Chronic treatment with 13-*cis*-retinoic acid changes aggressive behaviours in the resident-intruder paradigm in rats

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Abstract

Retinoids, vitamin A related compounds, have an established role in the development of the nervous system and are increasingly recognized to play a role in adult brain function. The synthetic retinoid, 13-c*is*-retinoic acid (13-*cis*-RA, Roaccutane) is widely used to treat severe acne but has been linked to an increased risk of neuropsychiatric side effects, including depression. Here we report that chronic administration with 13-*cis*-RA (1 mg/kg i.p. daily, 7-14 days) in adult rats reduced aggression- and increased flight-related behaviours in the resident-intruder paradigm. However, in the forced swim, sucrose consumption and open field tests treatment for up to 6 weeks with 13-*cis*-RA did not modify behaviour in adult or juvenile animals. The behavioural change observed in the resident-intruder paradigm is directly opposite to that observed with chronic antidepressant administration. These findings indicate that when a suitably sensitive behavioural test is employed then chronic administration of 13-*cis*-RA in adult rats induces behavioural changes consistent with a pro-depressant action.

1. Introduction

There is a growing body of evidence that retinoids, vitamin A-related compounds, play a functional role in the adult brain (Lane and Bailey, 2005; Mey and McCaffery, 2004), as well as their established role in the development of the nervous system (Maden, 2007; McCaffery et al., 2003). Retinoid-signalling regulates several behaviours including locomotor behaviours, learning and memory, sleep and depression (Lane and Bailey, 2005; O'Reilly et al., 2008; Tafti and Ghyselinck, 2007). Excessive consumption of vitamin A, hypervitaminosis A, has long been known to cause adverse psychiatric events (O'Donnell, 2004). One synthetic retinoid, 13-c*is*-retinoic acid (13-*cis*-RA, Roaccutane) has been widely used in the clinic to treat severe cystic or recalcitrant acne (Zouboulis and Piquero-Martin, 2003). Controversially, case reports have suggested that in 1-10% of patients 13-*cis*-RA treatment can induce depression, suicidal ideation and even completed suicide (Bremner and McCaffery, 2008; Hull and D'Arcy, 2005). While severe acne can itself have significant psychological and emotional impact (Kellett and Gawkrodger, 1999), one study in humans indicates that there are significant changes in metabolic activity in the orbitofrontal cortex of patients taking 13-*cis*-RA compared with antibiotic treated controls (Bremner *et al.*, 2005). Although these patients showed subtle changes in mood they were not clinically depressed according to the Hamilton rating scale (Bremner et al., 2005).

Animal models are beginning to be employed to elucidate the link between 13-*cis*-RA treatment and depression. In the forced swim test and tail suspension test an increased immobility was observed in juvenile mice treated chronically with 13-*cis*-RA (1mg/kg/day/ip for 6 wks) (O'Reilly et al., 2006). This finding is consistent with an increase in depression-related behaviours. However, behavioural studies in adult Wistar rats have shown that chronic treatment (> 7.5 mg/kg/day/gavage for 3-12 wks) with 13-*cis*-RA does not have a pro-depressive effect in the forced swim test or sucrose anhedonia paradigm (Ferguson et al., 2005; Ferguson et al., 2007). These studies suggest that the behavioural effects of 13-*cis*-RA may be age specific (adult vs juvenile), species specific (rats vs mice), or sensitive to

the different treatment regimes employed, such as the route of administration (oral gavage vs intraperitoneal injection)(Ferguson et al., 2005; Ferguson et al., 2007; O'Reilly et al., 2006).

In order to study the molecular mechanisms underlying a retinoid-induced increase in depression, it is essential to establish an appropriate animal model for investigating these effects. There are a number of well-established, pharmacologically validated paradigms for investigating depression-related behaviours. The resident-intruder paradigm provides an ethologically relevant animal model by which the effects of acute and chronic antidepressant treatment (including electroconvulsive shock) on rodent non-social, social and agonistic (i.e. aggression and flight) behaviours may be examined (Mitchell, 2005; Mitchell et al., 2003; Mitchell and Redfern, 1992). The ability of chronic antidepressant treatment to increase rodent aggressive behaviour is indicative of increased assertive behaviour and mirrors similar changes in human behaviour observed during recovery from depressive illness (Bond, 2005; Dixon et al., 1989; Eisen, 1989; Khan et al., 1989; Willner and Mitchell, 2002). In contrast, acute treatment with antidepressant drugs selectively reduces rodent aggression/assertiveness and may therefore predict increased depressive symptomatology, including suicide ideation, suicide attempts and self-harm (Bond, 2005; Mitchell, 2005; Möller et al., 2008). Thus the resident-intruder paradigm, coupled with ethological analysis, has the ability to predict whether drug treatment may induce behavioural changes consistent with either an antidepressant or a pro-depressant action.

In the present study we have tested whether 13-*cis*-RA can induce an increase in depression-related behaviours in adult and juvenile rats following chronic 13-*cis*-RA or vehicle administration. We have tested behaviour in adult rats in the resident-intruder paradigm and addressed whether, because of their developmental stage, juvenile rats may be particularly vulnerable to the effects of retinoid signalling compared with adult rat behaviour in the forced swim test and sucrose consumption test (Ferguson et al., 2005; Ferguson et al., 2007; O'Reilly et al., 2006). Locomotor behaviours were also examined to

control for any confounding effects of 13-*cis*-RA on locomotion since such behaviours have been reported to be influenced by retinoids (Krezel et al., 1998).

2. Experimental Procedures

2.1 Animals

All experiments were carried out under a project licence held under the Animals (Scientific Procedures) Act 1986 and in accordance with the UK Home Office guidelines. In all experiments, age-matched male Wistar rats were used. In the resident-intruder paradigm adult rats were maintained under reversed daylight conditions (12 h on/ 12 h off, lights on at 19:00 h) for at least 4 wks before the start of the experiment (8 wks old at start of experiment). These rats were group housed (n=4) with food and water provided *ad libitum*. Rats were designated 'resident' (220-340g pre-treatment weight, n= 8 per treatment group) and 'intruder' (230-300g pre-treatment weight, n= 8 per treatment group) and were obtained from different suppliers to ensure that resident animals (Charles River, UK) had never been in contact with intruder animals (University of Bath).

Animals used in the forced swim, sucrose consumption and open field tests were either juvenile (4 wks old at start of treatment, n= 8 per group) or adult (8 wks old, n= 8 per group) rats housed in groups of 4 (Charles River, UK). These animals were maintained under normal daylight conditions (12 h on/ 12 h off, lights on at 07:00 h) and food and water were provided *ad libitum*, except prior to undergoing the sucrose consumption test.

All animals were weighed upon arrival with subsequent measurements taken weekly throughout the course of the experiments. Consistent weight gain was used as an indicator of good general health and level of stress caused by handling or intraperitoneal injections.

2.2 Drug treatment regime

All animals (with the exception of intruder rats which were not treated) received daily intraperitoneal injections at a volume of 1ml/kg body weight. Vehicle control groups received sterile saline solution (0.9% w/v sodium chloride) with dimethyl sulphoxide (DMSO, Eur Ph, ICMD UK Ltd) at a ratio of 1:1 v/v. Drug treated groups received 1mg/kg 13-*cis*-RA (Sigma-

Aldrich, UK) dissolved in 1:1 v/v DMSO:saline. This dose of 13-*cis*-RA is in the range of doses widely used to treat acne in patients (0.5 to 2 mg/kg/day) and achieves plasma levels similar to those in patients (Kerr et al., 1982; O'Reilly et al., 2006). Solutions of 13-*cis*-RA solutions were prepared under red light because of its photosensitivity (O'Reilly et al., 2006). Treatment time was 14 days in total in the resident-intruder paradigm (see section 2.3). In all other protocols animals were treated for a total of 6 wks with behavioural testing occurring after 2 wks and 6 wks of treatment. In all experiments, rats received daily intraperitoneal injections, on alternating sides of the peritoneal midline to reduce irritation, at 16:00-17:00h to avoid any acute effect of the injections on behavioural testing.

2.3 Resident intruder paradigm

The resident-intruder paradigm is a pharmacologically validated model of depression-related behaviours in rats (Mitchell and Redfern, 1992, 2005). In all studies only the resident rats received drug or vehicle, and two groups of resident rats (and associated intruder conspecifics) were studied concurrently. Over the course of 4 wks, a group of 4 resident rats experienced 4 weekly encounters with 4 different intruder rats, such that each resident rat encountered each of 4 intruder rats. On the first occasion that each resident rat had a social encounter with an unfamiliar intruder rat, no treatment was given to provide a baseline behavioural profile (day 0). Following this baseline, resident rats were treated with either vehicle or 13-*cis*-RA daily for 14 days. Social encounters were performed after 7 and 14 days of treatment. The final social encounter was then performed 7 days after the cessation of drug treatment (day 21).

All social encounters were performed between 10:00 h and 16:00 h. Before each test day resident rats were separated from their group cages and housed individually for 3 days. At the start of each social encounter test the home cage containing the resident rat was positioned inside the recording cabinet for 30 min to allow habituation, following which the intruder conspecific was introduced (Mitchell and Redfern, 1992). The ensuing social

encounter was recorded on video tape for 10 min under low-intensity red light (2 lux at the cage floor). At the end of each recording session both resident and intruder rats were returned to their respective group cages.

Analysis of resident rat social behaviour, during video playback, involved scoring the occurrence of each of the various behaviours and postures summarised in Table 1 (Grant, 1963). The scores for each behaviour/posture were grouped according to their motivational category (Table 1) for each resident rat and the total score for each category expressed as a percentage of the total number of behaviours observed for that animal. All analysis was performed blind to the resident rat's treatment group.

2.4 Forced swim test

The forced swim test is a widely used pharmacologically validated behavioural model for assessing antidepressant and pro-depressant activity in rodents (Carlezon et al., 2006; Lucki, 1997; Porsolt et al., 1977). Adult and juvenile rats were treated daily for a total of 6 wks with either vehicle or 13-*cis*-RA (n=8 per treatment group) and were behaviourally tested after 2 and 6 wks of treatment. Animals experienced a 15 min pre-swim, followed the next day with a 6 min test session. For the swim sessions, rats were placed in a glass beaker (height 44cm, diameter 22cm) with water at a height of 34cm and a temperature of 25ºC (± 1 ºC). Behaviour was recorded for the duration of both the pre-swim and swim test sessions using a camcorder (Sony DCR-SR52). On completion of the swim session, rats were removed from the water, dried and returned to the group home cage. The water was replaced between trials. Analysis of behaviour during the 6 min swim test session, and during the first 6 min of the pre-swim test, was conducted blind to treatment and the time spent climbing, swimming or immobile was determined. Climbing was defined by vertical escape behaviour while swimming behaviour was defined by diving and circular paddling around the beaker. Immobility was scored as the minimal activity required to stay afloat.

2.5 Sucrose consumption test

The decreased consumption of palatable sweet solutions has been found to be an indicator of pro-depressant (anhedonic) effects, for example following chronic mild stress, that is reversed by treatment with antidepressants (Muscat et al., 1992a, b; Papp et al., 1991). Sucrose consumption was assessed by measuring the volume of a 1% (w/v) sucrose solution consumed over a period of 1 and 2 h following overnight (16h) food and water deprivation (Muscat et al., 1992a). During the test, rats were housed individually and the total amount of sucrose consumed was recorded, corrected for the body weight of each rat and expressed as g sucrose consumed /kg body weight.

2.6 Open field test

Given that the forced swim test relies on detecting changes in immobility with concomitant changes in swimming/climbing, it is important to demonstrate that drug treatment does not alter locomotor performance. The locomotor activity of vehicle and 13-*cis*-RA treated rats undergoing the forced swim and sucrose consumption tests was assessed in a circular open field divided into 8 segments by equally spaced radii (765 mm diameter x 185 mm high) and further divided with an internal circular perimeter (660 mm diameter). Rats were placed in the centre of the open field (facing towards the centre) and behaviour recorded for 10 min under low light conditions (10 lux) using a camcorder (Sony DCR-SR52). Analysis of behaviour in the open field was performed blind to treatment and both line crossings and vertical rearing behaviour were scored. A line crossing was defined as when all 4 paws were in one particular segment. Vertical rearing behaviour was defined as a lifting of the two front paws off the ground, but not for grooming.

The order of behavioural testing was sucrose consumption test, open field test and forced swim test with at least 16h elapsing between test sessions. Each of these behavioural tests was conducted during the light cycle between 09:00 and 16:00h. Drug or vehicle treatment

continued throughout the duration of behavioural testing. Individual rats were tested in a random order in each behavioural test.

2.7 Statistical analysis of resident-intruder studies

The data from the two groups of 4 resident rats were grouped (i.e. n = 8 for each treatment group) and the mean ± SEM for both the percentage values of each motivational category, and the total number of behaviours/postures observed, were calculated. All data were subjected to square root transformation prior to statistical analysis. 1-Way ANOVA (with 'treatment' as the dependent measure) with repeated measures over the four test sessions was employed to identify significant differences between the categories of behaviour of the drug and vehicle-treated resident rats. Within-treatment comparisons (following identification of time*treatment interactions or main effects of time) were further analysed by *post hoc* 1-way ANOVA tests (with 'time' as the dependent measure) following *a priori* decisions regarding appropriate multiple comparisons. Where appropriate, pre-treatment levels of behaviour (day 0) were compared to the levels of behaviour observed following 7 and 14 days of treatment and 7 days following the cessation of treatment (day 21). In addition, day 14 data (treatment) were compared to day 21 data (7 days post-treatment). Between-treatment comparisons (following identification of time*treatment interactions or main effects of treatment) were further analysed by *post hoc* 1-way ANOVA (with 'treatment' as the dependent measure) between the drug- and vehicle-treated resident rats at each time point for that behavioural category. In all cases, *P* values arising from repeated comparisons ANOVA are quoted following Huynn-Feldt correction.

2.8 Statistical analysis of all other behavioural studies

Two-way ANOVA (with 'treatment' and 'age' as dependent measures) with repeated measures over the two test sessions (wk 2 and wk 6) was performed on data from the forced swim, sucrose consumption and open field tests. Appropriate multiple comparisons were made and analysed by *post hoc* 1-way ANOVA tests. Values were taken to be significant

when $P<0.05$. All values are mean ± SEM unless otherwise stated. Differences in body weight between vehicle control and 13-*cis*-RA treated animals were compared using an unpaired *t*-test.

3. Results

3.1 13-*Cis*-RA treatment had no effect on weight gain

For all rats, body weight was measured weekly and the mean weight gain expressed as a percentage of each rats starting weight. In the resident-intruder experiment, the mean body weight of control and treated rats one wk prior to starting the experiment ("starting weight") was 269 ± 15 g and 269 ± 9 g respectively (n=8 per group). Over the course of the paradigm, there was no significant difference in weight gain during the resident-intruder experiment with weight gain at 21 days being 161 ± 9% and 155 ± 9 % for vehicle and 13-*cis*-RA treated rats, respectively. Likewise, 13-*cis*-RA had no effect on weight gain in rats undergoing the forced swim, sucrose consumption and open field tests. One week prior to starting chronic treatment ("starting weight"), the mean body weight of control and 13-*cis*-RA treated juvenile rats was 89 ± 2 g and 86 ± 2 g, respectively (n=8 per group). For adult vehicle and drug treated rats there was a significant difference in starting weight (290 ± 3 g and 281 ± 3 g, respectively, n=8 per group, $P< 0.05$, *t*-test). Despite this significant difference in starting weight, after 6 wks of treatment there was no significant difference in total mean percentage weight gain between vehicle and treated adult (155 ± 4% vs 155 ± 3%, respectively) or juvenile (447 ± 10% vs 444 ± 9%, respectively) rats. There is evidently a larger weight gain in juvenile rats, both control and treated groups, compared to adult animals corresponding to normal growth rates for Wistar rats (Charles River, UK).

3.2 Effects of 13-*cis*-RA treatment on resident rat behaviour in the resident-intruder paradigm

The results of the detailed analysis of social behaviour during encounters between unfamiliar intruder rats and resident rats treated with vehicle or 13-*cis*-RA are summarised in Table 2. 1-Way ANOVA with repeated measures revealed significant main effects of Treatment on Aggressive [$F(1,14)=54.661$, $P<0.0001$], Flight Submit [$F(1,14)=12.195$, $P=0.0036$] and Flight Escape [$F(1,14)=10.900$, $P=0.0052$] behaviours but not on any of the other categories of behaviour nor Total Behaviour score [all $Fs(1,14)\leq3.533$, $P\geq0.0811$ in all cases].

Furthermore, analysis revealed significant main effects of Time on Exploration [F(3,42)=5.047, *P*=0.0045], Flight Submit [F(3,42)=7.431, *P*=0.0004], Flight Escape [F(3,42)=13.660, *P*<0.0001] and Sexual [F(3,42)=6.267, *P*=0.0013] behaviours but not on any of the other categories of behaviour nor Total Behaviour score [all Fs(3,42)≤2.135, *P*≥0.1101 in all cases]. Finally, significant Treatment*Time interactions were identified for Aggression [F(3,42)=13.178, *P*<0.0001], Flight Submit[F(3,42)=3.279, *P*=0.0301] and Flight Escape [F(3,42)=14.003, *P*<0.0001] behaviours but not on any of the other categories of behaviour nor Total Behaviour score [all Fs(1,14)≤2.310, *P*≥0.0901 in all cases].

Within-Treatment comparisons revealed that resident rats treated with vehicle exhibited increased aggressive behaviour on Day 7 of treatment compared to the level of Aggression observed prior to treatment (i.e. Day 0; adjusted *P*<0.05). No other significant differences in Aggressive, Exploration, Flight Submit, Flight Escape or Sexual behaviours were observed in these control rats throughout the experiment (adjusted *P*s>0.05 in all cases). Within-Treatment comparisons also revealed that at days 7 and 14 of treatment, resident rats treated with 13-*cis*-RA exhibited significantly reduced aggressive behaviour (adjusted Ps<0.05 in both cases, Fig. 1A) concomitant with increased Flight Submit and Flight Escape behaviour (adjusted Ps<0.01 in all cases, Fig. 1B,C). By 7 days following the cessation of 13-*cis*-RA treatment the levels of all three behavioural categories had generally returned to baseline (Day 0 compared to post-dose day 7; adjusted *P*>0.05 in all cases, Fig. 1). Consequently, at post-treatment day 7 resident rats in the 13-*cis*-RA group exhibited increased Aggression with reduced Flight Escape behaviour compared to the respective levels observed at day 14 of treatment (*P*<0.01 in both cases). Further *post hoc* analysis revealed that 13-*cis*-RA treated resident rats exhibited reduced Exploration at day 7 (but not day 14) of treatment and 7 days following treatment (*P*<0.05 in both cases). No significant changes in sexual behaviour were observed throughout the course of the experiment in 13-*cis*-RA treated resident rats.

Between-Treatment comparisons showed that, at days 7 and 14 of treatment, 13-*cis*-RA treated resident rats exhibited significantly reduced Aggressive behaviour concomitant with significantly elevated Flight Submit and Flight Escape behaviour compared to the level of behaviour observed in vehicle treated resident rats ($P<0.01$ in all cases, Fig. 1). By 7 days following the cessation of treatment, however, the observed differences in the level of these behavioural categories between the vehicle and drug treated groups of resident rats had disappeared ($P>0.05$ in all cases).

3.3 Effects of 13-*cis*-RA treatment on behaviour in the forced swim test

The total amount of time spent in swimming, climbing or immobility behaviours during a 6 min swim test session is shown in Figure 2. There was no significant difference in the behaviour of adult or juvenile rats treated with 13-*cis*-RA compared to vehicle treated control animals. Two-way ANOVA was used to analyze the total time spent immobile and there was no significant main effect of Age [$F(1,7) = 0.381$, $P=0.557$] or any interaction between Age and Treatment [$F(3,21) = 1.281$, $P=0.328$]. A significant main effect of Treatment with repeated measures was revealed [$F(3,21)=14.672$, $P<0.001$], although *post hoc* analysis revealed that there was no significant effect of 13-*cis*-RA treatment, rather that repeated testing at 6wk compared to 2 wk significantly increased the time spent immobile in both adult and juvenile rats ($P<0.05$ in all cases). In adult rats (Fig 2A,B) tested after treatment for 2 wks, the mean time spent immobile for vehicle treated animals was 269 ± 14s and with 13-*cis*-RA 292 ± 8s, n= 8 per group. After 6 wks treatment, the mean time spent immobile for vehicle treated animals increased to 315 ± 7s and with 13-*cis*-RA to 306 ± 11s, n= 8 per group. Similarly, chronic administration of 13-*cis*-RA in juvenile rats (Fig 2 D,E) had no significant effect on behaviours in the forced swim test compared with vehicle treated control rats, although there was a significant effect of repeated testing after 2 wks treatment (mean time spent immobile for vehicle: 272 ± 12s and 13-*cis*-RA 272 ± 13s, n=8) and after 6 wks (mean time spent immobile for vehicle: 326 ± 7s and 13-*cis*-RA 332 ± 4s, n=8).

The forced swim test was preceded by a 15 min pre-swim 24 h prior to the 6 min swim test session. Analysis of the time spent immobile during the pre-swim session showed no significant main effect of Treatment or any interaction between Age and Treatment [Fs(3,21) ≤ 1.152, $P \geq 0.287$]. However there was a significant main effect of Age [F(1,7)=10.239, P= 0.018] such that juvenile animals exhibited behaviours that were different to those of the adult on the first performance of the task (2 wks), regardless of drug treatment (Fig 2 C,F). *Post-hoc* analysis revealed that during the first 6 min of the initial pre-swim session juvenile animals spent significantly less time immobile than adult animals (mean time spent immobile juvenile vs adult vehicle control: 143.5 ± 27.0 vs 221.1 ± 11.5 and juvenile vs adult 13-*cis*-RA treated: 161.1 ± 13.7 vs 209.5 ± 17.5 n = 8 per group, $P < 0.04$).

3.4 Effects of 13-*cis*-RA treatment on sucrose consumption

Sucrose consumption, following overnight food and water deprivation, was measured during a 2h test session. Two-way ANOVA was used to analyze the total sucrose consumption after 1h (Fig 3A) and after 2h (Fig 3B). For both measures, there was a significant main effect of Age [Fs (1,7) > 14.590, P <0.007] although there was no significant main effect of Treatment or any interaction between Age and Treatment [Fs (3,21) < 3.357, P> 0.130]. *Post-hoc* analysis using one-way ANOVA revealed that when animals were tested after 2 wks of treatment juvenile animals consumed more sucrose solution than adult animals (mean sucrose consumption (2h) for vehicle treated adult: 57.4 ± 2.8g and juvenile: 91.5 ± 7.6 g, P <0.001, n= 8), regardless of treatment (mean sucrose consumption (2h) for 13-*cis*-RA treated adult: 64.1 ± 5.2 g and juvenile: 92.2 ± 8.8 g, P <0.01, n= 8). While this trend was maintained after 6 wks of treatment there was no significant difference between juvenile and adult animals at this time point.

3.5 Effects of 13-*cis*-RA treatment on locomotor behaviour in the open field

To determine whether any behavioural effects of 13-*cis*-RA treatment could be attributed to a change in locomotor activity, rats were also tested in the open field. Two-way ANOVA was

used to analyze the total number of line crossings (Fig 4A) and the number of vertical rears (Fig 4B) for each group of animals. For both measures, there was a significant main effect of Age [Fs(1,7) ≥ 14.920 $P \leq 0.006$] and a significant main effect of Treatment with repeated measures [Fs(3,21) ≥ 11.460 $P \leq 0.015$] but no significant interaction between Age and Treatment [Fs (3,21) ≤ 4.287, $P \geq 0.120$]. *Post-hoc* analysis using one-way ANOVA revealed that, regardless of treatment, the mean total number of line crossings was significantly higher in juvenile treatment groups than in adult animals after both 2 wks (the mean number of line crossings for vehicle treated adult and juvenile treated rats was 108.9 ± 7.3 and 136.9 ± 7.4, respectively and for 13-*cis*-RA treated adult and juvenile rats was 106.9 ± 8.7 and 135.0 ± 8.5, respectively, $P < 0.002$, n= 8 per group, Fig 4A) and 6 wks of treatment (the mean number of line crossings for vehicle treated adult and juvenile treated rats was 59.6 ± 4.9 and 88.1 ± 5.4, respectively and for 13-*cis*-RA treated adult and juvenile rats was 71.5 ± 6.0 and 94.1 ± 7.3, respectively, $P < 0.03$, n= 8 per group, Fig 4A). Furthermore, there was a significant effect of repeated testing such that in all groups, regardless of treatment or age, locomotor activity was reduced when tested after 6 wks treatment to 60-70% of the activity levels recorded after 2 wks treatment ($P < 0.005$, paired *t*-test). This latter observation presumably reflects the decreased novelty of the open field when animals are exposed to the arena for a second test session (for example, Karrenbauer et al., 2009).

Similarly, p*ost-hoc* analysis of vertical rearing behaviour in the open field (Fig 4B) revealed that, in general, juvenile animals made significantly more vertical rears than adult animals after both 2 wks (the mean number of vertical rears for 13-*cis*-RA treated adult and juvenile rats was 47.3 ± 5.0 and 64.0 ± 3.7, respectively, $P = 0.04$, n= 8 per group) and 6 wks of treatment (the mean number of vertical rears for vehicle treated adult and juvenile treated rats was 34.5 ± 5.5 and 49.4 ± 3.9, respectively, $P=0.02$ and for 13-*cis*-RA treated adult and juvenile rats was 29.5 ± 3.2 and 53.3 ± 3.8, respectively, $P < 0.001$, n= 8 per group). In addition, there was a significant effect of repeated testing but only in adult animals, such that exposure to the open field arena after 6 wks of treatment significantly reduced vertical

rearing behaviour compared with 2 wks in both vehicle and 13-*cis*-RA treatment groups ($P < 0.005$, paired *t*-test).

Discussion

We have shown that 13-*cis*-RA, when chronically administered to rats, produces a behavioural effect in the resident-intruder paradigm directly opposite to that of antidepressant treatment (Mitchell, 2005). Specifically, after 7 or 14 days treatment with 13-*cis*-RA a significant reduction in aggressive behaviour towards intruder rats was observed, with a concomitant increase in submissive behaviours including flight-submit and flight-escape behaviour. Interestingly, both these changes in aggressive and flight behaviour were reversed to pre-treatment levels following one week cessation of drug treatment. These observations strongly indicate that the behavioural phenotype seen in the resident-intruder paradigm was related to the administration of 13-*cis*-RA. However, in the forced swim, sucrose consumption and open field tests there was no significant effect of 13-*cis*-RA treatment, after either 2 wks or 6 wks, in both adult and juvenile rats. This suggests that these behavioural tests are less sensitive measures of depression-related behaviour than the resident-intruder paradigm. One striking result of this study is the difference in behaviour between juvenile and adult rats, regardless of drug or vehicle treatment. Juvenile rats exhibited decreased time spent immobile in the forced swim test (pre-swim), increased sucrose consumption and increased locomotor and exploratory behaviour in the open field, compared with adult rats.

In this study we were only able to detect the behavioural effects of 13-*cis*-RA in the resident-intruder paradigm, but not in the forced swim test or sucrose consumption test. The forced swim test is pharmacologically validated for detecting the effects of numerous antidepressant drugs when administered acutely in both mice and rats (Carlezon et al., 2006; Lucki, 1997; Porsolt et al., 1977) . It has also been used to detect pro-depressant effects in a number of different situations (Alcaro et al., 2002; Carlezon et al., 2006; Carlezon et al., 2002; Dalla et al., 2004; Singewald et al., 2004). In juvenile mice 13-*cis*-RA increases the time spent immobile in the forced swim test (O'Reilly et al., 2006). However, in adult rats behaviour in the forced swim test, when both pre-swim and swim test data were compared, is not altered

by treating rats with 7.5mg/kg and 22.5mg/kg 13-*cis*-RA (via oral gavage, 48 days)(Ferguson et al., 2005; Ferguson et al., 2007). One possibility for this apparent behavioural difference in mice, but not in rats, is that there is a species difference in the response to 13-*cis*-RA. This is supported by the observation that learning and memory are impaired following chronic 13-*cis*-RA treatment in mice (Crandall et al., 2004) but not in rats (Ferguson and Berry, 2007). Alternatively, it may be that the forced swim test is not as sensitive a measure of the pro-depressant effects of 13-*cis*-RA treatment in rats, as it is in mice.

Increased sucrose consumption has been used as a measure of the anhedonic effects of exposure to chronic mild stress in rats, which can be reversed by chronic antidepressant treatment (Muscat et al., 1992a, b; Papp et al., 1991). Previous reports have suggested that 13-*cis*-RA treatment in adult rats has no effect on sucrose consumption (Ferguson et al., 2005; Ferguson et al., 2007). Here we confirm this finding and further show that juvenile rats are not more vulnerable to the effects of 13-*cis*-RA, at least as measured by behaviour in the sucrose consumption test and forced swim test. Since we were able to demonstrate a positive drug-induced effect in the resident-intruder paradigm, this indicates that this may be a more sensitive test of the pro-depressant actions of 13-*cis*-RA in rats than either the forced swim test or the sucrose consumption test.

In this study, irrespective of drug treatment, we found that the behaviour of the juvenile rats in the forced swim test (pre-swim), sucrose consumption and open field tests was markedly different to that of adult animals. Human adolescence is an important period of active neuronal development that represents a particularly vulnerable developmental stage during which external insults or emotional stress are likely to have important consequences for mental health (Andersen and Teicher, 2008; Paus et al., 2008; Spear, 2000). Modelling adolescence, a uniquely human experience, in animal models is contentious but in rodents juvenile animals 4-6wks of age have been suggested to represent a post-weaning period of sexual maturation that is associated with rapid growth and reproduces some of the

neurodevelopmental effects observed in human adolescence (Spear, 2000). Others have reported similar findings to us that juvenile animals show less immobility in the forced swim test and in the open field compared with adult animals (Hansen-Trench and Barron, 2005). In previous studies juvenile mice were shown to be susceptible to the pro-depressant behavioural effects of chronic treatment with 13-*cis*-RA in the forced swim and tail suspension tests (O'Reilly et al., 2006). Therefore we hypothesized that juvenile animals may be particularly vulnerable to the effects of 13-*cis*-RA because of the extensive neuronal maturation that continues during this post-natal period. So in this study, we examined behaviour in both juvenile and adult rats. Unfortunately we were not able to test the behaviours of juvenile animals in the resident-intruder paradigm tests because juvenile animals do not engage in the same range of aggression behaviours that adults rats do. Play fighting in rats starts at about 18 days of age (pre-weaning), peaks at about 30-36 days and subsides thereafter (Panksepp, 1981; Pellis and Pellis, 1987; Pellis and McKenna, 1992). Since the other behavioural tests we used were not sensitive to the pro-depressant actions of this drug, we cannot definitively say whether juvenile animals are more vulnerable to the effects of 13-*cis*-RA than adult animals.

Treatment of acne with 13-*cis*-RA has been controversially linked to an increase in depression, suicidal ideation and completed suicide in patients (Bremner and McCaffery, 2008; Hull and D'Arcy, 2005; Jacobs et al., 2001; Jick et al., 2000). The evidence for such an association is based on a number of case reports or retrospective analyses of larger data sets. In many of these studies, there is no clear assessment of mood or objective measure of depression before starting and after completing drug treatment so it is difficult to identify a causal link. Additionally, it is difficult to account for the psychosocial impact of severe acne itself on patient mood (Kellett and Gawkrodger, 1999). Since changes in depression associated with 13-*cis*-RA use are not well-defined, it is probable that underlying changes in aggression are also not recognized or under-reported. However, a few case reports have noted changes in aggression with 13-*cis*-RA treatment (Byrne et al., 1998; Duke and

Guenther, 1993). In the UK, in a review of adverse events associated with prescribing of 13-*cis*-RA to adolescents (13-18 yrs), the MHRA analysed 2114 spontaneous adverse event reports between 2001-2004 (MHRA and Agency, 2005). They reported that there were 210 reports of serious events (depression, completed suicide, suicidal ideation, suicidal attempt) and 118 reports of non-serious events (depression, aggression, mood swings). So both changes in depression and, to a lesser extent, aggression have been reported to be associated with 13-*cis*-RA use in humans.

Our findings that chronic treatment with 13-*cis*-RA alters aggressive behaviours in the resident-intruder paradigm not only reflect changes in aggression per se but are consistent with a pro-depressant action of this drug. The resident-intruder paradigm is a pharmacologically validated model that is sensitive to the effects of acute and chronic antidepressant treatment (Mitchell, 2005). A number of antidepressant agents including selective serotonin reuptake inhibitors, selective serotonin noradrenaline reuptake inhibitors and monoamine oxidase inhibitors, as well as non-drug treatments such as electroconvulsive shock, are known to specifically increase aggressive behaviour and reduce flight behaviour in resident rats (Mitchell, 2005). This reflects observations in patients that antidepressant treatment increases aggressive behaviour in submissive depressed patients (Bond, 2005; Dixon et al., 1989). Here, we have shown that 13-*cis*-RA decreases aggression behaviours in resident rats which is the opposite effect of antidepressant treatment. This suggests a pro-depressant action of retinoid treatment. Interestingly, the effects of 13-*cis*-RA on behaviour in the resident-intruder paradigm are reversed 7 days after cessation of treatment. While this observation supports the notion that any pro-depressive effects of 13-*cis*-RA treatment in humans may be readily reversible on ceasing treatment, our data were obtained with a relatively short treatment period. However, there are some reports in human patients taking 13-*cis*-RA that cessation of treatment led to an improvement in depression symptoms (Barak et al., 2005; Ng and Schweitzer, 2003). In one study, it was reported that the median recovery time following 13-*cis*-RA dechallenge was 4.5 days (Wysowski et al., 2001). This is

rapid in comparison to the reports for time to onset of depressive symptoms in patients which has been reported to be anything from a few days to several months reviewed by (Hull and D'Arcy, 2005).

What are the mechanisms by which 13-*cis*-RA might induce a change in aggression behaviours in adult rats? Numerous studies have implicated the serotonin system in controlling aggressive behaviour in both animal models and clinical studies where impulsivity, aggression and self-harm attempts (self-directed aggression) are linked with reduced serotoninergic function (Popova, 2008; Ryding et al., 2008; Siever, 2008). The pro-depressant actions of retinoids have been suggested to be mediated via changes in both serotoninergic and dopaminergic neurotransmitter systems, as well as effects on hippocampal adult neurogenesis (Bremner and McCaffery, 2008; O'Reilly et al., 2008). The rapid onset (7 days) and reversal (7 days) of the effects of 13-*cis*-RA in the resident-intruder paradigm indicate that changes in neurogenesis are unlikely to account for the behavioural changes. Our findings of changes in aggression behaviours in the resident-intruder paradigm indicate that serotoninergic mechanisms may be disrupted by chronic 13-*cis*-RA treatment. Retinoids act primarily via gene regulatory mechanisms and a number of neuronal genes have been identified as being amenable to retinoid regulation (Lane and Bailey, 2005). The expression of the 5-hydroxytryptamine 1A receptor (5-HT1AR) and the 5-HT reuptake transporter (5-HTT) have been reported to be increased following 13-*cis*-RA treatment both *in vitro* and *in vivo* (O'Reilly et al., 2007; Trent and Bailey, 2008). Interestingly the 5-HT1AR, alongside other 5-HT receptors, has been identified as a potential therapeutic target for the treatment of aggression (de Boer and Koolhaas, 2005; Mitchell, 2005). Specific 5-HT1AR agonists, mixed 5-HT1A/1BR agonists and selective 5-HT1BR agonists have anti-aggressive effects in rodents (de Boer et al., 2000; Fish et al., 1999; Olivier et al., 1995). Given the role of both the 5-HT1AR and 5-HTT in regulating 5-HT neuronal firing and synaptic 5-HT levels, then regulation of both of these proteins by 13-*cis*-RA may provide a mechanism by which

serotonin levels are disrupted and changes in aggression and depression behaviours occur.

Figure 1. 13-*Cis*-RA treatment altered aggression behaviours in the resident intruder paradigm. Drug treated (closed bars) or vehicle control (open bars) adult resident rats encountered intruder rats on each of four weekly occasions (Day 0 = pre-treatment, Day 7 = 7 days of treatment, Day 14= 14 days of treatment, Day 21 = post-treatment) (A) Ethological analysis revealed that drug treated resident rats displayed a reduced number of aggression behaviours (as a percentage of all behaviours) towards the intruder rat compared with vehicle treated control resident rats. Increases in flight-escape behaviours (B) and in flight-submit behaviours (C) are also evident. Data are mean ± sem of n=8 resident rats per treatment group. In all graphs significant effects of 13-*cis*-RA treatment compared with vehicle are indicated (*= P< 0.05). Within treatment groups, significant differences in behaviours compared with pre-treatment baseline values (Day 0) are also indicated (+ = P<0.05).

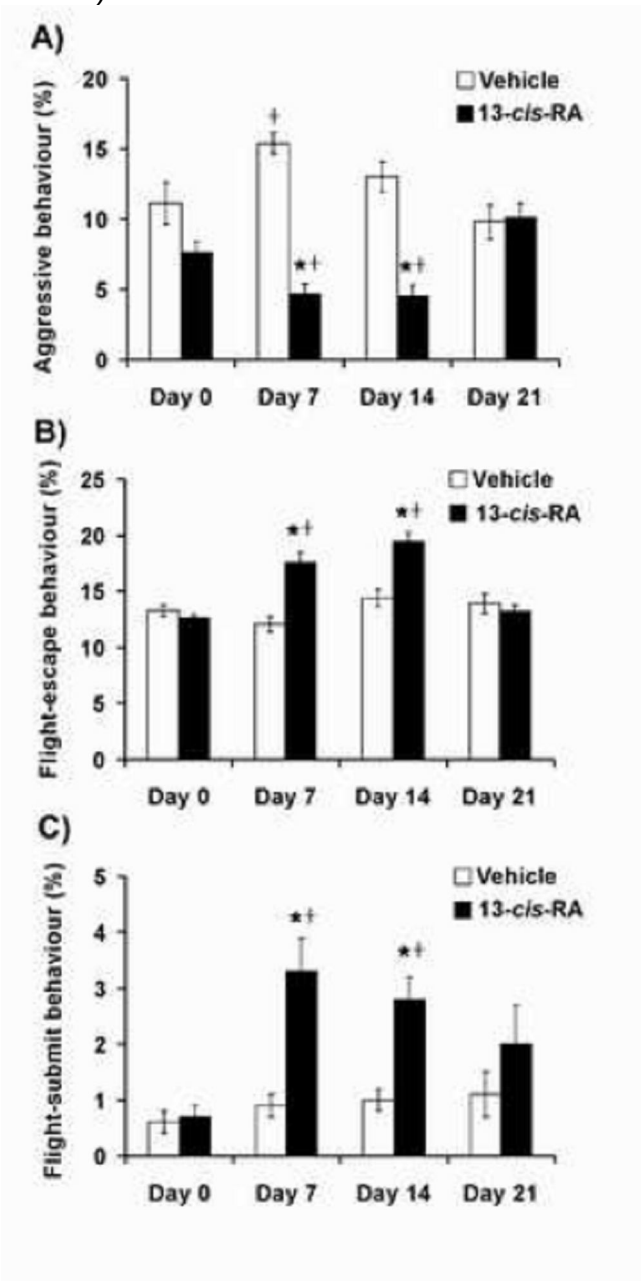


Figure 2. 13-*Cis*-RA had no effect on adult and juvenile rat behaviour in the forced swim test. Adult rat behaviour after 2 wks (A) and 6 wks (B) of vehicle control (open bars) or drug (closed bars) treatment. Juvenile rat behaviour after 2 wks (D) and 6 wks (E) of vehicle control (open bars) or drug (closed bars) treatment. Analysis of the total time spent immobile during the first 6 min of the pre-swim session, compared with swim test session, is also shown for both adult (C) and juvenile (F) rats. Data shown are mean ± sem of n=8 rats per treatment group.

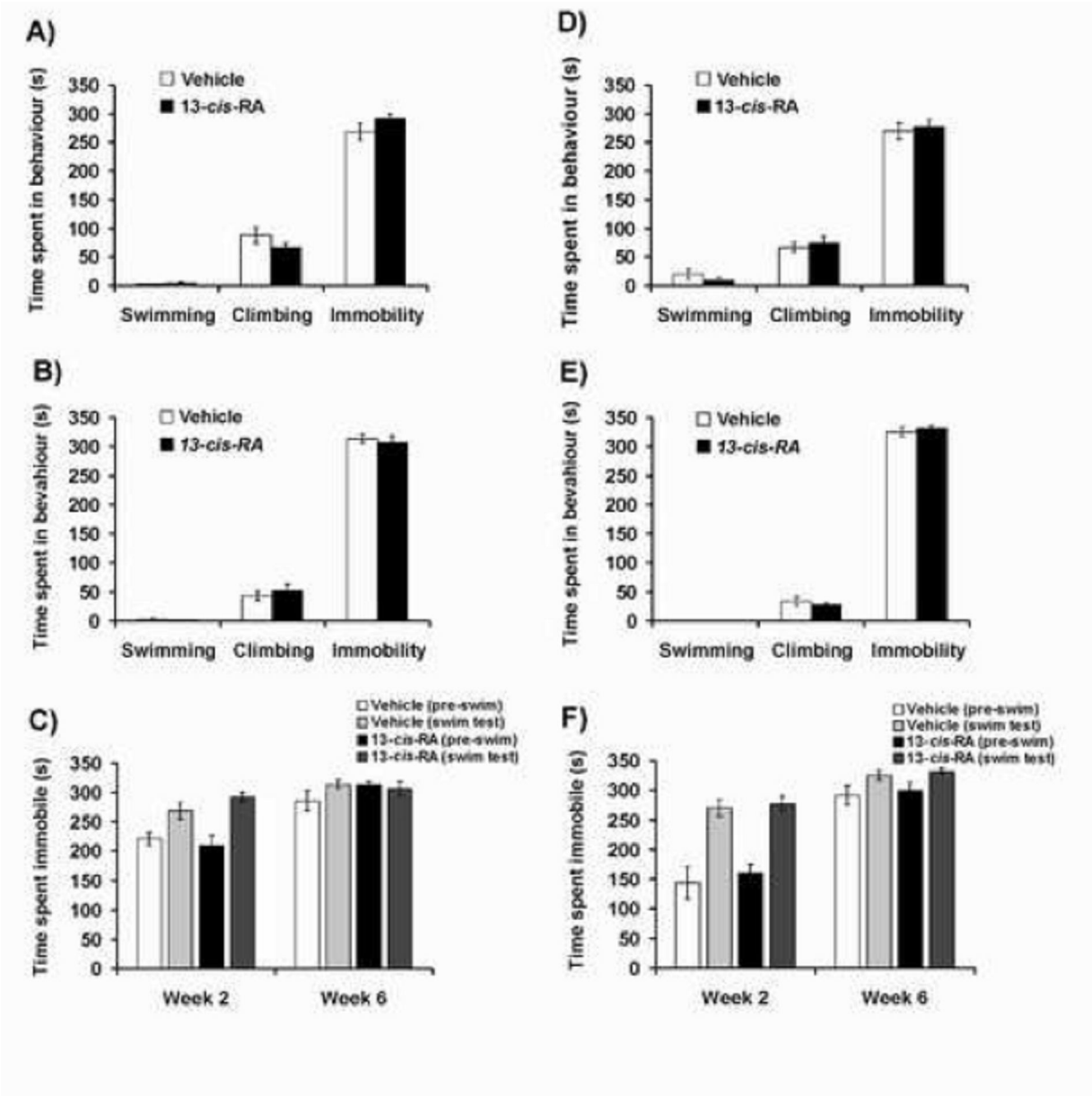


Figure 3. 13-*Cis*-RA treatment does not affect sucrose consumption in adult and juvenile rats. The mean total sucrose consumption (corrected for body weight) during a 1h (A) and 2h (B) test session is shown. In both adult and juvenile rats after 2wks or 6wks of treatment there was no significant effect of drug (closed bars) compared with vehicle controls (open bars). Data shown are mean ± sem of n=8 rats per treatment group.

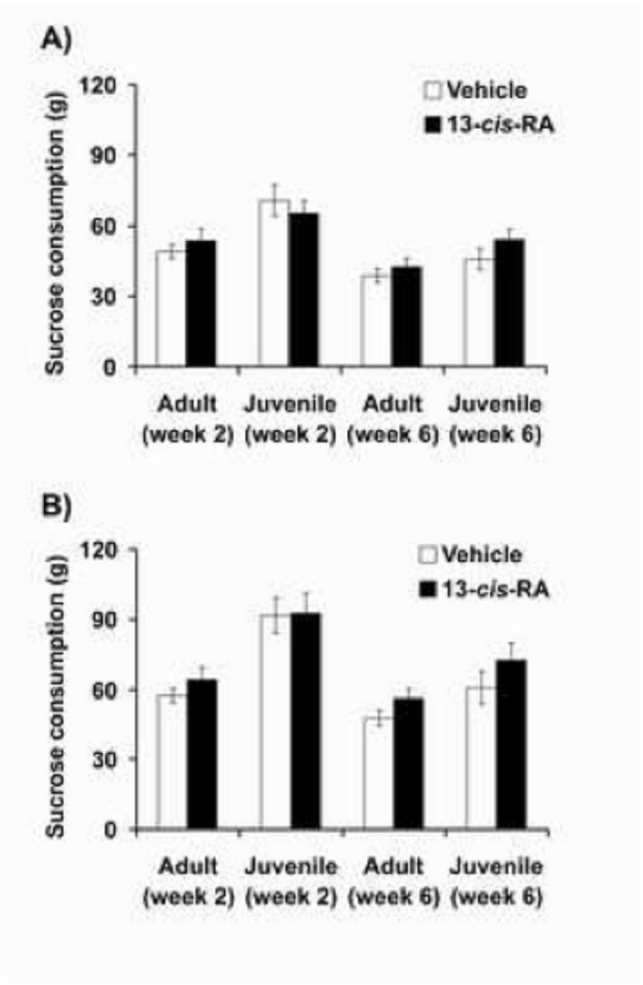
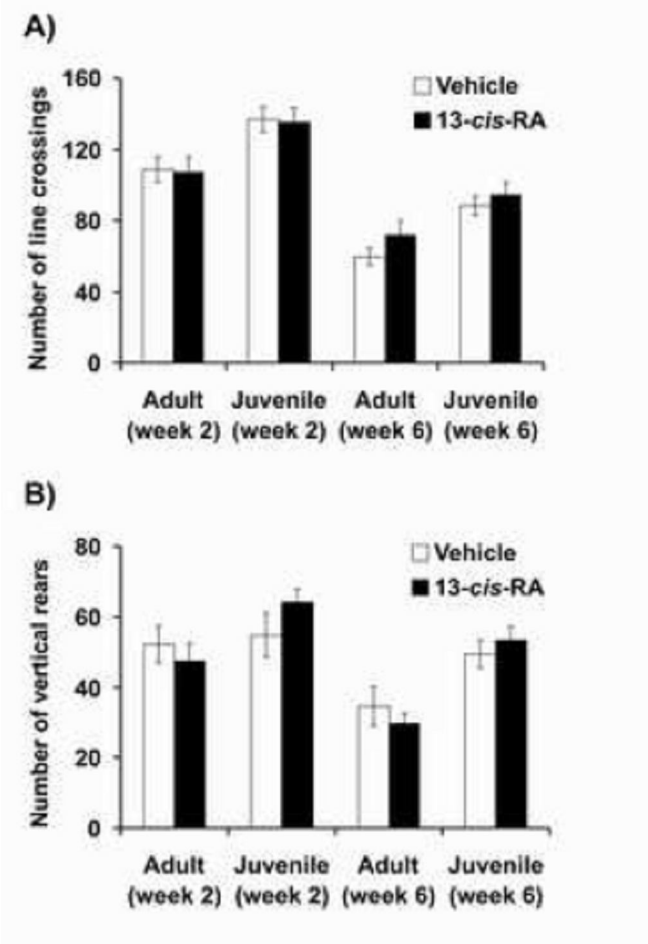


Figure 4. 13-*Cis*-RA does not affect locomotor activity of adult and juvenile rats in the open field test. Locomotor activity is assessed by the total number of line crossings (A) and the number of vertical rearings (B) in the open field during a 10 min test session. In both adult and juvenile rats after 2wks or 6wks of treatment there was no significant effect of drug (closed bars) compared with vehicle controls (open bars). Data shown are mean ± sem of n=8 rats per treatment group.



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Motivational category	Behavioural element
EXPLORATION	Locomotion, rearing
INVESTIGATION	Approach, follow, stretched attention, to-fro, walk round/circle/side, nose and investigate, sniff genitalia, tail rattle
SEXUAL	Mount*, attempt mount, lick penis
AGGRESSION	Aggressive groom, aggressive posture, attack, bite, offensive sideways, offensive upright, pull, threat/thrust
FLIGHT-SUBMIT	Defensive sideways, defensive upright, submit
FLIGHT-ESCAPE	Attend, crouch, elevated crouch, flag and evade, retreat, under food hopper
MAINTENANCE	Digging, drinking*, eating*, licking, scratching, head/body shake, washing

Table 1: Ethogram summarizing behaviours expressed by rats during social encounters (adapted from Grant, 1963).

*These behaviours were not recorded in the resident-intruder studies described here since full mating is not possible between male cohorts, while food and water were not available during each social encounter.

Behaviour	Vehicle-treated resident rats			
	Day 0 (Pre-dose)	Day 7	Day 14	Day 21 (7 days Post-dose)
Exploration	23.4±1.9	19.0±1.6	22.5±1.4	24.1±2.1
Maintenance	0.9±0.2	0.8±0.2	1.1±0.3	2.1±0.8
Investigation	50.0±0.9	50.6±2.0	47.7±1.0	48.7±1.8
Sexual	0.7±0.4	1.2±0.5	0.2±0.1	0.3±0.1
Aggression	11.1±1.5	15.4±0.8 *	13.0±1.1	9.8±1.2
Flight Submit	0.6±0.2	0.9±0.2	1.0±0.2	1.1±0.4
Flight Escape	13.3±0.5	12.1±0.7	14.4±0.8	13.9±0.9
Total Behaviours	1677.1±53.5	1706.6±80.5	1631.3±41.8	1500.9±74.5

Behaviour	13-*cis*-RA-treated resident rats			
	Day 0 (Pre-dose)	Day 7	Day 14	Day 21 (7 days Post-dose)
Exploration	29.6±2.1	23.1±1.4 *	25.5±1.1	22.9±1.5 *
Maintenance	0.8±0.3	0.8±0.2	1.5±0.4	0.9±0.4
Investigation	48.2±1.4	48.8±1.7	45.6±1.3	50.3±1.6
Sexual	0.6±0.4	1.7±0.5	0.7±0.3	0.5±0.2
Aggression	7.6±0.8	4.7±0.7 * ¥¥	4.5±0.8 * ¥¥	10.1±1.0 ††
Flight Submit	0.7±0.2	3.3±0.6 ** ¥¥	2.8±0.4 ** ¥¥	2.0±0.7
Flight Escape	12.6±0.3	17.6±0.9 ** ¥¥	19.4±0.9 ** ¥¥	13.2±0.6 ††
Total Behaviours	1527.1±91.3	1610.3±65.8	1589.8±48.5	1585.5±44.7

Table 2: Analysis of resident-rat behaviour on each of four encounters (Day 0, 7, 14, 21) in the resident-intruder paradigm. Values indicate Mean ± S.E.M percentage of Total Behaviour score, except Total Behaviour score = Mean ± S.E.M absolute observations. * $P<0.05$, ** $P<0.01$ c.f. Day 0 (Pre-dose). †† $P<0.01$ c.f. Day 14. ¥¥ P<0.01 c.f. vehicle-treated resident rats.

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