but also simultaneously attenuated AM 251-induced scratching. This profile mirrors earlier findings with naloxone/rimonabant and is consistent with the reported diversity of opioid–cannabinoid system interactions at a more molecular level. Although further studies are required (e.g. 'neutral' CB1 receptor antagonists), current data constitute further proof of concept regarding the anorectic efficacy, selectivity and added value of low-dose polytherapy with opioid and CB1 receptor antagonists.

1. Introduction

Given the current lack of truly effective pharmacological monotherapies for obesity (Clapham et al., 2001; Collins and Williams, 2001; Halford et al., 2003; Vickers and Cheetham, 2007; Wilding, 2007; Adan et al., 2008) the development of novel, safe and effective polytherapies for this disorder has become an increasingly attractive prospect (e.g. Neary et al., 2005; Talsania et al., 2005; Serrano et al., 2008; Tallett et al., 2008b; Vemuri et al., 2008). In this context, many G-protein-coupled receptors (GPCRs) are now known to function as dimers or oligomers i.e. physical associations between identical (homomers) or different (heterodimers) proteins (Rios et al., 2001; Milligan, 2004; Gurevich and Gurevich, 2008). For example, heterodimer formation between closely related GPCR monomers is necessary for the expression and function of $GABA_B$ receptors (i.e. dimerization of $GABA_{B(1)}$–$GABA_{B(2)}$ isoforms), while numerous examples of heterodimers between less closely related GPCR monomers have also been documented, e.g. D_2–D_3 dopamine receptors, μ- and κ-opioid receptors, and opioid and β_2-adrenergic receptors (George et al., 2002). Recent research has also pointed to the likely existence of μ-opioid/CB1-cannabinoid receptor heterodimers (Rios et al., 2001; Christie, 2006; Rios et al., 2006) and an associated range of functional interrelationships (i.e. non-additive, additive, synergistic, antagonistic interactions). These findings are particularly relevant to an emerging literature on potentially important system crosstalk between opioids and cannabinoids in the regulation of appetite and weight gain.

The remarkable parallels in the effects of μ-opioid and CB1-cannabinoid receptor manipulations on food intake have been recognised for many years (Cooper et al., 1988; Kirkham and Williams, 2001a; Yeomans and Gray, 2002; Cota et al., 2003; Bodnar, 2004; DiMarzo and Matias, 2005; Kirkham, 2005; Tucci et al., 2006). However, evidence for potentially important system interactions in the regulation of appetite is of much more recent origin. In part, this evidence derives from work showing that cannabinoid-induced increases in intake/food-reinforced responding in rodents can be blocked by the broad-spectrum opioid receptor antagonist naloxone and, reciprocally, that opioid-induced increases in intake/food-reinforced responding can be blocked by the CB1 receptor antagonist/inverse agonist rimonabant (Trojniar and Wise, 1991; Gallate et al., 1999; Williams and Kirkham, 2000, 2002; Verty et al., 2003; Solinas and Goldberg, 2005). Despite these pioneering findings, however, interpretative issues have arisen (e.g. Tallett et al., 2008b) regarding the intrinsic anorectic efficacy of the naloxone and rimonabant doses

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used in many of these studies. A complementary research strategy in this field has involved the low-dose co-administration of CB1 and opioid receptor antagonists. This work has suggested that the combination of rimonabant and naloxone produces a much greater decrease in food intake in rats than would be predicted by simple addition of the intrinsic effects of the two compounds (Kirkham and Williams, 2001b; Rowland et al., 2001). Comparable supra-additive or synergistic anorectic interactions have been reported in mice using the opioid antagonist nalmefene and the CB1 receptor antagonist/inverse agonist AM 251 (Chen et al., 2004). It should perhaps be noted, however, that neither the acute anorectic nor the chronic weight loss response to AM 251 is altered in mice completely lacking the μ-opioid receptor (Chen et al., 2006).

In view of the relative paucity of research on the anorectic potential of low-dose combinations of opioid and CB1-receptor antagonists, including the absence of detailed behavioural studies, we have recently profiled the effects of sub-anorectic doses of rimonabant and naloxone (alone and in combination) in non-deprived male rats (Tallett et al., 2008b). Our findings showed that only the *combination* of the two antagonists produced a significant suppression of food intake/feeding behaviour and an acceleration in the behavioural satiety sequence (BSS). However, in contrast to earlier research (Kirkham and Williams, 2001b; Rowland et al., 2001; Chen et al., 2004), the pattern of results suggested an additive rather than supra-additive interaction in the effects of the two compounds on ingestive behaviour. Intriguingly, our data also revealed that co-treatment with naloxone statistically abolished the compulsive scratching syndrome typically induced in rodents by higher doses of rimonabant (see also Tallett et al., 2007a). This serendipitous finding suggests that co-treatment with low doses of the two antagonists not only suppresses appetite but also concurrently attenuates an unwanted (side-) effect of CB1 receptor antagonists/inverse agonists that has recently been confirmed in humans (Addy et al., 2008; Kirkham, 2008).

To date, AM 251 is the only other CB1 receptor antagonist/inverse agonist with well-documented anorectic activity (Hildebrandt et al., 2003; McLaughlin et al., 2003; Shearman et al., 2003; Slais et al., 2003; Chambers et al., 2004; Zhou and Shearman, 2004; McLaughlin et al., 2005; Chambers et al., 2006; Tallett et al., 2007b), to have been used in interaction studies with any opioid receptor antagonist (Chen et al., 2004). Given the existence of potentially important pharmacokinetic, pharmacodynamic and behavioural differences relative to the parent molecule rimonabant (Gatley et al., 1996, 1997, 1998; Lan et al., 1999; Tallett et al., 2007b), our present aim was to assess the acute effects of AM 251, alone and in combination with naloxone, on food intake, behaviour and weight gain in male rats. We were specifically interested in the nature of any interactions on food intake/feeding behaviour (i.e. supra-additive or additive) and compulsive scratching (i.e. antagonism). Clearly, a similar pattern of results to that obtained with low-dose combinations of rimonabant and naloxone (Tallett et al., 2008b) would demonstrate not only the robustness but also the generality of the quite diverse interactions between opioid and cannabinoid systems in the regulation of behavioural processes.

2. Materials and methods

2.1. Subjects

Subjects were 10 adult male Lister hooded rats obtained from Charles River, U.K. On arrival in our laboratory (209.8±2.1 g), they were housed 5/cage (46×26.5×26 cm) for 1 week, following which they were transferred to individual cages (45×20×20 cm) for the remainder of the study. Single housing facilitated both initial familiarisation with the test diet and daily bodyweight tracking. Rats were maintained on a 12-h reversed light cycle (lights off: 0700 h) in a temperature (21±1 °C)- and humidity (50±2%)-controlled environment. The reversed light cycle permitted behavioural testing during the active (dark) phase of the light–dark cycle. Animals were handled regularly during routine husbandry and were thoroughly habituated to all experimental procedures prior to drug testing. Pelleted chow (Bantin & Kingman Universal Diet, UK; digestible energy value=14 kJ/g) and tap water were freely available in the home cages, with the exception of the injection-test interval during which home cage food was removed. Bodyweights were recorded at the same time daily (0900 h) throughout the experiment. This experiment was conducted under Home Office license in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.2. Drugs

AM 251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; Tocris Bioscience UK) was initially dissolved in a small volume of dimethyl sulfoxide (DMSO; Sigma-Aldrich, Poole, UK), and subsequently made up to required concentrations in 0.5% methylcellulose (Sigma-Aldrich, UK). A corresponding methylcellulose/DMSO mixture was employed for vehicle control injections, and all solutions had a final DMSO concentration of ≤1%. Naloxone hydrochloride (Sigma-Aldrich) was dissolved in a vehicle of physiological (0.9%) saline which, alone, served for control injections. The doses of AM 251 (0.5 and 1.0 mg/kg) and naloxone (0.1 mg/kg) were chosen on the basis of dose–response data obtained under local test conditions (Tallett et al., 2007b, 2008a,b). All solutions were freshly prepared on test days and administered intraperitoneally (IP) in a volume of 1 ml/kg. The injection-test interval for AM 251 (or vehicle) was 30 min and, for naloxone (or vehicle), 15 min.

2.3. Apparatus

Feeding tests were conducted in a large glass observation arena (60×30×45 cm), the floor of which was covered with wood shavings (Rodgers et al., 2001; Ishii et al., 2004, 2005; Tallett et al., 2007a,b, 2008a,b). A water bottle was suspended from one of the end-walls and a glass food pot, weighed immediately prior testing, was positioned in the centre of the arena and secured to the floor by an annular metal mounting. The test diet (mash) was prepared freshly each morning by adding water to a powdered form of the maintenance diet (Bantin & Kingman Universal Diet, UK; 1 g dry=3.125 g mash; digestible energy value=4.48 kJ/g). Portions of mash were then disbursed to individual pots, covered and refrigerated until required. Mash has the advantage of high palatability while its consistency minimises spillage and hoarding (e.g. Halford et al., 1998; Rodgers et al., 2001; Ishii et al., 2003). Two videocameras, one positioned vertically above the arena and the other horizontal to the front wall, were used to record the test sessions for subsequent detailed behavioural analysis. A dual-angle view of the test arena facilitates scoring accuracy by avoiding ambiguities that can arise from a single perspective. Camera signals were fed via an image merger to a nearby monitor and digital videodisk (DVD) recorder.

2.4. Procedure

All procedures (habituation and experimental) were conducted under dim red light (2 lux) during the dark phase of the LD cycle (0800–1600 h). Control food pots (2/day) were positioned adjacent to the test arena to assess loss of food mass through evaporation alone (average 1-h loss=0.15%; range 0.06–0.26%).

2.4.1. Habituation

After 2 weeks adaptation to local laboratory conditions, all subjects were familiarised (3-h/day on 2 consecutive days) with mash in their home cages. The following week, each animal was exposed daily for 5 days to a pseudo-experimental procedure comprising: removal of

home cage food, IP injection of AM 251 vehicle (and return to home cage for 15 min), IP injection of naloxone vehicle (and return to home cage for 15 min) and, finally, 1-h exposure to the test arena with pre-weighed mash and ad libitum tap water. Mash consumption (controlled for spillage) was accurately measured on each habituation trial. This pre-experimental phase familiarised animals with all aspects of the experimental protocol (diet, test environment, handling, injections) while simultaneously providing opportunity for the development of stable basal food intake prior to pharmacological manipulation.

2.4.2. Test phase

The test phase was conducted according to a within-subjects (crossover) design and began within 72 h of the final habituation trial. Treatment order was determined by Latin Square with a washout period of 7 days between successive treatments. We have previously shown that any persistent effect of AM 251 on weight gain dissipates within 72 h of acute treatment (Tallett et al., 2007b) whereas acute treatment with naloxone is without effect on post-treatment weight gain (Tallett et al., 2008a). On test days, animals were taken individually to a preparation room where they were sequentially treated (IP) either with vehicle (V) or one of two doses of AM 251 (0.5 mg/kg (AL) or 1.0 mg/kg (AH)) followed, 15 min later, by either vehicle (V) or naloxone (NX; 0.1 mg/kg). As such, there was a total of 6 treatment conditions: V–V, V–NX, AL–V, AL–NX, AH–V, and AH–NX. Consistent with the habituation procedures described above, animals were returned to their home cages (chow removed) for 15 min following each injection. Thirty minutes after the first injection, they were transferred to an adjacent laboratory and individually placed in the test arena with pre-weighed mash and ad lib tap water. Animals were then left undisturbed for the 1-hour DVD-recorded test session, following which any spillage was carefully retrieved and food pots accurately reweighed.

2.5. Behavioural analysis

DVDs were scored blind by a highly trained observer (intra-rater reliability ≥0.9), using an ethological software package ('Hindsight'; Weiss, 1995) that permits real-time scoring of behaviour by direct keyboard entry to a PC. A continuous observation method was employed in view of its advantages over time-sampling techniques (Halford et al., 1998). Based on previous research (Halford et al., 1998; Rodgers et al., 2001; Ishii et al., 2004, 2005; Tallett et al., 2007a,b, 2008a,b), measures recorded from DVD comprised: *latency to locate food source* (time in sec between the start of testing and first contact with the food pot), and *latency to feed* (time in sec between first contact with the food source and the first feeding episode), together with the frequency and duration of the following mutually exclusive behavioural categories: *feeding* (biting, gnawing, or swallowing food from food pot or from front paws); *drinking* (licking the spout of the water bottle); *grooming* (licking of the body, feet and genitals; stroking of face and whiskers with forepaws, biting the tail); *scratching* (repetitive ipsilateral hind paw scratching of flanks, neck and head); *sniffing* (rapid wrinkling of the nose/twitching of vibrissae at an aspect of the environment, head movements with rear limbs immobile); *locomotion* (walking around the cage or circling; movements involving all four limbs); *rearing* (forepaws raised from the cage floor, either supported against a wall or free standing); and *resting* (sitting or lying in a relaxed position with head curled to body or resting on the floor; animal inactive). We also measured the frequency and duration of a behavioural feature previously recorded as 'freezing' (e.g. Tallett et al., 2008b) but which does not accurately meet formal definitions of the classical defense response (e.g. Blanchard et al., 1993). The feature in question is best described as the cessation of and subsequent return to ongoing behaviour, an element that can be operationally distinguished from freezing, immobility and the orienting response and which we have parsimoniously called *stop*. Two further measures of feeding behaviour were derived from the recorded parameters: *average duration of feeding bouts* (total feeding duration in sec divided by total feeding frequency), and *average feeding rate* (total food intake in g divided by total feeding duration in min).

In addition to analysing treatment effects on global behavioural scores, each 60-min test period was divided into 12×5-minute time-bins thereby permitting analysis of treatment effects over time. Although testing in virtual darkness during the active (dark) phase of the LD cycle curtailed the display of postprandial resting, attention was nevertheless paid to the behavioural satiety sequence (BSS), i.e. the temporal relationship between feeding, grooming, and resting (Halford et al., 1998; Rodgers et al., 2001; Ishii et al., 2003, 2004, 2005; Tallett et al., 2007a,b, 2008a,b).

2.6. Test-day bodyweights and post-treatment bodyweight gain

Daily tracking of bodyweights (from day 1 of individual housing until 7 days post-dosing) was used both to confirm the equivalence of test-day bodyweights across treatment conditions and to detect any enduring effects of acute drug treatment on weight gain. In addition to analysing treatment effects on absolute weight gain over the 1 week period post-dosing, bodyweights for each post-treatment day were expressed as a percentage of test-day bodyweight (where test day = 100%), and analysed by drug condition.

2.7. Statistical analysis

Habituation intake data were analysed by one-way repeated measures analysis of variance (ANOVA) followed, where significant, by Bonferroni post-hoc tests. Treatment effects on food intake, 1 h behavioural totals and 7-day absolute weight gain were analysed by two-way (3×2) repeated measures ANOVA (factor 1 = AM 251; factor 2 = NX) followed, as appropriate, by Bonferroni tests. Treatment effects on behavioural change over time, as well as on percent bodyweight gain daily over the 7 days post-dosing, were analysed by three-way repeated measures ANOVA (factor 1 = AM 251; factor 2 = NX; factor 3 = timebin or day). Significant interactions were initially explored using two-way ANOVA for each time period/day followed, when significant, by Bonferroni tests. Where datasets failed Mauchly's Test of Sphericity, Greenhouse–Geisser significance levels are reported and, in all cases, findings were accepted as statistically-significant when $P \leq 0.05$.

3. Results

3.1. Habituation

Mean bodyweight for the sample ($N = 10$) was 209.8 ± 2.1 g on arrival and 543.2 ± 7.5 g by the end of the study; all animals remained healthy throughout the experiment. ANOVA showed that mash intake differed significantly over the course of habituation (trial 1: 14.08 ± 1.42 g; trial 2: 20.44 ± 1.84 g; trial 3: 19.26 ± 1.48 g; trial 4: 19.60 ± 1.33 g; trial 5: 20.07 ± 1.26 g; [$F_{(4,36)} = 12.31$, $P < 0.001$]). As might be expected, consumption on the first trial was significantly lower than on trials 2–5 ($P \leq 0.02$). The development of a stable intake pattern was shown by the lack of difference in intake scores over habituation trials 2–5, and further confirmed by the close similarity between these scores and those for the V–V control condition (21.47 ± 1.83 g) in the main experiment (see below).

3.2. Effects of AM 251 and naloxone, alone and in combination

3.2.1. Food intake

Test-day bodyweights did not differ significantly across treatment conditions [$F_{(5,45)} = 0.22$, $P > 0.05$]. A 3×2 repeated measures ANOVA

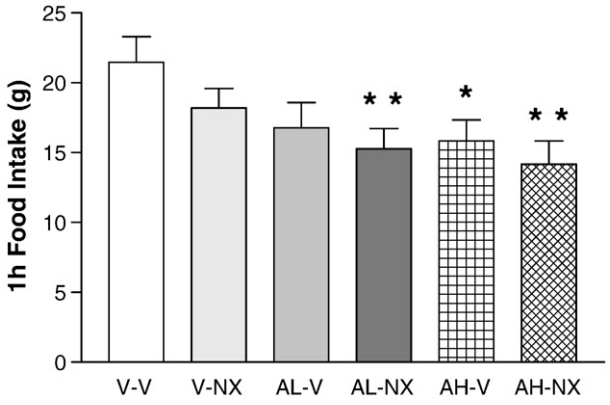

Fig. 1. Effects of naloxone and AM 251, alone and in combination, on food intake in non-deprived male rats exposed for 1-h to palatable mash. Data are mean values ± SEM. V = vehicle; NX = naloxone 0.1 mg/kg; AL = AM 251, 0.5 mg/kg; AH = AM 251, 1.0 mg/kg. *$P<0.05$, **$P<0.01$ vs V–V.

revealed a significant main effect on food intake for AM 251 [$F_{(2,18)}=16.74$, $P<0.001$] and a near-significant main effect of NX [$F_{(1,9)}=4.82$, $P=0.056$], but no significant interaction [$F_{(2,18)}=0.42$, $P>0.05$]. However, as previously noted (Tallett et al., 2008b), a significant interaction would not be expected where several treatments produce effects of a similar magnitude and in the same direction. Indeed, Fig. 1 shows that intake in all drug conditions was lower than that in the V–V control condition. Importantly, however, it was *significantly lower* only when animals received treatment with AH–V, AL–NX or AH–NX ($P\leq0.05$). Comparison of the absolute reductions for each treatment condition is—at best—indicative of an additive rather than supra-additive interaction between AM 251 and naloxone. This inference is supported by (i) the mean percentage changes from V–V control (V–NX = ↓15%; AL–V = ↓22%; AL–NX = ↓29%; AH–V = ↓26%; AH–NX = ↓34%), and (ii) the absence of statistically-significant differences between the AL–NX or AH–NX combinations and the constituent elements when given alone (i.e. V–NX, AL–V, or AH–V).

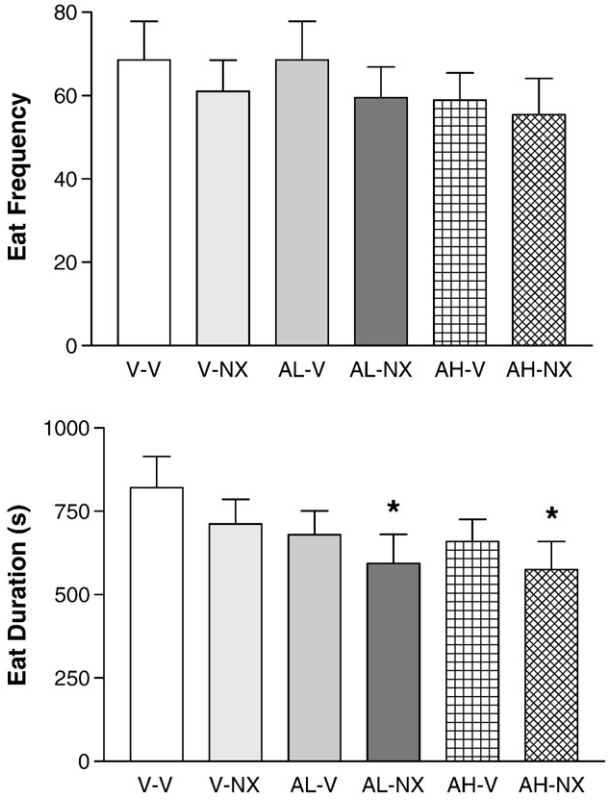

Fig. 2. Effects of naloxone and AM 251, alone and in combination, on the frequency (upper panel) and duration (lower panel) of feeding behaviour in non-deprived male rats during 1-h tests with palatable mash. Data are mean values + SEM. V = vehicle; NX = naloxone 0.1 mg/kg; AL = AM 251, 0.5 mg/kg; AH = AM 251, 1.0 mg/kg. See Table 1 for complementary data. *$P\leq0.05$, vs V–V.

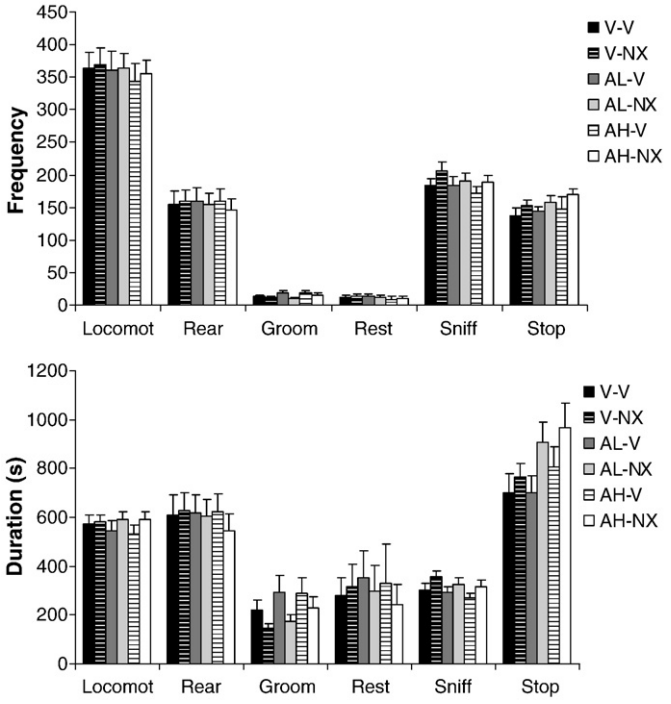

Fig. 3. Effects of naloxone and AM 251, alone and in combination, on the frequency (upper panel) and duration (lower panel) of non-ingestive behaviours in non-deprived male rats during 1-h tests with palatable mash. Data are mean values ± SEM. V = vehicle; NX = naloxone 0.1 mg/kg; AL = AM 251, 0.5 mg/kg; AH = AM 251, 1.0 mg/kg. See Fig. 4 for complementary data.

3.2.2. Total behavioural scores

Treatment effects on the total frequency and total duration of ingestive and non-ingestive behaviours are summarized in Figs. 2–4. Data for additional feeding-related parameters (latency to locate food source, latency to feed, average duration of feeding bouts, and average rate of feeding) are shown in Table 1.

There were no significant AM 251 × NX interactions [$F_{(2,18)}\leq2.38$, $P>0.05$]. Although most behavioural categories and all feeding-related parameters (Table 1) remained unaffected by AM 251 [$F_{(2,18)}\leq2.92$, $P>0.05$] and NX [$F_{(1,9)}\leq4.45$, $P>0.05$], several significant main effects were apparent. For AM 251, these comprised the duration of feeding [$F_{(2,18)}=6.92$, $P<0.01$] as well as frequency and duration of scratching [$F_{(2,18)}\geq5.28$, $P\leq0.02$]. Significant main effects of NX were found for the duration of feeding [$F_{(1,9)}=6.50$, $P<0.05$], the frequency and duration of sniffing [$F_{(1,9)}\geq11.28$, $P\leq0.01$], the frequency and duration of stop [$F_{(1,9)}\geq5.16$, $P\leq0.05$] and, marginally, the duration of scratching [$F_{(1,9)}=5.08$, $P=0.051$].

Post-hoc tests showed that, while all drug treatments tended to reduce time spent feeding relative to the V–V control condition, this effect reached statistical significance *only* for the AL–NX and AH–NX combinations ($P\leq0.05$). Consistent with the food intake pattern above, the mean percentage changes from V–V control were consistent with an additive rather than supra-additive effect of the two drugs (V–NX = ↓13%; AL–V = ↓17%; AL–NX = ↓28%; AH–V = ↓20%; AH–NX = ↓30%). This interpretation was confirmed by the absence of significant differences for feeding duration between the AL–NX or AH–NX combinations and each drug given alone (AL–V, AH–V, V–NX).

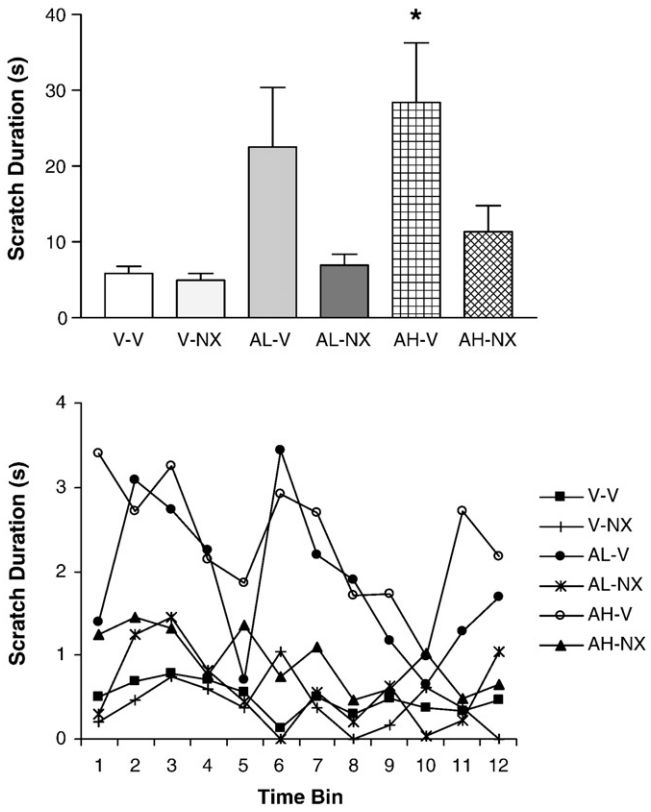

Fig. 4. Effects of naloxone and AM 251, alone and in combination, on the total duration (upper panel) and timecourse (lower panel) of scratching in non-deprived male rats during 1-h tests with palatable mash. Data are mean values ± SEM. V = vehicle; NX = naloxone 0.1 mg/kg; AL = AM 251, 0.5 mg/kg; AH = AM 251, 1.0 mg/kg. See Figs. 2, 3 and 5 for complementary data. *$P \leq 0.05$ vs V–V.

Although AM 251 appeared to dose-dependently increase both the frequency and duration of scratching, Bonferroni comparisons (vs V–V control) revealed significance only for the AH–V condition which increased the duration ($P<0.05$) but not the frequency of scratching. Co-treatment with NX seemed to block this response since scratching was not significantly elevated in AH–NX condition (Fig. 4, upper panel). However, despite the significant difference ($P<0.03$) between the AH–V and V–NX conditions, partial rather than complete antagonism was indicated by the absence of a significant difference between the AH–V and AH–NX conditions.

Follow up analysis of the main effects of NX on measures of sniffing and stop failed to reveal significant pairwise comparisons between the V–NX and V–V conditions, thereby confirming relatively weak effects of the opioid receptor antagonist, i.e. effects that emerge only as a function of substantially larger sample sizes (30 vs 10). Although the duration of stop was significantly enhanced by the combination of AH and NX ($P<0.05$ vs V–V; Fig. 3), the absence of significance relative to V–NX or AH–V would (as for food intake and feeding duration; see above) seem to indicate an additive effect of individually non-significant effects.

3.2.3. Behavioural timecourses and behavioural satiety sequence (BSS)

ANOVA failed to reveal any significant 3-way (time × AM 251 × NX; [$F_{(22,198)} \leq 1.41$, $P>0.05$]) or any additional 2-way (AM 251 × time: [$F_{(22,198)} \leq 1.42$, $P>0.05$]; NX × time: $F_{(11,99)} \leq 2.43$, $P>0.05$]) interactions. However, significant main effects of time were obtained for the majority of behavioural measures [$F_{(11,99)} \geq 4.55$, $P \leq 0.05$], except the duration of rearing, the frequency and duration of grooming, and the duration of scratching [$F_{(11,99)} \leq 2.11$, $P>0.05$]. These temporal patterns reflect well-documented reductions in active behaviour and increases in inactive behaviour over the course of the test session (e.g. Rodgers et al., 2001; Ishii et al., 2004; Tallett et al., 2007a,b, 2008a,b). Fig. 4 (lower panel) shows that the atypical scratching behaviour induced by AM 251 could be seen throughout the entire test session; this graph also confirms the relative absence of such behaviour in animals receiving combined treatment with AM 251 and NX.

Treatment effects on the behavioural satiety sequence (BSS) are summarized in Fig. 5. Behavioural structure was fully preserved under all treatment combinations, with feeding the predominant response during the first half of the test session. Although resting increased as the session progressed, it did not reach the high postprandial levels observed in earlier studies (e.g. Rodgers et al., 2001; Ishii et al., 2004). Recent unpublished observations have confirmed that this discrepancy in basal resting profile is entirely due to differences in maintenance light cycle (normal vs reversed), time of testing (light phase vs dark phase) and illumination at testing (bright vs dim). For these reasons, the feed-to-rest transition for most treatment conditions in the present study generally occurs towards the end of the test session. Nevertheless, the sharper decline in feeding observed over periods 3–6 in the AH–V and AH–NX conditions (relative to other treatments) is suggestive of a modest acceleration in behavioural satiety (lower left and right panels, Fig. 5).

3.2.3.1. Post-treatment bodyweight gain. A 2-way (AM 251 × NX) repeated measures ANOVA on 7-day absolute weight gain showed that there were no significant main effects of AM 251 [$F_{(2,18)}=0.08$, $P>0.05$] or NX [$F_{(1,9)}=0.68$, $P>0.05$], nor a significant drug interaction [$F_{(2,18)}=0.30$, $P>0.05$]. However, it is interesting to note that the least weight gain relative to V–V control (27.1 ± 2.0 g) was in the AH–NX condition (24.3 ± 1.9 g). A 3-way (AM 251 × NX × day) repeated measures ANOVA was used to analyse daily weight gain in the form of percentage change from test day (=100%). There was no significant 3-way interaction [$F_{(12,108)}=0.44$, $P>0.05$] nor were there any significant 2-way interactions [AM 251 × NX: $F_{(2,18)}=0.25$; AM 251 × day: $F_{(12,108)}=0.11$; NX × day: $F_{(6,54)}=0.70$; all $P>0.05$]. However, confirming normal growth patterns, there was a highly significant main effect for day [$F_{(6,54)}=402.80$, $P<0.01$].

4. Discussion

Recent work in our laboratory has indicated that while the acute anorectic response to CB1 receptor antagonists/inverse agonists (such

Table 1
Effects of AM 251 and naloxone, alone and in combination, on latency to locate food source, latency to commence feeding, average duration of feeding bouts and rate of feeding in non-deprived male rats presented with palatable mash

Measure	V–V	V–NX	AL–V	AL–NX	AH–V	AH–NX
Latency to locate food source (s)	6.56 ± 1.44	7.71 ± 2.93	5.77 ± 1.23	5.60 ± 1.48	6.71 ± 2.06	6.34 ± 1.15
Latency to commence feeding (s)	20.15 ± 5.77	29.05 ± 4.96	37.36 ± 11.60	30.20 ± 6.06	34.42 ± 5.97	21.32 ± 5.48
Average duration of feeding bouts (s)	13.62 ± 2.48	12.97 ± 2.15	13.79 ± 3.53	11.30 ± 2.48	12.69 ± 2.12	11.77 ± 2.00
Feeding rate (g/min)	1.70 ± 0.16	1.61 ± 0.11	1.50 ± 0.06	1.68 ± 0.14	1.46 ± 0.09	1.57 ± 0.13

Data are given as mean values ± SEM. s = seconds. V = vehicle; NX = naloxone, 0.1 mg/kg; AL = AM 251, 0.5 mg/kg; AH = AM 251, 1.0 mg/kg. No treatment significantly altered these parameters. See text for details and Fig. 2 for complementary data.

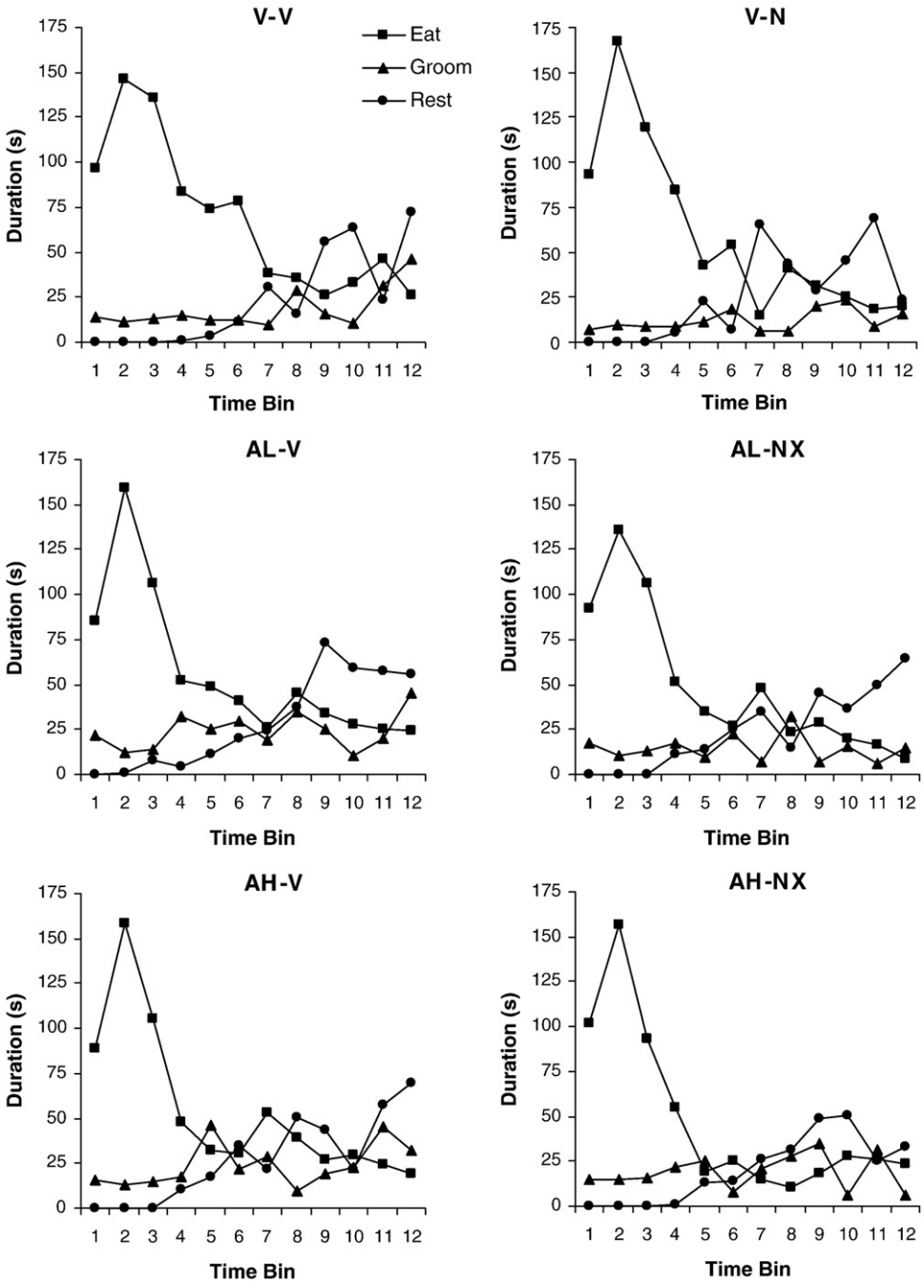

Fig. 5. Effects of naloxone and AM 251, alone and in combination, on the behavioural satiety sequence (BSS) in non-deprived male rats exposed for 1-h to palatable mash. Data are presented as mean duration scores in seconds. V = vehicle; NX = naloxone 0.1 mg/kg; AL = AM 251, 0.5 mg/kg; AH = AM 251, 1.0 mg/kg. The combination of AH–NX accelerated the BSS i.e. produced a shift to the left in the temporal sequence of behaviour.

as rimonabant and AM 251) may be largely due to response competition from compulsive scratching/grooming (Tallett et al., 2007a,b), the appetite suppressant effects of opioid receptor antagonists (such as naloxone) are behaviourally-selective (Tallett et al., 2008a). In view of the potential therapeutic advantages of drug polytherapy (e.g. Adan et al., 2008; Vemuri et al., 2008), we have recently examined the effects on appetite and weight gain of combined treatment with sub-anorectic doses of rimonabant and naloxone (Tallett et al., 2008b]. Our results demonstrated not only an additive effect of the two compounds on food intake and feeding behaviour but also clear naloxone antagonism of the scratching syndrome induced by the CB1 receptor antagonist/inverse agonist. As pruritus (itching and scratching) is now recognised as an unwanted effect of CB1 receptor antagonists/inverse agonists in humans (e.g. taranabant; Addy et al., 2008; Kirkham, 2008), we considered it crucial to assess the generality of our interaction findings using another CB1 compound of the same series, AM 251.

Differing from rimonabant only in respect of a single halogen substitution, AM 251 retains high affinity for brain CB1 receptors but displays substantially greater selectivity for CB1 relative to CB2 receptors (Gatley et al., 1996, 1997, 1998; Lan et al., 1999). Like the parent molecule, this compound (typically in dose range of 1–10 mg/kg) has consistently been found to reduce food intake and weight gain in rats and mice (Hildebrandt et al., 2003; McLaughlin et al., 2003; Shearman et al., 2003; Slais et al., 2003; Chambers et al., 2004; Zhou and Shearman, 2004; McLaughlin et al., 2005; Chambers et al., 2006). Although this anorectic activity is reported to occur in the absence of

deficits in general activity, exploration or food-handling ability, comparable doses (1–8 mg/kg) of the compound have been found to increase anxiety-like behaviour (e.g. Haller et al., 2004; Rodgers et al., 2005), elicit gaping (rejection) reactions in the taste reactivity test (McLaughlin et al., 2005; Jarrett et al., 2007), support conditioned taste aversion (McLaughlin et al., 2005; but see Chambers et al., 2006), and (like rimonabant), induce compulsive scratching and grooming (Tallett et al., 2007b). Furthermore, as synergistic interactions on food intake in mice have been reported for AM 251 and the μ-opioid receptor antagonist nalmefene (Chen et al., 2004), it was of considerable interest to determine whether a low (sub-anorectic) dose combination of AM 251 and naloxone would produce significant and behaviourally-selective reductions in food intake and feeding behaviour in rats.

Consistent with previous findings in our laboratory and elsewhere (Tallett et al., 2008a,b), present results show that 0.1 mg/kg naloxone did not significantly influence food intake, feeding behaviour, the BSS or post-treatment weight gain in male rats. Although the lower dose of AM 251 (0.5 mg/kg) was also without significant effect on these test parameters, the higher dose (1.0 mg/kg) of the compound significantly inhibited food intake and, while not affecting any measure of feeding behaviour or post-treatment weight gain, significantly increased the duration (but not frequency) of scratching behaviour. These results are very similar to those of a previous study in our laboratory (Tallett et al., 2007b) although, in that experiment, the inhibitory effects of 1.5 mg/kg AM 251 on food intake did not quite reach statistical significance. In this context, it is worth noting that, depending on the specific methodology used, the threshold anorectic dose of AM 251 in rats can be somewhat variable (Hildebrandt et al., 2003; McLaughlin et al., 2003; Shearman et al., 2003; Slais et al., 2003; Chambers et al., 2004; Zhou and Shearman, 2004; McLaughlin et al., 2005; Chambers et al., 2006). Nevertheless, these results fortuitously meant that the present study included both a sub-anorectic *and* an anorectic dose of AM 251.

In contrast to the lack of significant effect when given alone, co-administration of naloxone and the lower dose of AM 251 (0.5 mg/kg) produced statistically-significant reductions in mash consumption and in time spent feeding. In both cases, the data were consistent with an additive rather than supra-additive effect of the two drugs; in other words, the effect of the combined treatment could have been predicted from the simple sum of individual drug effects. This interpretation is further supported by the pattern of results obtained when animals received combined treatment with naloxone and the higher (intrinsically anorectic) dose of AM 251 (1.0 mg/kg). Here, the effects of combined treatment on food intake, feeding frequency and feeding duration, although greater than those of AM 251 alone, could also be readily explained in terms of simple addition. An additive effect would also account both for the significant increase in stop behaviour and the (non-significant) suppression of 7-day post-treatment weight gain when animals received this treatment combination. Current findings are clearly at variance with several reports of synergistic interactions between opioid and CB1 cannabinoid receptor antagonists (Kirkham and Williams, 2001a,b; Rowland et al., 2001; Chen et al., 2004). However, they fully replicate the pattern of results obtained in our earlier study with naloxone and rimonabant (Tallett et al., 2008b), including the lack of effect of combined treatment on a range of other feeding-related parameters (e.g. feeding latency, duration of feeding bouts, feeding rate). In passing, it is pertinent to note specifically the lack of treatment effect on feeding rate, reductions in which have been widely reported with other pharmacological manipulations, e.g. dopamine D2 receptor antagonists and serotonin releasers and/or reuptake inhibitors (e.g. Clifton, 2000; Lee and Clifton, 2002). The reason/s for the discrepancies between our findings and those reported by others are unclear but are unlikely to involve differences in species, palatability of the test diet, or doses of the test compounds. Of potential relevance are the results of several recent studies in mice. Thus, following initial demonstration of synergistic interactions between AM 251 and nalmefene on food intake and weight gain in mice (Chen et al., 2004), the same research group has since reported unaltered effects of AM 251 on intake and weight gain in mice lacking the μ-opioid receptor (Chen et al., 2006). These findings would suggest that, under some circumstances at least, these two systems can exert independent effects on appetite and energy homeostasis.

Present data clearly constitute further proof of concept in that significant anorectic activity can be obtained with low (intrinsically sub-anorectic) dose combinations of the opioid receptor antagonist naloxone and cannabinoid receptor antagonist/inverse agonists. In this context, it is pertinent to note that the significant anorectic activity observed in the AL–NX condition was not associated with any other significant behavioural change or with any signs of behavioural disruption: examination of Fig. 5 confirms the structural integrity of the BSS in V–NX, AL–V and AL–NX treatment conditions. Of equal, if not greater, importance is the finding that NX at least partially antagonised the scratching syndrome induced by the higher dose of AM 251 (1.0 mg/kg); although not significant, a similar pattern is evident for the AL–NX combination. As was the case for food intake and feeding behaviour (vide supra), this finding replicates our earlier observation regarding the ability of naloxone to counter the scratching response induced by rimonabant (Tallett et al., 2008b). Thus, not only is the anorectic response to low-dose combinations of NX and AM 251 behaviourally-selective, NX substantially attenuates an unwanted behavioural effect of higher doses of the CB1 receptor antagonist/inverse agonist. In view of these findings, it is perhaps relevant that opioid receptor antagonists are used clinically to treat a variety of human pruritic skin conditions (Terra and Tsunoda, 1998; Cies and Giamalis, 2007). Since methyl naltrexone and topical naltrexone are especially effective in this regard (Friedman and Dello Buono, 2001; Bigliardi et al., 2007), cutaneous opioid receptors are clearly implicated both in the itching/scratching syndrome and the therapeutic response. As itching and scratching are well-documented opioid receptor antagonist-sensitive responses to opiates in animals and humans (Ballantyne et al., 1988; Kuraishi et al., 2000; Miyamoto et al., 2002), one interpretation of present findings is that CB1 receptor antagonist/inverse agonists, through a mechanism/s currently unknown, stimulate the peripheral release of opioid peptides. Overall, present findings, along with our earlier report on naloxone/rimonabant interactions (Tallett et al., 2008b), are particularly significant in view of the recent confirmation of itching and scratching (pruritus) as a significant side-effect of CB1 receptor antagonist/inverse agonists in humans (taranabant; Addy et al., 2008; Kirkham, 2008). More specifically, our data suggest that polytherapy with low doses of a (long-acting) opioid receptor antagonist and a CB1 receptor antagonist/inverse agonist would not only suppress appetite but would also attenuate/prevent at least one of the unwanted effects of CB1 receptor antagonist/inverse agonist monotherapy.

In conclusion, the current study has provided further evidence of additive interaction between low doses of naloxone and CB1 receptor antagonist/inverse agonists in the regulation of food intake and feeding behaviour. The lack of effect of such treatments on latencies to find the food source and to commence feeding, as well their lack of effect on the rate of consumption, suggests an action on mechanisms (e.g. reward and/or satiety) other than primary motivation. The effects of combined treatment are not only behaviourally-selective but have the added advantage that naloxone significantly attenuates a prominent side-effect (scratching) of CB1 receptor antagonist/inverse agonists. This profile of additive (feeding) and antagonistic (scratching) interactions between endogenous opioids and cannabinoids, while complex, is nevertheless consistent both with the diversity of opioid–cannabinoid interactions at the molecular level and with the crucial importance to such interactions of the specific transmitter system/s involved (e.g. GABA vs glutamate; Rios et al., 2006; Schoffelmeer et al., 2006). As both rimonabant and AM 251 have significant inverse

agonist activity at CB1 receptors (Pertwee, 2005; Bergman et al., 2008), further progress in this area would ideally involve exploration of opioid antagonist interactions with 'neutral or silent' CB1 receptor antagonists, including ligands with poor (e.g. LH-21; Pavon et al., 2006) and good (e.g. AM 4113; Chambers et al., 2007; Sink et al., 2008a,b) CNS penetration. Although difficult to routinely accommodate within the design of most behavioural studies, isobolographic techniques (e.g. Roth and Rowland, 1999; Rowland et al., 2001) could in future be used to statistically characterise/confirm the additive or supra-additive nature of such treatment interactions.

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