Involvement of 2-arachidonoylglycerol signaling in social challenge responding of male CD1 mice

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Abstract

Rationale

Endocannabinoids are strong modulators of emotionality and present a novel target for psychotropic drug development. Increasing evidence suggests that endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) affect behavior differentially. While the roles of anandamide have been investigated extensively, studies regarding the specific roles of 2-AG became possible only recently, and its involvement in social behaviors has not yet been studied.

Objective

We studied the impact of 2-AG signaling on aggression as a first attempt to characterize the role of this endocannabinoid in social behaviors.

Methods

2-AG signaling was enhanced by the monoacylglycerol lipase inhibitor JZL184 (8, and 16 mg/kg) in mice later submitted to the resident/intruder paradigm.

Results

JZL184 near completely abolished aggressiveness in residents and increased victimization (i.e., attacks by the opponent). Interestingly, the level of defensiveness remained unaltered, despite the large increase in bites received. The CB1 receptor blocker AM251 (0.5 mg/kg) did not influence the effects of JZL184. In intruders, JZL184 near completely suppressed bites and offensive behavior in a fashion similar to residents, but it also increased agitation and defensiveness during, and the corticosterone response to, aggressive encounters. Experiments involving the corticosterone synthesis inhibitor metyrapone (30 mg/kg) suggest that the suppression of biting and offensive behavior is directly influenced by JZL184, whereas increased agitation and defensiveness (seen in intruders only) are a secondary development of the stress-endocrine effects of JZL184.

Conclusions

2-AG signaling emerges as a surprisingly strong negative modulator of aggressiveness, which warrants further studies into its general role in social behavior and the target receptors involved.

Keywords

2-arachidonoylglycerol
Aggression
AM251
CB1R
Corticosterone
Endocannabinoid
JZL184
Metyrapone

Monoacilglycerol lipase
Social behavior

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Abbreviations

2-AG	2-arachidonoylglycerol
AEA	Anandamide
ANOVA	Analysis of variance
CB1R	Cannabinoid receptor type-1
HD	Head-dipping
MAGL	Monoacylglycerol lipase
SAP	Stretched attend posture

Introduction

The discovery of retrograde signaling by endocannabinoids, which regulates ion channel activity and neurotransmitter release, has triggered an avalanche of experimental studies implicating the endocannabinoid system in a growing number of functions, and providing a pharmacological target of unique therapeutic possibilities (Pacher and Kunos 2013; Piomelli 2003; Wilson and Nicoll 2002). The role of cannabinoid signaling in emotional behavior is well established, and its utility as a target for anxiolytic and antidepressant drug development was recurrently proposed (Hill et al. 2009; Tambaro and Bortolato 2012; Valverde 2005). Findings, however, are often controversial with cannabinoid receptor ligands, which also show important side effects (Moreira et al. 2009; Zanettini et al. 2011). Therefore, attention gradually shifted towards indirect cannabinoid modulators, which alter the metabolism of the main endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG). Such compounds include inhibitors of the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), which selectively enhance brain levels of anandamide and 2-AG, respectively (Long et al. 2009; Piomelli et al. 2006).

The involvement of cannabinoid signaling in the control of social behavior in general and, in aggression in particular, received relatively little attention so far, but a series of studies demonstrate that altered CB1 receptor signaling alters aggressiveness (Miczek 1978; Rodriguez-Arias et

al. 2013; van Ree et al. 1984). The overall conclusion of these studies is that increased CB1 functions inhibit aggressive behavior, but as with studies related to anxiety and depression, effects depend largely on experimental conditions, and are often conflicting. Findings on the impact of FAAH inhibitors on aggressiveness are sparse and negative. While the indirect enhancement of anandamide signaling increased amiable social interactions, no effects on aggression were detected so far with FAAH inhibitors (Cassano et al. 2011; Manduca et al. 2014; Moise et al. 2008). These reports, combined with findings on the efficacy of cannabinoid ligands in tests of aggression raise the possibility that the effect of cannabinoids on aggression are mediated by 2-AG, the other main endocannabinoid in the brain.

Studies into the specific behavioral roles of 2-AG were made only recently possible by the development of the MAGL inhibitor JZL184 (Long et al. 2009). This compound was shown to affect locomotion and emotionality in a series of non-social tests (Aliczki et al. 2012, 2013; Busquets-Garcia et al. 2011; Sciolino et al. 2011), confirming that 2-AG is relevant for behavioral control but also suggesting that its roles are different from those played by anandamide. The compound was not yet studied in tests relevant for social behavior, and as such, the roles of 2-AG are unknown in this respect at present.

In the present study, we studied the impact of the MAGL inhibitor JZL184 in the resident/intruder test, where—in contrast to the social interaction test which is run in a neutral arena—the dyadic encounter takes place in the home cage of one of the contestants, which enhances the expression of aggressive behavior. As the blockade of 2-AG degradation by JZL184 increases corticosterone secretion (Aliczki et al. 2013), which may per se affect aggressiveness (Mikics et al. 2004), we also studied glucocorticoid responses to the resident/intruder test and the impact of the corticosterone synthesis inhibitor metyrapone. In non-social tests, the behavioral effects of enhanced 2-AG signaling depended largely on context (Aliczki et al. 2013; Busquets-Garcia et al. 2011; Sciolino et al. 2011); therefore, we studied the effects of the compound in both residents and intruders, for which the very same resident/intruder test is contextually highly different. Finally, we investigated molecular mechanisms by employing the CB1 receptor blocker AM251.

Methods

Subjects

Subjects were 2-month-old male CD1 mice weighing 30–35 g (Charles River laboratories, Budapest, Hungary). They were kept under a light/dark cycle of 12/12 h with lights on at 0700 hours. Food and water were freely available; temperature and humidity were kept at 23 ± 2 °C and 60 ± 10 %, respectively. In contrast to rats that are highly social, individual housing is not stressful for the mouse, which is a solitary species (Arndt et al. 2009; Benton and Brain 1981; Capanna et al. 1984). Mice establish strong dominance hierarchies (Capanna et al. 1984; Poshivalov 1980), which would have constituted a confounding factor in this experimental design if the mice were group housed. Therefore, both residents and intruders were housed individually for 2 weeks before experimentation. Mice were experimentally naive, had no drug history, and were used in one experiment only.

Animal welfare and ethical statement

Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Drugs

The MAGL inhibitor JZL184 (Cayman Chemical, Ann Arbor, MI) and the cannabinoid receptor type-1 (CB1R) antagonist AM251 (Sigma Aldrich, Saint Louis, MO) were dissolved in 0.2 ml dimethylsulfoxide and were diluted to the final volume with saline containing 0.4 % methylcellulose. Both compounds were administered intraperitoneally in a volume of 10 ml/kg body weight. JZL184 was injected at the doses of 0 (vehicle), 8, and 16 mg/kg body weight, respectively, while AM251 was administered in the doses of 0 (vehicle), 0.5, and 1.0 mg/kg body weight, respectively. Both JZL184 and AM251 doses were selected based on earlier studies (Aliczki et al. 2012, 2013; Haller et al. 2007; Long et al. 2009; Sciolino et al. 2011). The corticosterone synthesis blocker metyrapone (2-Methyl-1,2-di-3-pyridyl-1-propanone) (Sigma Aldrich, Saint Louis, MO) was dissolved in saline containing 5 % Tween 80 and administered in doses 0

(Vehicle) and 30 mg/kg intraperitoneally in a volume of 5 ml/kg body weight. The selection of the metyrapone dose was based on previous studies (Aliczki et al. 2013). When drugs were co-administered, they were delivered in two separate injections in rapid succession.

Behavioral tests

Behavioral tests were conducted in the early light phase of the day in a separate quiet room under ~400 lx light intensity, which was similar to that employed in the maintenance rooms. Behavior was video recorded in all tests by a Sony DCR-SR75 digital video camera. Video recordings were analyzed with the custom-made H77 event recording software (J. Haller, Institute of Experimental Medicine, Budapest, Hungary). As bites are very fast, these were scored separately at slow motion (frame-by-frame when necessary). All behaviors were scored by one and the same experimenter (MA). Intra-rater reliability was over 90 %.

In the resident/intruder test, an intruder of smaller size was placed into the home cage of residents for 10 min. An experimenter blind to the treatments recorded the frequency of bites delivered to, and received from opponents. The duration of the following behaviors were also recorded: *aggressive grooming* (pushing down the opponent, while it is standing or trying to escape, nibbling the fur and the skin with quick movements of the head); *tail rattling* (rapid rattling of the tail while the subject faces its opponent); *wrestling* (wrestling movements often associated with biting); *defensive upright* (trials of keeping the opponent at distance with forepaws while rising on hind legs); *avoidance* (evading the approaching opponent); *flight* (quickly moving away from the chasing opponent). *Aggressive grooming*, *tail rattling*, and *wrestling* were summed up as *offensive behaviors*, while *defensive upright*, *avoidance*, and *flight* were summed up as *defensive behaviors*.

The open-field was a white non-transparent plastic box of 45 × 45 × 25 cm (height). Subjects were placed in one of the corners of the open-field and were allowed to explore it for 5 min. The apparatus was covered with a transparent Plexiglas lid during testing and was cleaned with tap water and paper towel between subjects. Locomotor activity was scored by counting the crossing of lines that divided the open-field into 16 equal squares. Exploration in the central area (i.e., the four squares in the center of the apparatus) was also scored. Increased exploration of the central area

indicates lower levels of anxiety-like behavior. The grid was drawn on the video screen; thus, it was invisible to subjects.

The elevated plus-maze was made of black-painted aluminum. It consisted of two open arms (30 × 7 cm) and two closed arms (30 × 7 cm with 30 cm high walls) that were connected by a central platform (7 × 7 cm). The plus-maze was elevated to 70 cm from the floor. Subjects were placed on the central platform facing one of the open arms and were allowed to explore the apparatus for 5 min. The apparatus was cleaned with tap water and paper towel between tests. The number of entries into the closed arms is considered a measure of locomotor activity, whereas more time spent in the open arms indicates lower levels of anxiety (Pellow et al. 1985). Subjects were considered to enter a compartment when all four legs crossed the lines separating the compartments. Risk-assessment activities were also analyzed as ethological measures of anxiety (Cole and Rodgers 1993). Particularly, we scored the frequency and duration of *head-dipping* (exploratory movement of head/shoulders over the side of the maze) and *stretched attend posture* (exploratory posture in which the body is stretched forward then retracted to the original position without any forward locomotion). Risk assessment behaviors were differentiated based on the location of their occurrence. As risk assessment from protected areas (i.e., from the closed arms or central platform) were shown to correlate negatively with open arm exploration (Cole and Rodgers 1993; Cruz et al. 1994; Fernandez Espejo 1997), protected head dipping and stretch-attend posture were studied here as ethological indicators of anxiety-like behavior. This procedure and interpretation is widely employed (Aliczki et al. 2012, 2013; Cruz et al. 1994; Navarro et al. 2006; Rodgers et al. 1992; Wall et al. 2003).

Blood sampling and corticosterone measurement

Corticosterone levels were measured from trunk blood sampled on ethylenediaminetetraacetic acid (EDTA)-containing plastic tubes after the resident/intruder test. After sampling, blood was centrifuged at 4 °C and the blood plasma was separated and stored at −20 °C till analysis. Plasma corticosterone was measured by radioimmunoassay as described earlier (Toth et al. 2011). The corticosterone antiserum was raised in rabbits against corticosterone-carboxymethyloxime bovine serum albumin. ^{125}I-labeled corticosterone-carboxymethyloxime-tyrosine-methyl esther was

used as tracer. The interference with plasma transcortin was eliminated by inactivating transcortin at low pH. The sensitivity of the assay was 1 pmol/ml. Intra- and inter-coefficient of variation was 10 and 25 %, respectively.

Experimental design

In *experiment 1*, we studied the effects of JZL184 on territorial aggression and social stress-induced plasma corticosterone levels in residents that were faced with an intruder for 10 min. Subjects were treated with 0 (vehicle), 8, or 16 mg/kg JZL184 in a random order. After 40 min, a smaller, non-treated mouse was placed into their home cage for 10 min. After the encounter, trunk blood was collected from residents for corticosterone measurements. Sample sizes were 10 animals in each group.

In *experiment 2*, we investigated the effects of JZL184 on intruder behavior and corticosterone levels in a different set of animals. The experimental design was identical with that employed in *experiment 1*, but this time treatments were administered to, and blood was sampled from, intruders. Sample sizes were 10 subjects in the vehicle control group and nine animals in each the 8 mg/kg and the 16 mg/kg JZL184 treated group.

Experiment 3 investigated the interaction between the behavioral effects of JZL184 and plasma corticosterone by employing the corticosterone synthesis inhibitor metyrapone. This study was performed in intruders because plasma corticosterone was not affected by JZL184 in residents, for which synthesis inhibition in such subjects was considered meaningless. The JZL184 dose employed was 16 mg/kg; metyrapone was co-administered in the dose 0 (vehicle) and 30 mg/kg. Doses were based on previous experience with the compound (Aliczki et al. 2013). Subjects were studied 40 min later in the resident/intruder test as intruders. Sample sizes were 10 subjects in each group.

In e*xperiment 4*, we assessed the CB1R-dependence of JZL184 effects by co-administering the CB1R antagonist AM251 and JZL184 (16 mg/kg). We excluded intruders from this study for two reasons: (i) plasma corticosterone was increased by JZL184 in these subjects, and data indicated that this endocrine effect was relevant for agitation and defensiveness, which were induced specifically in intruders by JZL184. This circumstance may have complicated the interpretation of AM251

findings; (ii) biting and offensive behaviors were influenced similarly in residents and intruders and neither effect seemed to depend on plasma corticosterone. As such, we hypothesized that the CB1R-dependence of behavioral effects can be better studied in residents than in intruders, and findings obtained in the former can be extrapolated to the latter as it regards biting and offensive behavior. In the first experiment (*experiment 4a*) we administered 1 mg/kg AM251 to subjects. Despite the fact that this dose was ineffective earlier in non-social tests (Haller et al. 2004), it strongly affected aggressive behavior in the present studies. Therefore, AM251 was also tested at the 0.5 mg/kg dose (*experiment 4b*). The results of both experiments were presented, because the behavioral effects of JZL184 and the large AM251 dose were highly similar, which is per se relevant for the mediation of JZL184 effects. Sample sizes were 9–10 per group in both experiments.

In *experiment 5*, we studied the effects JZL184 in the open-field and elevated plus-maze to establish whether changes in aggressive behavior were secondary to altered locomotor activity or anxiety. Subjects were treated with 0 (vehicle), 8, and 16 mg/kg JZL184. After 40 min, they were submitted to the open-field test for 5 min, which was followed immediately by a 5 min-long plus-maze test. Sample sizes were 6–7 animals per group.

As the behavioral effects of pharmacological treatments may be influenced by the context, we also investigated locomotion and social behaviors in the resident/intruder tests that were described above (experiments 1 and 2). Locomotion was evaluated by counting line crossings in a manner similar to that employed in the open-field test and was considered an indicator of locomotor activity in the context of a social challenge. Social behaviors consisted in sniffing directed towards opponents (any body part), which is commonly used as a sign of anxiety-like behavior in comparable test situations (File and Hyde 1978). We scored these behaviors for the first 2 min of the encounter, to avoid interference from aggressive behavior. Within this time window, the majority of subjects neither attacked, nor was attacked by opponents. In the few cases where they did, we used the latency of the first attack as a cutoff time and extrapolated the data. Both behaviors were expressed as frequencies/min. Sample sizes were identical with those shown for experiments 1 and 2.

Statistical analysis

Data were presented as mean ± standard error of the mean, and were evaluated by one-, two-, or three-factor analysis of variance (ANOVA) as shown in "Results". ANOVA assumptions were checked by the Levene's test. Where assumptions were not fulfilled, data were square root transformed. The Duncan test was used for post-hoc comparisons. *P*-values lower than 0.05 were considered statistically significant.

Results

Experiment 1 and 2: the effects of JZL184 on aggression in residents and intruders

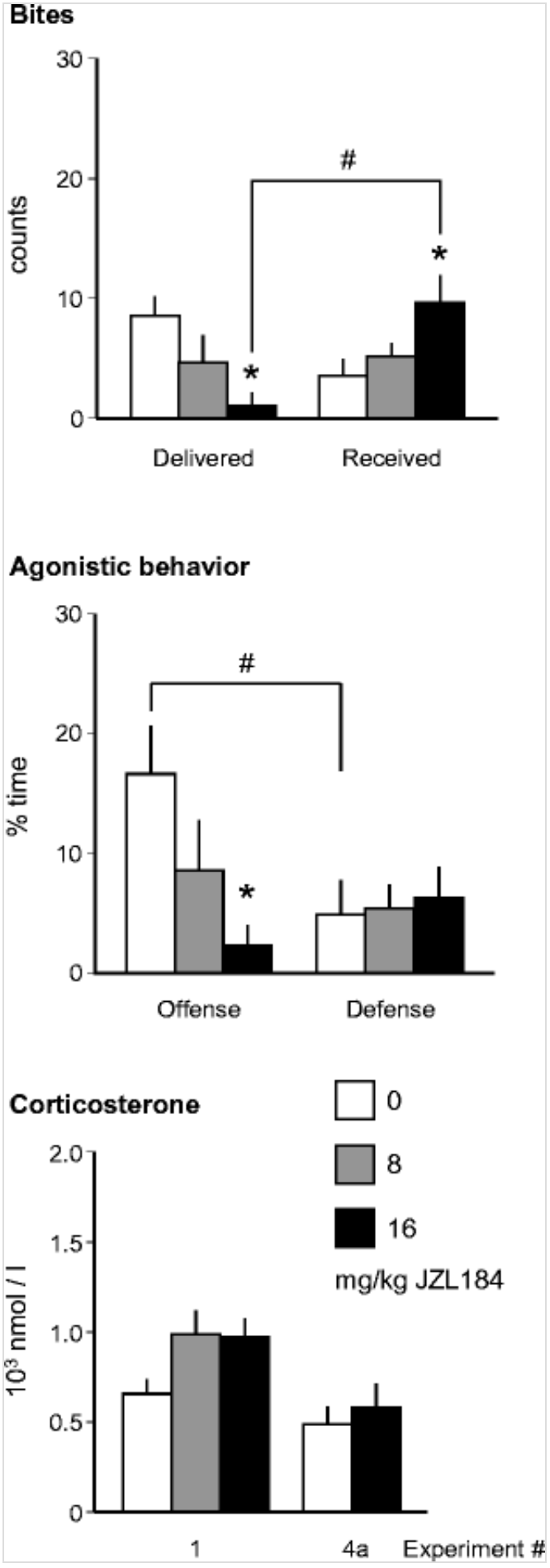
We observed that JZL184 not only affected aggressiveness in the treated subjects but also dramatically changed the relationship between contestants. To study this relationship, we performed two repeated measure ANOVAs. In one ANOVA, the number of bites was analyzed as a function of JZL184 dose given to the resident (factor 1: 0, 8, and 16 mg/kg) and the nature of the bites (factor 2, a repeated measure: delivered by the resident vs. received by the resident). In another ANOVA, the number of agonistic behaviors was analyzed as a function of JZL184 dose delivered to the resident (factor 1: 0, 8, and 16 mg/kg) and the nature of the behavior (factor 2, a repeated measure: offensive vs. defensive).

In residents (experiment 1), bite counts depended on the interaction between factors ($F_{\text{interaction}}(2,27) = 5.94$, $p = 0.007$). Note that intruders were not treated in this study. JZL184 dramatically decreased the number of bites delivered and increased the number of bites received; moreover, the roles of contestants reversed at the large dose as shown by the fact that treated residents received considerably more bites than they delivered (Fig. 1, upper panel). Agonistic behavior was changed in a comparable but not entirely similar manner ($F_{\text{interaction}}(2,27) = 3.41$, $p = 0.047$). Offense was dramatically decreased by JZL184, but defense remained unaltered, suggesting that the effects of the compound were restricted to offensive behaviors (Fig. 1, middle panel). Due to changes in offense, the relationship between the two behaviors was still affected. Vehicle-treated controls showed significantly more offense than defense, which difference disappeared in both JZL184-treated groups, where time devoted to offense and defense was statistically similar. The compound did not alter plasma corticosterone significantly as shown in Fig. 1 (lower panel).

Nevertheless, there was a relatively large difference between control and JZL184-treated animals (even if non-significant). To ascertain that the lack of significance was not due to a type 2 error, the lower panel also shows plasma corticosterone levels measured in Experiment 4, where subjects were also residents and were studied under highly similar conditions. JZL184 failed to affect plasma corticosterone significantly in this study as well.

Fig. 1

Resident mice: the effects of JZL184 on aggressive behavior and plasma corticosterone (experiment 1). Note that intruders were not treated in this study. The *lower panel* also shows plasma corticosterone data from experiment 4a, to substantiate the finding that plasma corticosterone is not altered by JZL184 in residents. Data were shown as mean ± standard error of the mean. *, significant difference from vehicle control; $^{\#}$, significant difference between behaviors of opposing valence (e.g., bites delivered vs. bites received as well as offense vs. defense) ($p < 0.05$ at least)

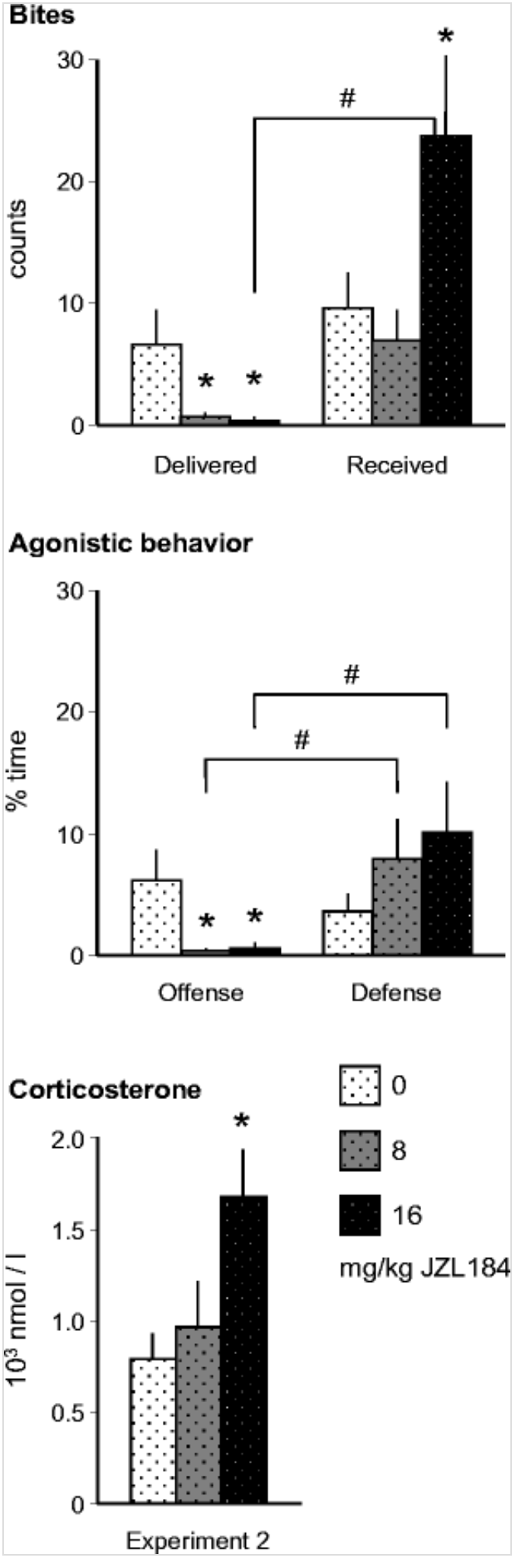


In intruders treated with JZL184 (Experiment 2), the interaction between factors was significant for both treatment and bites ($F_{\text{interaction}}(2,25)$ =

4.29, $p = 0.025$) as well as for treatment and agonistic behavior ($F_{\text{interaction}}(2,27) = 3.41$, $p = 0.047$). Note that residents were not treated in this study. Intruders not only received more bites than they delivered, but also showed more defense than offense (Fig. 2). In addition, JZL184 increased plasma corticosterone significantly in this category of subjects ($F(2,24) = 4.51$, $p = 0.02$). Thus, the effects of JZL184 were similar in residents and intruders as regard to bites and offense, but different as regard to defensive behavior and plasma corticosterone. While defensive behavior was not altered by the compound in residents (they only lost the preponderance of offensive over defensive behaviors that was seen in controls), the behavior of intruders treated with JZL184 shifted clearly towards defensiveness. In addition, intruders showed elevated levels of plasma corticosterone when they had been treated with JZL184, an effect not seen in residents.

Fig. 2

Intruder mice: the effects of JZL184 on aggressive behavior and plasma corticosterone (experiment 2). Note that residents were not treated in this study. Data were shown as mean ± standard error of the mean. *, significant difference from vehicle control; $^{\#}$, significant difference between behaviors of opposing valence (e.g. bites delivered vs. bites received as well as offense vs. defense) ($p < 0.05$ at least)


Bites
counts
30
20
10
0
#
*
*
*
Delivered
Received
Agonistic behavior
% time
30
20
10
0
#
#
*
*
Offense
Defense
Corticosterone
10^3 nmol / l
2.0
1.5
1.0
0.5
0
*
0
8
16
mg/kg JZL184
Experiment 2

AQ2

Experiment 3: interactions with glucocorticoid production

The impact of metyrapone was investigated in intruders, where plasma glucocorticoids were increased by JZL184. Metyrapone affected plasma corticosterone in the expected manner: it significantly decreased plasma glucocorticoids in both vehicle- and JZL184-treated mice (Table 1). The behavioral effects of metyrapone, JZL184, and their combined administration were very similar. All three decreased the number of bites delivered and increased the number of bites received over those that were delivered. In addition, the duration of offensive behaviors was also decreased by all three treatments (for data and statistics, see Table 1). Surprisingly, however, metyrapone abolished the effects of JZL184 on defensiveness, despite the fact that both compounds made subjects defensive when administered alone. This strange finding is addressed in "Discussion."

Table 1

Intruders: interactions between the effects of JZL184 and those of the corticosterone synthesis inhibitor metyrapone

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Treatment		Bites		Agonistic behavior		CORT
1	2	Delivered	Received	Offense	Defense	
Vehicle	Vehicle	6.60 ± 2.91	9.70 ± 2.77	6.11 ± 2.65	3.54 ± 1.55	783.9 ± 144.5
Vehicle	Metyrapone 30 mg/kg	1.10 ± 0.62[a]	18.30[b] ± 2.95	0.66 ± 0.66[a]	9.60[b] ± 3.00	438.0 ± 32.5[a]
JZL184 16 mg/kg	Vehicle	0.33 ± 0.33[a]	23.78[b] ± 8.18	0.52 ± 0.50[a]	10.20[b] ± 4.04	1670.5 ± 258.3[a]
JZL184 16 mg/kg	Metyrapone 30 mg/kg	0.30 ± 0.21[a]	14.90[b] ± 4.97	0.00 ± 0.00[a]	4.71 ± 2.21	446.2 ± 33.9[a]
$F_{interaction}(1,35)$		5.09		6.41		10.07
p		0.03		0.015		0.003
Data were shown as mean ± standard error of the mean. JZL184 and metyrapone doses were 16 and 30 mg/kg, respectively						

[a]Significantly different from control (vehicle + vehicle injections)

[b]Significant difference between bites delivered and received as well as between offense and defense ($p < 0.05$ at least)

Experiment 4: interactions between JZL184 and the CB1 receptor blocker AM251

This study was performed in residents, because corticosterone production was affected by JZL184 in intruders and in addition, this endocrine effect seemed to interact to a certain extent with the behavioral effects of the drug. Therefore, CB1 mechanisms were investigated in residents, where JZL184 did not affect corticosterone production significantly, and as such, interference from endocrine factors was minimal.

The large dose of AM251 (1 mg/kg) inhibited aggressiveness significantly, an effect that was highly similar to that observed with JZL184 (Table 2). The three factors (factor 1: JZL184 treatment; factor 2: AM251 treatment; repeated measurements factor 3: type of behavior) showed significant interaction for both bites and agonistic behavior (see Table 2 for statistics). All three JZL184, AM251, and their combined administration reduced the number of bites delivered and the duration of offensive behaviors. In addition, the preponderance of bites delivered over those received and of offense over defense disappeared in all treatment groups. Thus, the effects of JZL184 and 1 mg/kg AM251 were highly similar, which made it unlikely that the effects of the former were mediated by the CB1 receptor. This assumption was strongly supported by the study where AM251 was administered at a lower dose.

Table 2

Residents: interactions between the effects of JZL184 and the large dose (1 mg/kg) of the CB1 blocker AM251

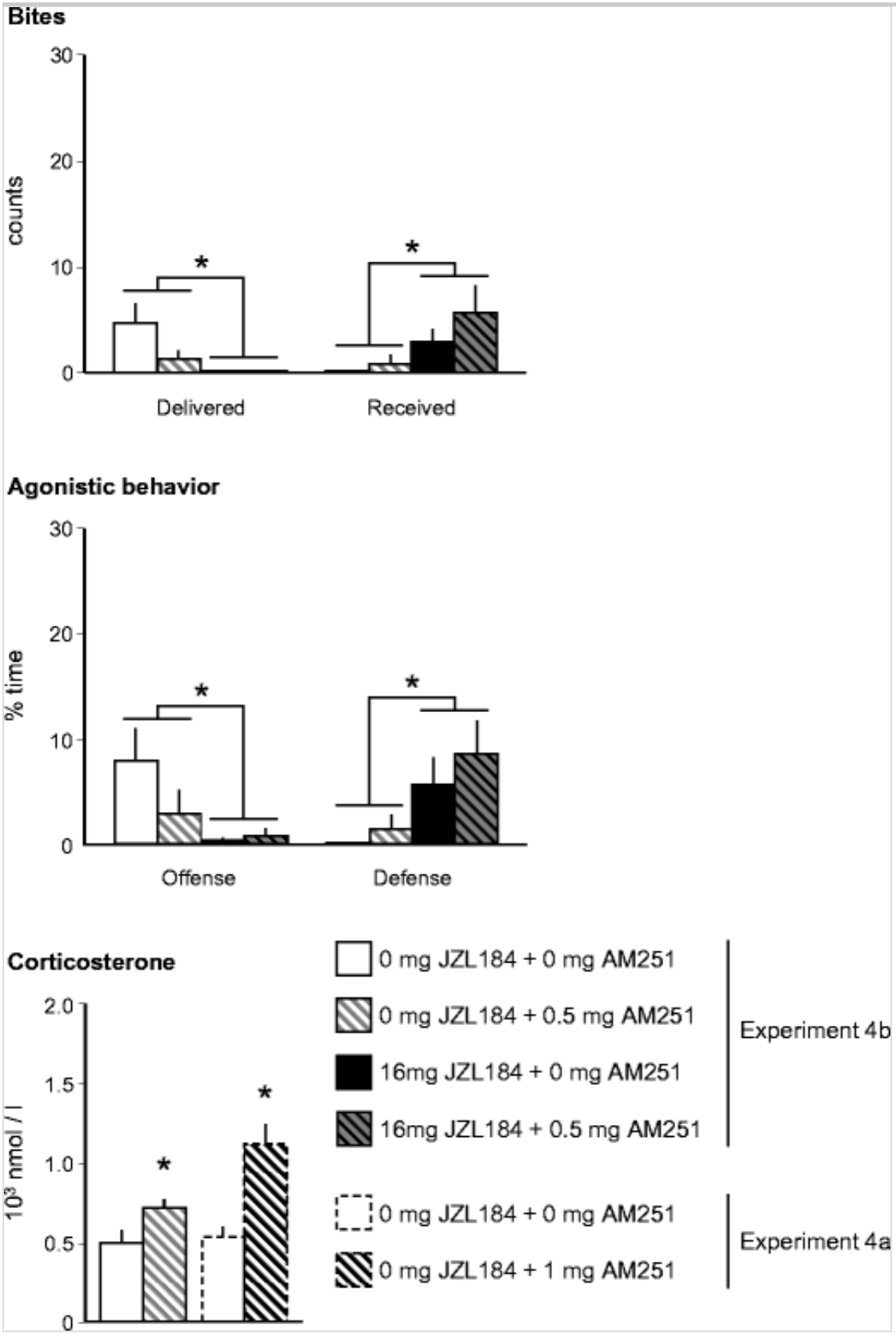
Treatment		Bites		Agonistic behavior	
1	**2**	**Delivered**	**Received**	**Offense**	**Defense**
Vehicle	Vehicle	6.75 ± 1.71	0.62[b] ± 0.49	13.62 ± 3.63	0.1[b] ± 0.1
Vehicle	AM251 1 mg/kg	1.00 ± 0.60[a]	2.44 ± 1.65	4.61 ± 2.05[a]	0.98 ± 1.30
JZL184		0.75 ±	0.0 ±	1.31 ±	0.03 ±

16 mg/kg	Vehicle	0.49[a]	0.00	0.75[a]	0.03
JZL184 16 mg/kg	AM251 1 mg/kg	1.00 ± 0.78[a]	0.00 ± 0.00	2.37 ± 1.64[a]	0.01 ± 0.01
$F_{interaction}(1,33)$		7.79		5.92	
p		0.009		0.02	
Data were shown as mean ± standard error of the mean. JZL184 and AM251 doses were 16 and 1 mg/kg, respectively					
[a]Significantly different from control (vehicle + vehicle injections)					
[b]Significant difference between bites delivered and received as well as between offense and defense ($p < 0.05$ at least)					

In experiment 4b, 0.5 mg/kg AM251 affected behavior neither alone (*bites*: $F_{AM251}(1,34) = 0.1$, $p > 0.9$; *agonistic behavior*: $F_{AM251}(1,34) = 0.1$, $p > 0.9$) nor in interaction with other factors (*bites*: $F_{AM251*JZL184}(1,34) = 1.95$, $p = 0.17$; *agonistic behavior*: $F_{AM251*JZL184}(1,34) = 1.35$, $p > 0.25$; *bites*: $F_{AM251*JZL184*bite\ type}(1,34) = 0.26$, $p = 0.61$; *agonistic behavior*: $F_{AM251*JZL184*behavior\ type}(1,34) = 0.51$, $p > 0.47$). By contrast, there was an interaction between JZL184 treatment and the type of behavior for both bites ($F_{JZL184*bite\ type}(1,34) = 14.32$, $p = 0.0005$) and agonistic behavior ($F_{JZL184*behavior\ type}(1,34) = 15.35$, $p > 0.0004$). Particularly, JZL184 dramatically decreased the number of bites delivered and offense, and increased the number of bites received and defense (Fig. 3). The lack of behavioral effects of AM251 was not due to a loss of biological activity at 0.5 mg/kg, because this compound preserved its effects on corticosterone production (Fig. 3). The figure includes data obtained in experiment 4a, where 1 mg/kg AM251 also increased plasma corticosterone.

Fig. 3

Resident mice: the effects of 16 mg/kg JZL184, 0.5 mg/kg AM251, and their combination on aggressive behavior (experiment 4b). JZL184 treatment was the only factor that provided significant differences; AM251 did not affect behavior significantly. The *lower panel* demonstrates that the low dose of AM251 (0.5 mg/kg) preserved its biological activity because it was able to significantly increase plasma corticosterone. For comparison, we also showed plasma corticosterone data obtained experiment 4a where AM251 was studied at a larger dose. *, significant difference compared to vehicle control ($p < 0.05$ at least)



AQ4

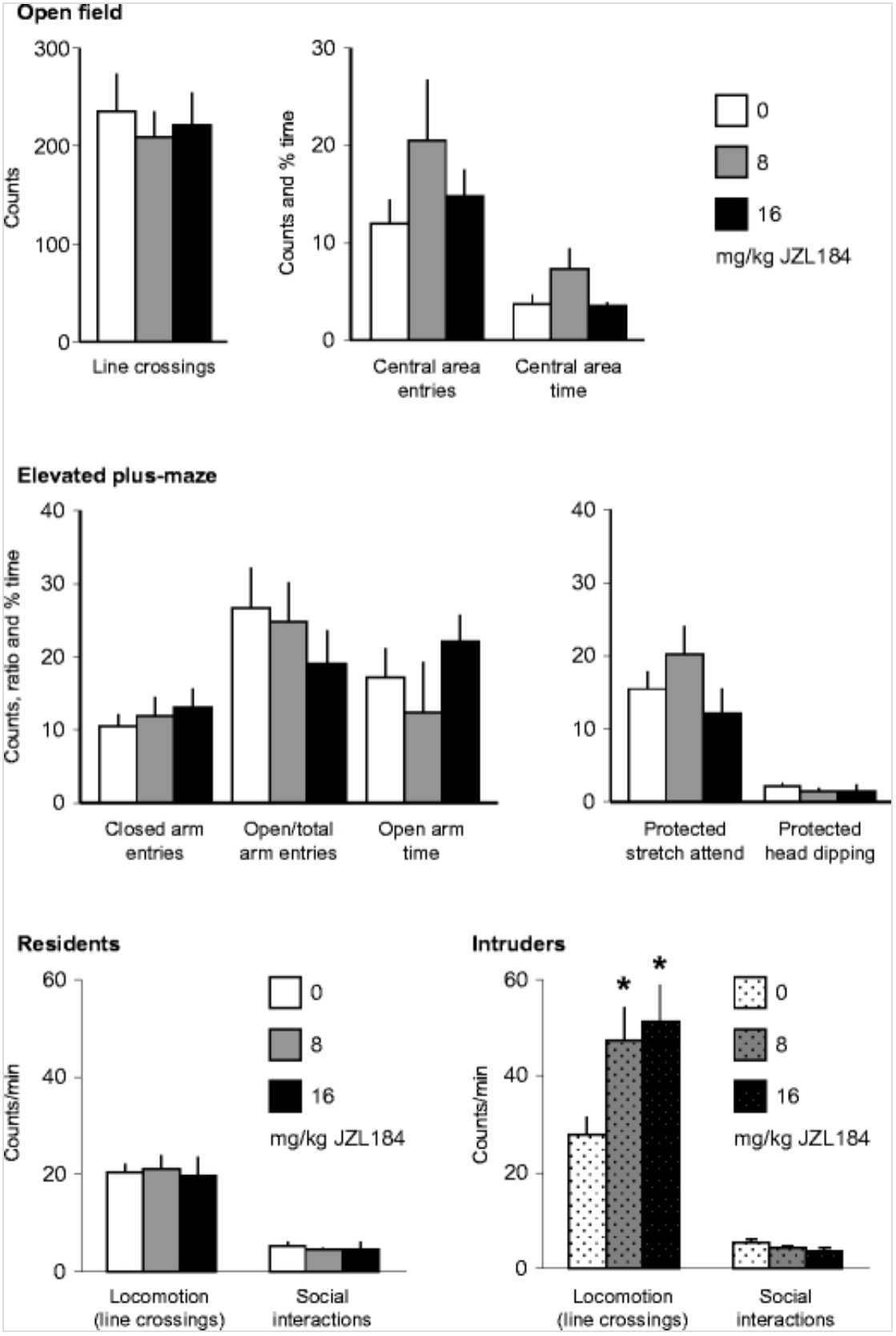
Experiment 5: possible confounds from non-specific effects

JZL184 did not affect locomotor activity or central area exploration in the open-field (line crossings: $F(2,13) = 0.15$, $p = 0.86$; central area entries:

$F(2,13) = 2.56$, $p = 0.31$; central area time %: $F(2,13) = 2.27$, $p = 0.14$) (Fig. 4, upper panels). Locomotor activity and anxiety-like behavior in the elevated plus-maze were also unaltered by JZL184-treatment (*closed arm entries*: $F(2,13) = 0.31$, $p = 0.74$; % *open arm entries*: $F(2,13) = 1.8$, $p = 0.2$; *open arm time* %: $F(2,13) = 0.75$, $p = 0.49$; *protected stretch attend posture frequency*: $F(2,13) = 1.91$, $p = 0.19$; *protected head dipping frequency*: $F(2,13) = 0.43$, $p = 0.66$; *protected stretch attend posture*, *time* %: $F(2,13) = 1.47$, $p = 0.27$; *protected head dipping*, *time* %: $F(2,13) = 0.63$, $p = 0.55$) (Fig. 4, middle panels).

Fig. 4

The effects of JZL184 in tests relevant to locomotion and anxiety (open-field and elevated plus-maze, experiment 5). The *lower panels* show the frequency of line crossings (locomotion) and social interactions in the first 2 min of resident/intruder tests, when these behaviors were not yet affected by the emergence of biting attacks (experiments 1 and 2; for details see "Methods and Results"). *, significant difference from vehicle-treated controls ($p < 0.05$ at least)



Since one can reasonably assume that behavior in non-social situations, e.g., those shown in the open-field and elevated plus-maze is not adequate control for the effects of the same drugs in a social context, we scored locomotor behavior and social interactions in the resident-intruder tests. To avoid confounds from repeated defeats which were especially common in JZL184-treated subjects, behavior was scored for the first 2 min of the

conflict (see "Methods"). In residents, neither locomotion nor social interactions were affected by JZL184 (*line crossings*: $F(2,27) = 0.1$, $p = 0.9$; *social interactions*: $F(2,27) = 0.13$, $p = 0.9$), which supports the findings obtained in non-social tests and suggests that changes in aggressiveness are not secondary to changes in locomotion or anxiety in residents (Fig. 4, lower left-hand panel). A somewhat different picture emerged in intruders, where social interactions were not affected by JZL184 ($F(2,25) = 1.95$, $p = 0.16$), but locomotion counts increased considerably in both groups treated with the compound ($F(2,25) = 4.22$, $p = 0.02$) (Fig. 4, lower right-hand panel). Thus, JZL184 increased agitation in intruders in the early phases of the conflict. Interestingly, metyrapone abolished the effect of JZL184 on locomotion, suggesting that agitation was increased by the compound via its effects on corticosterone production (Table 3). Social interactions were affected by metyrapone but not by JZL184.

Table 3

Intruders: the effects of JZL184 and metyrapone on the frequency of line crossings (locomotion) and social interactions when facing non-treated residents

Treatment		Line crossings	Social interactions	
1	2		Collapsed over the significant factor	All groups
Vehicle	Vehicle	27.50 ± 3.60	4.34 ± 0.57	5.31 ± 0.83
JZL184 16 mg/kg	Vehicle	50.11[a] ± 6.98		3.27 ± 0.62
Vehicle	Metyrapone 30 mg/kg	37.65 ± 3.90	2.54[a] ± 0.45	2.12 ± 0.41
JZL184 16 mg/kg	Metyrapone 30 mg/kg	34.79 ± 10.97		2.96 ± 0.80
$F_{JZL184}(1,35)$		1.25	0.66	
p		0.25	0.42	
$F_{metyrapone}(1,35)$		0.35	6.14	
p		0.57	0.018	
$F_{interaction}(1,35)$		5.49	3.19	
p		0.024	0.083	

Data were shown as mean ± standard error of the mean. JZL184 and metyrapone doses were 16 and 30 mg/kg, respectively. The frequency of behaviors was shown for the first 2 min of encounters, i.e., in the period when bites did not occur yet
[a]Significantly different from control (vehicle + vehicle injections) ($p < 0.05$ at least)

Discussion

MAGL blockade by JZL184 strongly suppressed aggressiveness in residents without affecting defensiveness and plasma glucocorticoid responses to aggressive encounters. Behavioral effects were not due to disrupted locomotion or anxiety and, surprisingly, were not mediated by the CB1 receptor. In intruders, effects of JZL184 on biting and offense were similar to those seen in residents, but unlike residents, intruders showed increased defensiveness and glucocorticoid responses when treated with JZL184. In addition, JZL184-treated intruders showed increased agitation at the beginning of aggressive encounters. Behavioral effects differentiating intruders from residents appear to be due to the differential effects of JZL184 on aggression-related glucocorticoid production.

Earlier findings on the relationship between cannabinoid signaling and aggression are contrasting (see "Introduction") but share two features: (i) the observed effects—irrespective to their direction—strongly depend on conditions (e.g., the stressfulness of testing conditions, the status of the individuals, the timing of testing, the duration of treatment, etc.; (Miczek 1978; Rodriguez-Arias et al. 2013; van Ree et al. 1984) and (ii) are typically low in magnitude. JZL184 detaches from other cannabinoid treatments in both respects: biting and offensive behavior were almost totally eliminated in treated subjects, and this was seen in both residents and intruders which are of largely different status in the paradigm employed. It occurs that the effects of JZL184 on aggression are the strongest reported so far with cannabinoid treatments.

Differences did occur between residents and intruders: the latter uniquely showed increased agitation at the beginning of, and increased defensiveness during the aggressive encounter. Our data suggest that these effects are secondary to increased glucocorticoid stress responses elicited by JZL184 in intruders. Agitation—manifested as increased locomotion at the beginning of the encounter—was clearly abolished by the

corticosterone synthesis inhibitor metyrapone, which demonstrates the involvement of glucocorticoids in this behavioral response to JZL184. The situation is more complex with defensiveness which was increased by both JZL184 and metyrapone, but the increase was abolished by the combined treatment. This suggests that JZL184 and corticosterone influence defensiveness by interacting but mutually inhibiting mechanisms. While the nature of this interaction is unclear at present, this finding still shows that the two mechanisms are independent of each other. Taken together, our findings suggest that the effects of JZL184 on biting and offense did not depend on glucocorticoids, because (i) effects on these behaviors were similar in residents and intruders, while corticosterone production was affected in the latter only; (ii) combined treatment with metyrapone and JZL184 did not alter the behavioral response. By contrast, effects on agitation and defensiveness were at least partly due to enhanced glucocorticoid production because (i) these behaviors were not affected by JZL184 in residents where glucocorticoid production was normal, and (ii) combined JZL184 and metyrapone ameliorated these behavioral effects of the former in intruders.

Intriguingly, aggression-related glucocorticoid production was increased by JZL184 neither in the residents studied here nor in the same strain of mice submitted to forced swimming stress as reported earlier (Aliczki et al. 2013). One can assume that the endocrine effects of JZL184 emerge in especially critical situations only, but the phenomenon certainly needs further studies.

JZL184 did not disrupt locomotion and did not affect anxiety, which suggests that the aggression-related effects noticed here were not compromised by non-specific behavioral effects. In one of our earlier studies performed under highly similar conditions, locomotor behavior and anxiety were increased by JZL184 80 and 120 min but not 40 min after treatment (Aliczki et al. 2012) which is in line with the present findings, where testing started 40 min after treatment.

Surprisingly, the CB1 antagonist AM251 did not influence the effects of JZL184 at a dose, which did not affect aggressive behavior per se, but which preserved its biological activity as shown by its effects on plasma corticosterone. In addition, a larger dose of the CB1 antagonist AM251 and JZL184 affected behavior in a highly similar manner, which further

supports the notion that the mechanisms mediating the effects of JZL184 are other than the CB1 receptor. 2-AG was recently shown to be a strong endogenous activator of the transient receptor potential vanilloid type 1 (TRPV1) receptor (Zygmunt et al. 2013), while TRPV1-KO mice dominated their wild types in the tube test of social dominance (You et al., 2012). These findings raise the possibility that 2-AG affects aggressiveness by a mechanism involving the TRPV1 receptor. Naturally, one cannot exclude the involvement of other 2-AG-sensitive targets, e.g., the CB2 receptor which was recently shown to be expressed in the brain or the yet poorly known GPR35 and GPR55 receptors (Onaivi et al. 2006; Zhao and Abood 2013). The lack of observable involvement of the CB1 receptor in the effects of JZL184 may be explained by assuming that (i) off-target effects 2-AG on aggression are considerably stronger than those mediated by the CB1 receptor. This assumption is supported by the relatively week and controversial effects of CB1 ligands on aggressive behavior; (ii) brain circuits activated by aggressive behavior involve neurons where the relative importance of CB1 mechanisms is smaller than that of off-target mechanisms. Noteworthy, the indirect enhancers of cannabinoid signaling (e.g., enzyme inhibitors) augment ongoing cannabinoid signaling in neurons that are activated in particular contexts without inducing non-specific activations in silent neurons, which may confer them functional selectivity that is absent in the case of other cannabinoid treatments (Zanettini et al. 2011) and may render the second assumption formulated above viable.

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Taken together, our findings show that JZL184 is an unusually strong negative modulator of aggressive behavior. The molecular mechanisms mediating its effects as well as its more general effects on social behavior and competitiveness require further studies, which are fully warranted by the strong effects of the compound on aggression.

Acknowledgments

The authors thank the help of Christina Miskolczi with language editing. This research was supported by Hungarian Scientific Research Fund (OTKA) grants no. 112787 and 112907.

Conflict of interest

The authors declare no conflict of interest.

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