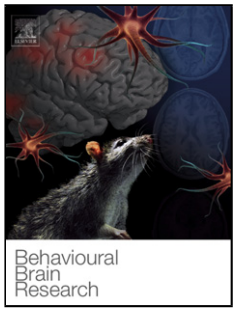


Title: Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive- like behaviours in the rat

Authors: Philip J. Ryan, Elena Büchler, Fazel Shabanpoor, Mohammad Ahkter Hossain, John D. Wade, Andrew J. Lawrence, Andrew L. Gundlach

PII: S0166-4328(13)00053-3
DOI: doi:10.1016/j.bbr.2013.01.034
Reference: BBR 8114

To appear in: *Behavioural Brain Research*

Received date: 21-12-2012
Revised date: 22-1-2013
Accepted date: 27-1-2013

Please cite this article as: Ryan PJ, Büchler E, Shabanpoor F, Hossain MA, Wade JD, Lawrence AJ, Gundlach AL, Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive- like behaviours in the rat, *Behavioural Brain Research* (2010), doi:10.1016/j.bbr.2013.01.034

Behavioural Brain Research BBR-D-12-01225R1, 31 January 2013

Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive- like behaviours in the rat

Philip J. Ryan[a,d], Elena Büchler[a]*, Fazel Shabanpoor[a,c]**, Mohammad Ahkter Hossain[a,b,c], John D. Wade[a,b,c], Andrew J. Lawrence[a,b,d], Andrew L. Gundlach[a,b,d]†

[a]The Florey Institute of Neuroscience and Mental Health, and [b]Florey Department of Neuroscience and Mental Health, [c]School of Chemistry, [d]Department of Anatomy and Neuroscience, The University of Melbourne, Victoria, Australia

*Current address: Department of Psychopharmacology, Central Institute of Mental Health, Mannheim, Germany

**Current address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK

Running title: Central RXFP3 activation alters affective behaviour in rats

† **Correspondence**: A/Pr Andrew L. Gundlach
The Florey Institute of Neuroscience and Mental Health
The University of Melbourne, Victoria 3010, Australia
Phone: +61-3-9035-6507
Fax: +61-3-9035-3201
E-mail: andrewlg@unimelb.edu.au

Abstract

Relaxin-3 is a recently discovered neuropeptide and the results of earlier anatomical and pharmacological studies suggest it plays a physiological role in modulating functions such as arousal, learning and memory, food intake and neuroendocrine homeostasis. Relaxin-3 is also postulated to modulate affective behaviour, based on high densities of the relaxin-3 G-protein coupled receptor (RXFP3) in brain areas involved in stress and mood/anxiety, including the central amygdala, bed nucleus of the stria terminalis and hypothalamic paraventricular nucleus (PVN); and strong activation of relaxin-3 neurons by stressors, via activation of corticotropin-releasing factor receptor-1 (CRF_1). This study assessed the effect of central administration of a newly developed RXFP3-selective agonist, on anxiety- and depressive-like behaviour in rats. Adult, male Sprague-Dawley rats administered 5 μg [R3A(11-24,C15→A)B] (referred to as *RXFP3-A2*), intracerebroventricularly, demonstrated decreased anxiety-like behaviour in the light-dark box and elevated plus maze, but not in the open field. Notably, in the repeat forced swim test, central RXFP3-A2 administration decreased immobility in rats that had been subjected to the 'stress' of former exposure to the anxiety tests, but not in experimentally naïve rats. These data implicate relaxin-3/RXFP3 signalling in the modulation of effects of acute (anxiety) and cumulative (depression) neurogenic stressors on behaviour; and suggest a potential for RXFP3 agonists as anxiolytic and anti-depressant agents. In addition, our results demonstrate that exposure of adult Sprague-Dawley rats to tests of anxiety-like behaviour (~10-14 days prior) can significantly increase immobility time in the repeat forced swim test.

Highlights

- Icv RXFP3-A2 decreased anxiety-like behaviour in adult male Sprague-Dawley rats
- Testing in anxiety-like tests 14 days earlier increased depressive-like behaviour
- Icv RXFP3-A2 decreased depressive-like behaviour in 'pre-tested' rats

Keywords

Anxiety; Corticotropin-releasing factor; Depression; Relaxin-3; Stress

Abbreviations used in text

aCSF, artificial cerebrospinal fluid; ACTH, adrenocorticotropic hormone; BNST, bed nucleus of stria terminalis; CRF, corticotropin-releasing factor; CRF_1, corticotropin-releasing factor-1 receptor; EPM, elevated plus maze; FST, forced swim test; GABA, γ-aminobutyric acid; HPA, hypothalamus-pituitary-adrenal (axis); icv, intracerebroventricular(ly); i.p., intraperitoneal; LDB, light-dark box; LOF, large open field; mRNA, messenger ribonucleic acid; PVN, hypothalamic paraventricular nucleus; R3/I5, relaxin-3/insulin-like peptide 5 chimeric peptide (a RXFP3 selective agonist); RXFP1/3, relaxin-family peptide receptor 1/3; RXFP3-A2, RXFP3-selective relaxin-3 analogue 2 ([R3A(11-24,C15→A)B])

1. Introduction

Anxiety and depression are mental health conditions that are widespread in the community, with a lifetime prevalence estimated at 29% for anxiety disorders, and 21% for mood disorders (including depression) in the USA [1]. Depressive disorders are currently the leading cause of 'years-lost-to-disability' worldwide [2]. For many patients, current anti-anxiety and anti-depressant medications are (or become) ineffective, and even if helpful, they reduce symptoms without eliciting recovery [3]. Therefore, more effective, targeted therapies are required [4].

Corticotropin-releasing factor (CRF) and its major endogenous receptor, corticotropin-releasing factor-1 receptor (CRF_1), are critical mediators in the physiological response to stressors and in the pathophysiology of anxiety and depression [5, 6]. A recently discovered neuropeptide, relaxin-3 [7, 8], has been postulated to modulate anxiety-like and depressive-like behaviour, based on the strong expression of CRF_1 by relaxin-3 neurons in the nucleus incertus [8, 9], a nucleus located in the midline periventricular grey of the hindbrain [10-12], and the responsiveness of these neurons to CRF and stressors [13]. Intracerebroventricular (icv) CRF and psychogenic stressors similarly activate relaxin-3 expressing neurons, as reflected by neuronal activation of c-*Fos* [9], and stressors such as repeat forced swim have been shown to increase relaxin-3 mRNA expression in the nucleus incertus, an effect blunted by pre-treatment with the CRF_1 antagonist, antalarmin [14].

Relaxin-3 immunoreactive nerve fibres and RXFP3 mRNA are highly concentrated in brain regions involved in stress responses and anxiety-like behaviour, including the amygdala, bed nucleus of the stria terminalis (BNST) and the hypothalamic paraventricular nucleus (PVN) [9, 15-17]. Relaxin-3 expression is also negatively regulated by serotonin/5-HT_{1A} receptor signalling, which is strongly implicated in both the aetiology and/or treatment of anxiety- and depressive-like behaviour [18-23] and there are strong interactions between serotonin (5-HT) and CRF signalling (e.g. [24]). Treatment of rats with p-chlorophenylalanine, an inhibitor of serotonin synthesis, increased relaxin-3 mRNA expression in nucleus incertus neurons [25].

Prior to this report, no experimental studies had clearly described effects of increased endogenous relaxin-3 on complex behaviour during stressful or anxiety-provoking situations. Several studies have suggested that acute central native relaxin-3 administration *promotes* the hypothalamic stress response, and therefore may increase anxiety-like behaviour. For example, Fos protein and CRF expression were increased in the PVN 1-2 h after icv relaxin-3

administration [26], and plasma levels of ACTH and corticosterone were increased after acute icv relaxin-3 treatment [26, 27]. A recent study also reported that relaxin-3 knockout mice displayed decreased anxiety-like behaviour in the elevated plus maze (as reflected by percentage open arm entries of total time, but no other test of anxiety-like behaviour), suggesting a possible role for endogenous relaxin-3 in *promoting* anxiety-like behaviour [28], although this relatively small effect was not replicated in an independently derived line of relaxin-3 knockout mice [29]. In contrast, other studies suggest relaxin-3 may *reduce* the stress response. For example, injection of an RXFP3-selective agonist, R3/I5 (200 ng) [30] into the central amygdala reduced the characteristic freezing response displayed following fear conditioning in rats [31].

Therefore, a role for relaxin-3 signalling in the response to stress is predicted; and the goal of the present study was to examine further whether RXFP3 signalling is involved in promoting or blunting stress signalling pathways. We examined the effect of icv administration of a 5 µg dose (~1 nmol) of the RXFP3-selective agonist peptide, RXFP3-A2 [32], on the behaviour of adult, male Sprague-Dawley rats in several tests of affective behaviour, including the light-dark box (LDB), the large open field (LOF), and the elevated plus maze (EPM), to assess anxiety-like behaviour [33]; and the repeat forced swim test (FST) to assess the potential efficacy of the peptide as an antidepressant [34-37].

2. Materials and methods

2.1 Animals

Experiments were conducted with the approval of the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee and according to the guidelines issued by the National Health and Medical Research Council of Australia. All efforts were made to minimise the number of animals used. Male Sprague-Dawley rats supplied by the Animal Resources Centre, Perth, WA, Australia, weighed 250-300 g on arrival. Rats were single-housed under ambient conditions (21°C) and maintained on a 12 h light:dark cycle (lights on 0700-1900) with access to food (laboratory chow) and water *ad libitum*. Rats were acclimatised to the animal facility for at least 1 week prior to any experimentation.

2.2 Relaxin-3 peptide analogues

Native relaxin-3 has been shown to activate RXFP3 *and* RXFP1, the cognate receptors for relaxin-3 and relaxin, respectively [38-41], an effect that has confounded its use *in vivo* and the interpretation of such data. Hence chimeric peptides have been developed that selectively activate RXFP3 relative to RXFP1 and are preferred for *in vivo* studies [30, 40]. In this study we used a newly developed RXFP3 agonist peptide, [R3A(11-24,C15→A)B] (referred to as 'RXFP3-selective agonist, analogue 2' or 'RXFP3-A2'), which is highly selective for RXFP3 over RXFP1 *in vitro*. RXFP3-A2 has very high affinity for RXFP3 (binding $pK_i = 7.87 \pm 0.12$ M; and activation potency $pEC_{50} = 8.43 \pm 0.09$) and a lack of activity at RXFP1 (binding pK_i <5 M; activation potency, no activity) [32]. Briefly, RXFP3-A2 ([R3A(11-24,C15→A)B]) was synthesized using solid phase peptide synthesis and purified using reverse phase HPLC (e.g. [42]). The identity and purity of the peptide was confirmed by reverse phase HPLC, MALDI-TOF mass spectrometry and NMR analysis and amino acid composition was checked [32].

Recent studies have demonstrated that icv injection of 5 μg RXFP3-A2 increased food intake by ~3-fold during the first hour [32], in a similar fashion and potency to the chimeric agonist, R3/I5 [30, 40], and native relaxin-3 [38]. In addition, it has been shown that RXFP3-agonist-induced food intake is blocked by prior administration of an RXFP3-selective antagonist, RXFP3-A3 [32], demonstrating the selectivity of the agonist for RXFP3 *in vivo* (see also [40]). The 5 μg dose of RXFP3-A2 was used because it demonstrated robust *in vivo* effects in the food intake paradigm. RXFP3-A2 was dissolved in aCSF to a concentration of 1 μg/μl, and 5 μg (~1 nmol) in 5 μl was injected into the 'agonist-treated' groups of rats.

2.3 Stereotaxic implantation of a guide cannula into lateral ventricle

Each rat was deeply anaesthetised with 4% isoflurane in room air, 2 L/min (Delvet, Seven Hills, NSW, Australia) and maintained with ~2% isoflurane in room air, 0.2 L/min. The head was positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and the surgical region was shaved and cleaned with Riodine® (Povidone-Iodine 10% (w/v); ORION Laboratories Pty Ltd, Balcatta, WA, Australia) and an 80% ethanol (Merck Pty Ltd (Australia), Kilsyth, VIC, Australia) solution. A small incision was made in the skin, and the area cleaned and dried. The incisor bar was adjusted so that the level of bregma matched the level of lambda. Three pits were drilled into the skull in the bone adjacent to the eventual cannulation site. Screws (1.4 mm diameter × 3 mm length; Mr Specs, Mordialloc, VIC, Australia) were inserted to anchor the cannula in place. A small hole was drilled through the skull, through which a stainless-steel guide cannula (22 gauge, cut 5 mm below pedestal; Plastics One, Roanoke, VA, USA) was implanted using the following coordinates relative to Bregma: anteroposterior, -0.8 mm; mediolateral, -1.5 mm; dorsolateral -3.5 mm [43]. Cannulae were affixed to the screws and the skull using dental cement (Vertex-Dental, Zeist, The Netherlands). A 30-gauge obturator was inserted into the cannula to maintain patency.

After surgery, each rat was placed under a heat lamp until regaining consciousness and housed individually in clean cages. Meloxicam (3 mg/kg, i.p.; Troy Laboratories, Smithfield, NSW, Australia) was administered to provide acute post-operative analgesia; and 0.5 mg/ml paracetamol in 5% sucrose/water (Panadol® Rapid Soluble, GlaxoSmithKline, Ermington, NSW, Australia) was given for 3 days as ongoing post-operative analgesia. An antibiotic injection was also administered peri-operatively (5 mg enrofloxacin, Bayer Australia Ltd, Pymble, NSW, Australia). Rats were single-housed and allowed to recover for 7 days, during which time they were handled and weighed daily to habituate them to the experimenter.

2.4 Icv infusions and verification of cannula location and patency

Lateral ventricle infusions of peptides or vehicle were made using 29-gauge hypodermic tubing (Small Parts Inc., Miramar, FL, USA) connected to a 10-µl microsyringe (Hamilton Instruments, Reno, NV, USA) by polyethylene tubing (0.80 mm outer and 0.40 mm internal diameter; Microtube Extrusions, North Rocks, NSW, Australia). Rats were gently held in a towel, and the injector, was inserted into the protruding guide cannula. Infusions of 5 µl were delivered manually over the course of ~20 s, with care to ensure that all the solution was delivered. The injector was left in place for ~10 s after infusion.

Correct cannula positioning was verified in each rat by testing the acute dipsogenic response to an injection of angiotensin II (5 µl of a 4 ng/µl solution; Auspep, Parkville, VIC, Australia) in artificial cerebrospinal fluid (aCSF; 1470 mM NaCl; 40 mM KCl; 8.5 mM $MgCl_2$; 23 mM $CaCl_2$). Dipsogenesis was defined as repeated drinking episodes of ≥5 sec that commenced ≤1 min after angiotensin II administration. The dipsogenic response to angiotensin II was re-tested after each behavioural test and the behavioural data in response to RXFP3-A2 injection were excluded if the rat did not display a positive angiotensin II elicited drinking response.

2.5 Behavioural testing

Behavioural tests were conducted during the light phase. Rats were habituated to the behavioural test room overnight prior to testing. Rats that underwent several anxiety-like tests had a minimum of 3 days separation between paradigms. Initially, rats were randomly assigned to peptide or vehicle groups, but where rats in a cohort underwent several tests, peptide and vehicle treatments were alternated in a counter-balanced manner. The majority of the behavioural data were generated by automated software analysis, and so were unaffected by any possible observer interference/bias. Tests that required scoring by an observer were conducted under blinded conditions.

2.5.1 Light-dark box

Experiments were conducted between ~1000-1600 h. Rats were placed in the 42 cm (length) × 42 cm (width) × 40 cm (height) automated locomotor cell (Tru Scan Photobeam Rat Arena, E63-20; Coulbourn Instruments, Whitehall, PA, USA). Half of the locomotor cell was covered by the 'dark' box made of plastic opaque to visible light, but transparent to photo beams. A small opening (7 cm × 7 cm) enabled rats to enter or leave the 'dark' side. The light side was lit by an array of light-emitting diodes (400 lux in the centre), creating an aversive stimulus. Horizontal and vertical movements (rearing) of rats were tracked using Tru Scan 2.03 software (Coulbourn Instruments), which provided 5 min time bins and total session data outputs. The height for vertical plane (rearing) recordings was set at 19 cm. Rats received icv injection 10 min prior to being placed in the dark side of the box at the beginning of the experiment and sessions lasted 20 min. Boxes were cleaned with warm water and dried between experiments.

Parameters measured included number of entries into the light compartment, time spent in light compartment, number of moves in the light compartment and latency to enter the light compartment (emergence). Rats that did not enter the light compartment at all were excluded,

which resulted in 5 rats being excluded from both vehicle- and agonist-treated groups (e.g. see [44]).

2.5.2 Large open field

The large open field (LOF) was a 1.2 m diameter circular arena on the floor of an experimental room, surrounded by a 61 cm high aluminium wall. The arena was divided into three regions, the 'centre' which was 0.6 m in diameter, surrounded by the 'middle' area which was 0.9 m diameter, and the 'outer' area which formed a band around the edge of the LOF. The arena was lit by a flood light, which was mounted on the ceiling above the centre of the arena, and provided light of about 1200 lux in the centre, and 1000 lux at the periphery. Rats were tracked using EthoVision®, Version 3.0.15 (Noldus Information Technology, Wageningen, The Netherlands), which provided measures of time spent and number of entries into the centre, middle and outer regions, in addition to the latency to exit the centre region and locomotor activity. Rats received an icv injection 10 min prior to being placed in the centre of the arena at the start of the trial, and recorded for 10 min. The floor and walls of the arena were cleaned with warm water between trials. Between experiments, the flood light was turned off to ensure no overheating of the apparatus.

2.5.3 Elevated plus maze

The elevated plus maze (EPM) consisted of four arms (44 cm long × 12 cm wide) projecting from a central square (12 cm × 12 cm), with a height of 72 cm from the ground. Two opposing arms had high 10 cm walls and were designated 'closed' arms. A small 0.6 cm ledge was present on either side of the 'open' arms to prevent rats falling off as they turned around. The apparatus was placed in the middle of the experimental room under low lux (~50 lux closed arms, ~70 lux open arms). Rats received an icv injection 10 min prior to being placed in the central square, facing an open arm, and were allowed to explore the apparatus for 10 min, while being recorded by EthoVision® software (EthoVision®, Version 3.0.15, Noldus Information Technology, Wageningen, The Netherlands).

Parameters measured included time spent in and entries into the open and closed arms. Centre and arms boundary thresholds were defined as all four limbs must enter an arm to be recorded [45]. The apparatus was cleaned and dried with 80% ethanol, followed by water, between rats.

2.5.4 Repeat forced swim test

Rats were placed in a tall glass vessel (21 cm × 30 cm × 51 cm with curved corners) filled with tap water to a height of 33 cm and a temperature between 22-24°C. Rats were kept in the

water for 10 min before being removed and towelled dry, and placed under a heat lamp for about 30 min. The forced swim session was repeated at the same time the following day and rats received any icv injection 10 min prior to the start. This protocol has been used in our laboratory to assess effects on relaxin-3 mRNA levels in the nucleus incertus [14].

Rats were filmed using a Panasonic Colour CCTV Camera (Matsushita Electric Industrial Co Ltd, Osaka, Japan), and were manually scored by a blinded investigator using the EthoVision® program (Noldus Information Technology) for the total amount of time spent in the Porsolt (immobile) posture, the frequency of adopting the Porsolt posture, and latency to enter the Porsolt posture. The Porsolt posture was defined as immobility in all four limbs for ≥1 s. If the front paws moved to steady the rat during its immobile posture, this was not counted as a break in immobility.

Two cohorts of rats were used in the repeat forced swim test (FST) studies. An initial cohort (termed 'pre-tested') underwent multiple behavioural tests - first in the LDB, followed 4-5 days later by the LOF, then the repeat FST, 13-14 days later. Another cohort (termed 'experimentally naïve') underwent testing only in the repeat FST. These data were analysed by a two-way analysis of variance (ANOVA) for two factors (drug treatment and prior history of testing), with Bonferroni *post hoc* tests for 'main' effects, and *simple effects analysis* for 'interaction' effects using IBM SPSS Statistics 20.0 (see [46]). Importantly, rats which underwent multiple testing procedures were administered injections in a counter-balanced manner, i.e. rats that received RXFP3-A2 in the first test received vehicle in the next test.

Due to differences in the behaviour of control rats in the 'experimentally naïve' and 'pre-tested' cohorts in the repeat FST, a separate cohort of rats was investigated. These *uncannulated* rats were divided into two groups: one underwent testing in the LDB and the LOF, prior to testing in the repeat FST (over the same timeline as the previous cohort of 'multiple-test' rats) and another ('experimentally naïve') group had no testing prior to the repeat FST, but was handled regularly (daily) prior to testing (**Fig 1**).

2.5.5 Locomotor cell

Rats were placed in a 42 cm (length) × 42 cm (width) × 40 cm (height) clear walled locomotor cell (Tru Scan Photobeam Arena, E63-10; Coulbourn Instruments, Whitehall, PA, USA) for 60 min, with photobeam detectors to track the horizontal and vertical movements (rearing) of rats, providing 5 min time bin and session total data outputs. Testing was performed under low light conditions (~15-20 lux). Parameters measured included number of

moves and distance moved. Locomotor cells were cleaned with warm water between experiments.

Rats underwent testing over four separate days and were injected on two separate occasions: first on day one, just prior to entering the locomotor cell, to test the effect of a novel environment; and secondly on day 4 after 30 min habituation, to test the effect of the agonist in a habituated environment.

2.6 Data collection and statistical analysis

Data analysis and generation of histograms were routinely performed using GraphPad Prism Version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Results are expressed as mean ± SEM. Statistical significance was routinely evaluated using a Student's t-test, unless otherwise stated. If any data point was ≥2 standard deviations from the mean, it was excluded as an outlier (www.statsoft.com/textbook/).

3. Results

3.1 Icv RXFP3-A2 decreased anxiety-like behaviour in the light-dark box

Icv injection of 5 µg (~1 nmol) of the selective RXFP3 agonist peptide, RXFP3-A2, decreased anxiety-like behaviour in the LDB. Specifically, relative to control, icv infusion of RXFP3-A2 significantly decreased the latency to enter the light compartment by 59% ($t(33) = 2.19$, $p = 0.036$), and increased the number of entries into the light compartment by 35% ($t(33) = 2.09$, $p = 0.044$), time spent in the light compartment by 87% ($t(33) = 3.34$, $p = 0.002$) and number of moves in the light compartment by 94% ($t(33) = 3.62$, $p = 0.001$) (**Fig. 2**). Icv RXFP3-A2 had no significant effect on general activity in the LDB, suggesting that the results were related to anxiolytic effects and not to changes in activity. Specifically, there was no difference in total number of floor plane moves (control 1940 ± 167 vs RXFP3-A2 1896 ± 113) and floor plane distance (control 7376 ± 1318 vs RXFP3-A2 6584 ± 953).

3.2 Icv RXFP3-A2 had no effect on behaviour in the large open field

In the same cohort of rats tested 4-5 days later, acute icv injection of 5 µg RXFP3-A2 had no significant effect on anxiety-like behaviour in the LOF by any parameter measured (centre entries, centre duration, latency to exit centre), and no significant effect on locomotor activity (**Fig. 3**). Importantly, this lack of effect was not the result of tolerance to RXFP3-A2, since vehicle-injected rats from the light-dark test were treated with agonist in the LOF and *vice versa* in the cross-over design used.

3.3 Icv RXFP3-A2 decreased anxiety-like behaviour in the elevated plus maze

A separate cohort of rats underwent testing in the EPM, due to differences observed in the outcomes from the LDB and LOF tests, and icv injection of 5 µg RXFP3-A2 decreased anxiety-like behaviour. Specifically, icv infusion of RXFP3-A2 increased the percentage entries into the open arms by 66% ($t(22) = 3.28$, $p = 0.0035$) and percentage time spent in the open arms by 66% ($t(22) = 2.19$, $p = 0.039$) relative to control values (**Fig. 4**). There was no difference in the number of entries into the closed arms of the EPM, which is often used as an indirect measure of general activity [47, 48] (**Fig. 4**).

3.4 Icv RXFP-A2 decreased immobility in forced swim test, in rats pre-exposed to anxiety-like behaviour tests

Rats that had been tested in the LDB and LOF were housed in their home cages for 13-14 days before exposure to the repeat FST. No treatments were given on the first day of the test. On the second day, acute icv injections were administered 10 min prior to the second forced

swim. A separate cohort of rats was tested that were 'experimentally naïve' prior to the repeat FST, i.e. had undergone no previous behavioural testing.

Comparison of the performance of the two cohorts of rats in the FST using a two-way ANOVA, comparing history (naïve versus pre-tested) and treatment (control versus RXFP3-A2) as factors, demonstrated a significant interaction between history and treatment in the total duration in the immobility (Porsolt) posture ($F(1,36) = 4.76$, $p = 0.036$). In terms of treatment groups, a simple effects analysis revealed that in the control group the immobility time was significantly higher in the pretested compared to the naïve by ~3-fold ($p = 0.002$), whereas the immobility time was not significantly different in the RXFP3-A2 treated rats. In terms of history, a simple effects analysis also revealed that immobility time was significantly higher in the control vs RXFP3-A2 in the pretested rats by ~2-fold ($p = 0.009$; **Fig. 5a**), but not different in the naïve rats. There were no significant main effects of history or treatment. There were no significant differences in immobility frequency or latency to first immobility (data not shown).

In addition, rats in the pre-tested control group exhibited a broad range of individual variation in immobility, with an immobility range 19 to 256 s, while rats in the pre-tested RXFP3-A2-treated rats exhibited a more 'restricted' immobility range of 4.4 to 85 s (**Fig. 5b**).

3.5 Increased immobility in rats which had undergone previous tests of anxiety-like behaviour versus experimentally naïve rats

In order to confirm that the increase in immobility during the FST was caused by previous testing in anxiety-like paradigms, a separate cohort of rats was tested. This cohort, which did not undergo icv cannulation, was divided into two groups: one group underwent testing in the both the LDB and the LOF prior to testing in the FST (following the same timeline as rats described in Section 3.4); the other 'experimentally naïve' group had no behavioural testing prior to the FST, but was handled regularly by the experimenters.

There was a significant difference between these two groups in immobility in the FST, with a 60% decrease in the total time spent in the Porsolt (immobile) posture in the 'experimentally naïve' group ($t(17) = 2.69$, $p = 0.016$) (**Fig. 6**). There was also a trend for a decrease in the frequency of immobile posture ($t(17) = 2.00$, $p = 0.062$), but no difference in latency to enter the immobile posture (data not shown).

3.6 Icv RXFP3-A2 had no effect on general activity in the locomotor cell

Male Sprague-Dawley rats were tested in locomotor cells to investigate the effect of RXFP3-A2 on general activity. On day 1 of the locomotor cell test (a novel environment), rats injected with 5 µg RXFP3-A2 displayed no significant difference in any measures of general activity compared to control rats, including total moves ($t(28) = 0.17$, $p = 0.87$) and total distance travelled ($t(28) = 0.50$, $p = 0.62$) (**Fig. 7a,b**). On day 4 of locomotor cell testing (an habituated environment), rats injected with 5 µg RXFP3-A2 again displayed no significant difference in any measures of general activity relative to control rats, including total moves ($t(22) = 0.88$, $p = 0.39$) and total distance moved ($t(22) = 0.94$, $p = 0.36$) (**Fig. 7c,d**).

4. Discussion

Our findings demonstrate the ability of central administration of an RXFP3-selective agonist peptide to decrease anxiety-like behaviour in adult male rats and produce an antidepressant-like response in the FST, especially in rats with a history of prior behavioural testing. These preclinical data suggest that drugs that activated RXFP3 might have potential as anxiolytic agents and may also have an antidepressant action. Importantly, these data also demonstrate long-lasting consequences of anxiety testing in Sprague-Dawley rats that impact upon other affective behavioural profiles. Divergent effects of RXFP3 activation in the Porsolt swim test in naïve and 'pre-tested' rats suggest a potential role for relaxin-3 signalling following cumulative low-level stressors, possibly related to coping and resilience.

4.1 Anxiety-like behaviour

4.1.1 Light-dark box

The LDB test is based on the conflict for a rodent between its inclination to explore a novel environment and its aversion to brightly illuminated areas [49]. The amount of exploratory activity in the light compartment is considered an index of anxiolytic activity. While the LDB test was initially developed for mice [50], it has also been used to test rat behaviour [51]. In the current study, there was a clear decrease in anxiety-like behaviour in rats treated with RXFP3-selective agonist compared to control. Icv infusions of RXFP3-A2 significantly altered several parameters measured in the LDB: the latency to enter the light compartment was reduced; while the number of entries, the time spent, and the number of moves in the light compartment, were increased. There was no significant difference in the total number of moves, suggesting differences observed were due to altered levels of anxiety, rather than reflecting any altered locomotor behaviour.

4.1.2 Elevated plus maze

The EPM test is based on the natural aversion of rodents for open spaces and reflects the conflict between exploration and aversion to elevated open places [45, 52, 53]. Central injection of the RXFP3-selective agonist produced a clear anxiolytic effect in the EPM, including an increased percentage of open arms entries and time spent in the open arms, which are considered measures of anxiety-like behaviour [52]. There was no difference in the number of entries into the closed arms, which is considered a measure of motor activity in this test [47, 48], again suggesting that the differences between groups are due to changes in anxiety-like behaviour, rather than changes in activity.

The protocol used here was a 10 min session, whereas a widely used protocol employs a 5 min session [54]. For this reason, we examined the first 5 min of the EPM data, which similarly demonstrated that RXFP3-A2 treatment decreased anxiety-like behaviour (data not shown).

4.1.3 Large open field

In the LOF test, rats are exposed to an unknown environment from which escape is prevented by surrounding walls. In contrast to the clear effects observed in the LDB and the EPM, there was no significant effect of RXFP3-A2 treatment on anxiety-like measures or locomotor activity in the LOF test. Importantly, this lack of effect was not the result of tolerance to RXFP3-A2, since control rats from the light-dark test were treated in a cross-over design with peptide agonist in the LOF, and *vice versa*. The LOF has been criticised in terms of predictive validity, as it is not sensitive to certain compounds that act as anxiolytic agents, and it may be better thought of as a model of 'normal' anxiety rather than 'pathological' anxiety [55].

Another distinguishing feature of the LOF compared to the other approach-avoidance paradigms is the impossibility of 'escape' to a safe enclosure, i.e. the LOF confronts rodents with a novel environment from which there is no escape; while the LDB and EPM provide a safe enclosure (dark box or closed arms) to which the rodent can 'escape' (e.g. [56]). It is possible, therefore, that the relaxin-3/RXFP3 system is involved in preparing behavioural strategies to escape from a 'stressful' situation, similar to the proposed role of the 'nucleus incertus' (the major relaxin-3-expressing brain region) [12, 57]. It is also possible that these different results were due to the experimental history of the rats, i.e. previous exposure to the LDB test. A difference between naïve and previously tested mice has been noted in the LOF paradigm, with pre-tested mice demonstrating more anxiety-like behaviour [58]; but in our study none of the rats froze in the centre, and the majority of rats explored the central area at some point during the test, suggesting they were not 'extremely' anxious.

4.2 Forced swim test

Although the FST in rats does not induce symptomatology similar to human depression, it has been demonstrated to be an effective screening test for antidepressant drugs [34-36]. In the current study, RXFP3-selective agonist treatment produced a significant decrease in immobility, a measure of antidepressant-like behaviour, in the repeat FST, but only in rats which had undergone previous tests of anxiety-like behaviour, not in experimentally naïve rats. If an overall score of immobility of <100 s is termed 'resilient' and ≥100 s 'depressive'

(which is approximately the mean duration of time spent immobile in the pre-tested rats), rats in the pre-tested control group exhibited a broad range of individual variation in immobility, with a 50:50 distribution of resilient/depressive rats (i.e. period of immobility range 19 to 256 s; 45% 'resilient' and 55% 'depressive'), whereas *all* pre-tested RXFP3-A2-treated rats exhibited 'resilient' behaviour (immobility range 4.4 to 85 s) (**Fig 5b**). These data suggest RXFP3 activation promotes 'resilient' behaviour and a resultant decreased variation in immobility. Naturally, this hypothesis requires further experimental testing, but nevertheless it is clear that RXFP3 activation effectively reduced the inter-animal variability in this assay.

4.2.1 Effect of experimental history on the forced swim test

Our results demonstrate that prior testing in paradigms of anxiety-like behaviour significantly increases immobility in the FST. Control rats which had undergone earlier tests of anxiety-like behaviour displayed increased immobility time in the FST compared to naïve counterparts (**Fig 5a**). Similar results were obtained in uncannulated/non-injected rats, where the 'pre-tested' group demonstrated increased immobility compared to the 'experimentally naïve' rats (**Fig 6**), indicating that prior history of testing causes the difference in immobility, rather than other factors, such as prior history of agonist injections.

The uncannulated 'pre-tested' group also displayed a broad variation in immobility (range 14 to 179 s; 40% 'resilient': 60% 'depressive'), similar to the cannulated 'pre-tested' control rats, whereas *all* rats in the uncannulated 'experimentally naïve' group exhibited 'resilient' behaviour (immobility range 5 to 89 s) (**Fig. 6b**).

It has been demonstrated that pre-exposure of rats to stressors such as cold, restraint or foot-shock, 24 h prior to the test [59] can increase immobility in the FST, but in this study, the LOF test was performed almost 2 weeks prior to the FST. These results agree with a recent report that an acute restraint stress 10 days before was more 'anxiogenic' in the EPM than a restraint stress 24 h before. The restraint stress given 10 days before was also accompanied by an increase in spine density on principal neurons of the basolateral amygdala, suggesting a longer period of time might be necessary to allow changes at both synaptic and behavioural levels [60, 61]. These results also suggest that pre-testing may be similar to other studies using 'sub-chronic unpredictable stress' to test resilience-like behaviour [62]. Our data re-emphasise the importance of experimental history on performance in some subsequent tests.

4.3 Locomotor activity

Central administration of RXFP3-A2 in rats did not alter general locomotor activity in either the novel or habituated environment, suggesting that the anxiolytic activity observed in the LDB and EPM was not a false positive result due to increased general activity. The apparent lack of effect of an RXFP3-selective agonist on general activity in the locomotor cell varies somewhat from a report of an increase in locomotor activity following RXFP3 activation. The RXFP3 agonist, R3/I5, was reported to dose-dependently increase 'locomotor activity' when given 2-3 h after lights on, whereas an RXFP3 antagonist had no effect on locomotor activity under these conditions [63]. However, in contrast to the current study, Wistar not Sprague-Dawley rats were used, and the experiment was performed in a different locomotor system, the Motor Monitor (Hamilton Kinder Software, 2000) which measured the summation of floor (horizontal) and vertical plane moves compared to the Tru Scan Photobeam Arena, (Coulbourn Instruments) employed here, which measured floor plane and vertical plane separately. Furthermore, other studies that assessed the effect of relaxin-3-related peptides on locomotor activity in a number of different paradigms, did not report a significant effect. For example, human relaxin-3 infused chronically into male Wistar rats (600 pmol/day, icv) produced no marked difference in locomotor activity in a Plexiglas box at different time points during the light and dark phases [64].

4.4 Anxiety, depression and relaxin-3

The present results suggest endogenous relaxin-3/RXFP3 signalling may be involved in regulating anxiety-like behaviour and can presumably influence equivalent circuits to those that are dysfunctional in clinical depression. There are, however, existing data which appear inconsistent with this idea. On one hand, the *decrease* in anxiety-like behaviours following icv injections of the RXFP3-selective agonist suggests that relaxin-3 signalling may provide 'negative feedback' to the hypothalamic-pituitary-adrenal (HPA) axis, as it is assumed that HPA activity is related to anxiety-like behaviour [65]. On the other hand, centrally (icv) administered relaxin-3 has been demonstrated to increase CRF expression in the PVN, and increase plasma ACTH and corticosterone levels, which might be predicted to *increase* anxiety-like behaviour [26, 27]. Interestingly, central administration of other neuropeptides, such as neuropeptide Y and neuropeptide S, produces a similar 'incongruous' pattern of *increased* HPA axis activity combined with *decreased* anxiety-like behaviour, suggesting that increased CRF in the PVN and activation of the HPA axis does not always or necessarily equate to increased anxiety-like behaviour [66-69].

In addition, while central administration of CRF is anxiogenic in several animal behavioural tests, including the unfamiliar open field [70], the elevated plus maze [71] and the acoustic startle response test [72], several findings suggest that this effect is independent of the HPA axis [73]. For example, lesions of the PVN do not block the activation of the acoustic startle reflex by CRF, unlike lesions of the central amygdala [74]; blockade of the HPA axis induced by peripheral immunoneutralisation of CRF does not affect the behavioural response to social defeat stress in rats [75]; and CRF-induced changes in responding in a conflict test remain unaltered despite dexamethasone blockade of the HPA axis [76].

Another possible explanation is that the differences in responses were due to activation of different receptors, i.e. the increased stress response in the HPA axis was induced by icv *native* relaxin-3, which activates RXFP1 as well as RXFP3, whereas the anxiolytic behaviour was induced by selective RXFP3 activation. This could be further explored by pre-treating rats with selective RXFP1 and RXFP3 antagonists prior to injection of *native* relaxin-3, and determining the response of the HPA axis.

If RXFP3 activation in PVN does not strongly alter anxiety-like behaviour, the effect may be via actions within the amygdala and/or BNST, which express high levels of RXFP3 [16], and are implicated in mediating behavioural responses to threats [17, 77, 78]. In particular, the BNST is implicated in slower-onset, longer-lasting behavioural responses to actual or perceived threats, which are hypothesised to be involved in anxiety-like behaviour [17, 77, 78]. It is possible that the RXFP3-selective agonist alters GABA transmission to produce an inhibitory (anxiolytic) effect in areas such as the amygdala and BNST, which 'overrides' any stimulation of the HPA axis.

4.5. Relaxin-3, GABA and serotonin in anxiety and depressive disorders

The inhibitory neurotransmitter, GABA, has been well studied in the context of anxiety, particularly as benzodiazepines (which act on GABA receptors) are first-line treatments for most anxiety disorders [79]. Relaxin-3 neurons in the nucleus incertus co-express GABA [16], and it is likely that relaxin-3 is co-released with GABA and/or modulates GABAergic functions pre- or post-synaptically. Further investigations are required to determine the modulatory effect of relaxin-3/RXFP3 signalling on GABAergic function in anxiety- and depressive-like behaviours. This could be done at many levels, for example by reducing or 'knocking down' levels of transmitter GABA in the nucleus incertus or other relaxin-3 neuron populations using viral vectors targeting GABA synthesising or transporting proteins in the

rat (see e.g. [80]), or by using suitable mouse knockout models (e.g. [81]), and assessing the effects of RXFP3 agonists on of anxiety- and depressive-like behaviour. Interactions of relaxin-3 and GABA signalling could also be investigated using electrophysiology, as the release of classical neurotransmitters and peptides can be differential and dependent on frequency and patterns of firing, due to different subcellular storage sites (see [82, 83] for review).

In addition, relaxin-3 expression in nucleus incertus neurons has been reported to be negatively regulated by serotonin via $5HT_{1A}$ receptors, based on findings in serotonin depletion studies [25]; and moderate levels of relaxin-3-like immunoreactivity and RXFP3 expression are detected in several serotonin-expressing regions, including the dorsal raphé nucleus [16], which is strongly implicated in the modulation of anxiety- and depressive-like behaviours [18, 21]. The precise nature of serotonin and relaxin-3 interactions under normal physiological conditions or after acute or chronic elevated stress conditions are not known and remain an important area for future study.

4.6 Acute versus chronic stress/anxiety and relaxin-3

Rats that had undergone previous tests of anxiety displayed a marked increase in immobility time in the FST compared to experimentally naïve rats and RXFP3 activation was able to reduce these heightened levels of immobility. One possible explanation for this finding is that pre-tested rats have an up-regulation of RXFP3 expression compared to acutely tested rats, in which case 'additional' exogenous RXFP3 agonist may have a greater effect in pre-tested rats. This may occur in parallel with heightened CRF and other stress-related signalling. It is also possible that elevated CRF signalling in chronic stress may not be effectively 'regulated' by endogenous relaxin-3 signalling and the exogenous agonist may restore the balance. These possibilities require further investigation (see e.g. [14]).

4.7 Future directions

The site(s) of actions of the RXFP3 agonist require characterisation and this could be achieved by observing the effects of targeted injections of RXFP3 agonist into specific brain regions. Neurons which are targeted by the RXFP3 agonist could be 'chemically phenotyped' using immunohistochemical labelling techniques, which would provide information on the types of neurons activated, and signal transduction mechanisms.

In order to further clarify the role of relaxin-3-related peptides in anxiety-like behaviour, the activity of RXFP3 selective agonists could be tested in other paradigms of anxiety-like

behaviour that do not involve approach-avoidance conflict (reviewed in [84]). Further clarification of the antidepressant-like effects of relaxin-3/RXFP3 signalling could be tested in paradigms which use chronic administration of a relaxin-3-related peptide, which is a more accurate representation of the human clinical situation [84]. For example, the chronic social defeat paradigm is a rodent model which can only be 'normalised' by chronic, not acute, administration of an antidepressant [85].

5. Conclusions

Our findings provide the first experimental evidence that an RXFP3-selective agonist has a potential role as an anxiolytic and/or antidepressant agent. Acute injections of 5 μg RXFP3-A2 decreased anxiety-like behaviours of adult rats in the light-dark box and elevated plus maze. RXFP3-A2 also decreased immobility in the forced swim test, but only in rats that had previously undergone tests of anxiety-like behaviour. Our results also demonstrate that previous testing in paradigms of anxiety-like behaviour two weeks prior can significantly increase immobility time in the forced swim test.

Acknowledgements

This research was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia, the Pratt Foundation and the Besen Family Foundation, and the Florey Neuroscience Institutes Trust Foundation. During these studies, P.J.R. was the recipient of an Australian Postgraduate Award and a Dowd Foundation Neuroscience Scholarship. E.B. was a visiting undergraduate scholar from the University of Applied Sciences, Gelsenkirchen, Germany. F.S. and M.A.H. were Florey Neuroscience Institutes Foundation Fellows and J.D.W., A.J.L. and A.L.G. are NHMRC Research Fellows. The authors wish to thank Brett Purcell and members of the Florey Integrated Neuroscience Facilities (INF) for their advice and assistance with behavioural testing, and Elena Krstew for help with statistical analysis.

References

[1] Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry. 2005;62:593-602.

[2] World Health Organization. The global burden of disease: 2004 update. Geneva, WHO Press, World Health Organization; 2008.

[3] Wang PS, Insel TR. NIMH-funded pragmatic trials: moving on. Neuropsychopharmacology. 2010;35:2489-90.

[4] Covington HE, III, Vialou V, Nestler EJ. From synapse to nucleus: novel targets for treating depression. Neuropharmacology. 2010;58:683-93.

[5] Bale TL, Vale WW. CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol. 2004;44:525-57.

[6] Binder EB, Nemeroff CB. The CRF system, stress, depression and anxiety-insights from human genetic studies. Mol Psychiatry. 2010;15:574-88.

[7] Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Claasz AA, Reytomas IG, et al. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. J Biol Chem. 2002;277:1148-57.

[8] Burazin TCD, Bathgate RAD, Macris M, Layfield S, Gundlach AL, Tregear GW. Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. J Neurochem. 2002;82:1553-7.

[9] Tanaka M, Iijima N, Miyamoto Y, Fukusumi S, Itoh Y, Ozawa H, et al. Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. Eur J Neurosci. 2005;21:1659-70.

[10] Goto M, Swanson LW, Canteras NS. Connections of the nucleus incertus. J Comp Neurol. 2001;438:86-122.

[11] Olucha-Bordonau FE, Teruel V, Barcia-Gonzalez J, Ruiz-Torner A, Valverde-Navarro AA, Martinez-Soriano F. Cytoarchitecture and efferent projections of the nucleus incertus of the rat. J Comp Neurol. 2003;464:62-97.

[12] Ryan PJ, Ma S, Olucha-Bordonau FE, Gundlach AL. Nucleus incertus - an emerging modulatory role in arousal, stress and memory. Neurosci Biobehav Rev. 2011;35:1326-41.

[13] Bittencourt JC, Sawchenko PE. Do centrally administered neuropeptides access cognate receptors? An analysis in the central corticotropin-releasing factor system. J Neurosci. 2000;20:1142-56.

[14] Banerjee A, Shen PJ, Ma S, Bathgate RA, Gundlach AL. Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF_1 activation. Neuropharmacology. 2010;58:145-55.

[15] Sutton SW, Bonaventure P, Kuei C, Roland B, Chen J, Nepomuceno D, et al. Distribution of G-protein-coupled receptor (GPCR)135 binding sites and receptor mRNA in the rat brain suggests a role for relaxin-3 in neuroendocrine and sensory processing. Neuroendocrinology. 2004;80:298-307.

[16] Ma S, Bonaventure P, Ferraro T, Shen PJ, Burazin TCD, Bathgate RAD, et al. Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. Neuroscience. 2007;144:165-90.

[17] Davis M, Walker DL, Miles L, Grillon C. Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacology. 2010;35:105-35.

[18] Barnes NM, Sharp T. A review of central 5-HT receptors and their function. Neuropharmacology. 1999;38:1083-152.

[19] Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, et al. Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci. 2003;23:8788-99.

[20] Millan MJ. The role of monoamines in the actions of established and "novel" antidepressant agents: a critical review. Eur J Pharmacol. 2004;500:371-84.

[21] Ressler KJ, Nemeroff CB. Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. Depress Anxiety. 2000;12 Suppl 1:2-19.

[22] Akimova E, Lanzenberger R, Kasper S. The serotonin-1A receptor in anxiety disorders. Biol Psychiatry. 2009;66:627-35.

[23] Richardson-Jones JW, Craige CP, Nguyen TH, Kung HF, Gardier AM, Dranovsky A, et al. Serotonin-1A autoreceptors are necessary and sufficient for the normal formation of circuits underlying innate anxiety. J Neurosci. 2011;31:6008-18.

[24] Forster GL, Pringle RB, Mouw NJ, Vuong SM, Watt MJ, Burke AR, et al. Corticotropin-releasing factor in the dorsal raphe nucleus increases medial prefrontal cortical serotonin

via type 2 receptors and median raphe nucleus activity. Eur J Neurosci. 2008;28:299-310.

[25] Miyamoto Y, Watanabe Y, Tanaka M. Developmental expression and serotonergic regulation of relaxin 3/INSL7 in the nucleus incertus of rat brain. Regul Pept. 2008;145:54-9.

[26] Watanabe Y, Miyamoto Y, Matsuda T, Tanaka M. Relaxin-3/INSL7 regulates the stress-response system in the rat hypothalamus. J Mol Neurosci. 2011;43:169-74.

[27] McGowan BM, Stanley SA, Ghatei MA, Bloom SR. Relaxin-3 and its role in neuroendocrine function. Ann N Y Acad Sci. 2009;1160:250-5.

[28] Watanabe Y, Tsujimura A, Takao K, Nishi K, Ito Y, Yasuhara Y, et al. Relaxin-3-deficient mice showed slight alteration in anxiety-related behavior. Front Behav Neurosci. 2011;5:50.

[29] Smith CM, Hosken IT, Sutton SW, Lawrence AJ, Gundlach AL. Relaxin-3 null mutation mice display a circadian hypoactivity phenotype. Genes Brain Behav. 2012;11:94-104.

[30] Liu C, Chen J, Kuei C, Sutton S, Nepomuceno D, Bonaventure P, et al. Relaxin-3/insulin-like peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR)135 and GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. Mol Pharmacol. 2005;67:231-40.

[31] Ma S, Kastman H, Olucha-Bordonau FE, Capogna M, Hossain MA, Wade JD, et al. Relaxin-3 receptor activation in central amygdala enhances fear extinction in the rat: Implications for relaxin-3 control of emotion. Soc Neurosci Abstr. 2010;809.24.

[32] Shabanpoor F, Akhter Hossain M, Ryan PJ, Belgi A, Layfield S, Kocan M, et al. Minimization of human relaxin-3 leading to high-affinity analogues with increased selectivity for relaxin-family peptide 3 receptor (RXFP3) over RXFP1. J Med Chem. 2012;55:1671-81.

[33] Belzung C, Le Pape G. Comparison of different behavioral test situations used in psychopharmacology for measurement of anxiety. Physiol Behav. 1994;56:623-8.

[34] Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature. 1977;266:730-2.

[35] Porsolt RD, Anton G, Blavet N, Jalfre M. Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol. 1978;47:379-91.

[36] Borsini F, Meli A. Is the forced swimming test a suitable model for revealing antidepressant activity? Psychopharmacology (Berl). 1988;94:147-60.

[37] Cryan JF, Valentino RJ, Lucki I. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci Biobehav Rev. 2005;29:547-69.

[38] McGowan BM, Stanley SA, Smith KL, White NE, Connolly MM, Thompson EL, et al. Central relaxin-3 administration causes hyperphagia in male Wistar rats. Endocrinology. 2005;146:3295-300.

[39] Bathgate RAD, Lin F, Hanson NF, Otvos LJ, Guidolin A, Giannakis C, et al. Relaxin-3: Improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo. Biochemistry. 2006;45:1043-53.

[40] Kuei C, Sutton S, Bonaventure P, Pudiak C, Shelton J, Zhu J, et al. R3(BΔ23-27)R/I5 chimeric peptide, a selective antagonist for GPCR135 and GPCR142 over relaxin receptor LGR7: in vitro and in vivo characterization. J Biol Chem. 2007;282:25425-35.

[41] Otsubo H, Onaka T, Suzuki H, Katoh A, Ohbuchi T, Todoroki M, et al. Centrally administered relaxin-3 induces Fos expression in the osmosensitive areas in rat brain and facilitates water intake. Peptides. 2010;31:1124-30.

[42] Haugaard-Jonsson LM, Hossain MA, Daly NL, Bathgate RAD, Wade JD, Craik DJ, et al. Structure of the R3/I5 chimeric relaxin peptide, a selective GPCR135 and GPCR142 agonist. J Biol Chem. 2008;283:23811-8.

[43] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. Sixth Ed. London, Academic Press; 2007.

[44] Kliethermes CL, Cronise K, Crabbe JC. Anxiety-like behavior in mice in two apparatuses during withdrawal from chronic ethanol vapor inhalation. Alcohol Clin Exp Res. 2004;28:1012-9.

[45] Featherby T, van den Buuse M, Lubman DI, Lawrence AJ. Persistent downregulation of hippocampal CREB mRNA parallels a Y-maze deficit in adolescent rats following semi-chronic amphetamine administration. Br J Pharmacol. 2008;154:417-28.

[46] Field A. Discovering Statistics Using SPSS. Third Ed. London, U.K., SAGE Publications Ltd; 2009.

[47] Fernandes C, File SE. The influence of open arm ledges and maze experience in the elevated plus-maze. Pharmacol Biochem Behav. 1996;54:31-40.

[48] File SE. Factors controlling measures of anxiety and responses to novelty in the mouse. Behav Brain Res. 2001;125:151-7.

[49] Bourin M, Hascoet M. The mouse light/dark box test. Eur J Pharmacol. 2003;463:55-65.

[50] Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav. 1980;13:167-70.

[51] Chaouloff F, Durand M, Mormede P. Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. Behav Brain Res. 1997;85:27-35.

[52] Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods. 1985;14:149-67.

[53] Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl). 1987;92:180-5.

[54] Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc. 2007;2:322-8.

[55] Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol. 2003;463:3-33.

[56] Dulawa SC, Grandy DK, Low MJ, Paulus MP, Geyer MA. Dopamine D4 receptor-knock-out mice exhibit reduced exploration of novel stimuli. J Neurosci. 1999;19:9550-6.

[57] Goto M. Hodological and functional study of the nucleus incertus and pars alfa of the pontine periventricular gray. Sao Paulo: University of Sao Paulo, Brazil; 1998.

[58] McIlwain KL, Merriweather MY, Yuva-Paylor LA, Paylor R. The use of behavioral test batteries: effects of training history. Physiol Behav. 2001;73:705-17.

[59] Borsini F, Lecci A, Sessarego A, Frassine R, Meli A. Discovery of antidepressant activity by forced swimming test may depend on pre-exposure of rats to a stressful situation. Psychopharmacology (Berl). 1989;97:183-8.

[60] Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S. Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. Proc Natl Acad Sci U S A. 2005;102:9371-6.

[61] Roozendaal B, McEwen BS, Chattarji S. Stress, memory and the amygdala. Nat Rev Neurosci. 2009;10:423-33.

[62] Russo SJ, Murrough JW, Han MH, Charney DS, Nestler EJ. Neurobiology of resilience. Nat Neurosci. 2012;15:1475-84.

[63] Sutton SW, Shelton J, Smith CM, Williams J, Yun S, Motley T, et al. Metabolic and neuroendocrine responses to RXFP3 modulation in the central nervous system. Ann N Y Acad Sci. 2009;1160:242-9.

[64] Hida T, Takahashi E, Shikata K, Hirohashi T, Sawai T, Seiki T, et al. Chronic intracerebroventricular administration of relaxin-3 increases body weight in rats. J Recept Signal Transduct Res. 2006;26:147-58.

[65] Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB. The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol. 1999;160:1-12.

[66] Heilig M, McLeod S, Koob GK, Britton KT. Anxiolytic-like effect of neuropeptide Y (NPY), but not other peptides in an operant conflict test. Regul Pept. 1992;41:61-9.

[67] Suda T, Tozawa F, Iwai I, Sato Y, Sumitomo T, Nakano Y, et al. Neuropeptide Y increases the corticotropin-releasing factor messenger ribonucleic acid level in the rat hypothalamus. Brain Res Mol Brain Res. 1993;18:311-5.

[68] Smith KL, Patterson M, Dhillo WS, Patel SR, Semjonous NM, Gardiner JV, et al. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. Endocrinology. 2006;147:3510-8.

[69] Okamura N, Reinscheid RK. Neuropeptide S: a novel modulator of stress and arousal. Stress. 2007;10:221-6.

[70] Takahashi LK, Kalin NH, Vanden Burgt JA, Sherman JE. Corticotropin-releasing factor modulates defensive-withdrawal and exploratory behavior in rats. Behav Neurosci. 1989;103:648-54.

[71] Baldwin HA, Rassnick S, Rivier J, Koob GF, Britton KT. CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. Psychopharmacology (Berl). 1991;103:227-32.

[72] Swerdlow NR, Geyer MA, Vale WW, Koob GF. Corticotropin-releasing factor potentiates acoustic startle in rats: blockade by chlordiazepoxide. Psychopharmacology (Berl). 1986;88:147-52.

[73] Thorsell A. Brain neuropeptide Y and corticotropin-releasing hormone in mediating stress and anxiety. Exp Biol Med (Maywood). 2010;235:1163-7.

[74] Liang KC, Melia KR, Campeau S, Falls WA, Miserendino MJ, Davis M. Lesions of the central nucleus of the amygdala, but not the paraventricular nucleus of the hypothalamus, block the excitatory effects of corticotropin-releasing factor on the acoustic startle reflex. J Neurosci. 1992;12:2313-20.

[75] Pich EM, Heinrichs SC, Rivier C, Miczek KA, Fisher DA, Koob GF. Blockade of pituitary-adrenal axis activation induced by peripheral immunoneutralization of corticotropin-releasing factor does not affect the behavioral response to social defeat stress in rats. Psychoneuroendocrino. 1993;18:495-507.

[76] Britton KT, Lee G, Vale W, Rivier J, Koob GF. Corticotropin releasing factor (CRF) receptor antagonist blocks activating and 'anxiogenic' actions of CRF in the rat. Brain Res. 1986;369:303-6.

[77] Davis M, Shi C. The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety? Ann N Y Acad Sci. 1999;877:281-91.

[78] Walker DL, Toufexis DJ, Davis M. Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. Eur J Pharmacol. 2003;463:199-216.

[79] Shader RI, Greenblatt DJ. Use of benzodiazepines in anxiety disorders. N Engl J Med. 1993;328:1398-405.

[80] Horvath L, van Marion I, Tai K, Nielsen TT, Lundberg C. Knockdown of GAD67 protein levels normalizes neuronal activity in a rat model of Parkinson's disease. J Genet Med. 2011;13:188-97.

[81] Tong Q, Ye CP, Jones JE, Elmquist JK, Lowell BB. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. Nat Neurosci. 2008;11:998-1000.

[82] Hokfelt T, Broberger C, Xu ZQ, Sergeyev V, Ubink R, Diez M. Neuropeptides - an overview. Neuropharmacology. 2000;39:1337-56.

[83] van den Pol AN. Neuropeptide transmission in brain circuits. Neuron. 2012;76:98-115.

[84] Cryan JF, Holmes A. The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov. 2005;4:775-90.

[85] Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, et al. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science. 2006;311:864-8.

Figure Legends

Fig. 1. Schematic of the number and order of behavioural tests of different cohorts of rats in the light-dark box (LDB), large open field (LOF) and forced swim test (FST).

Fig. 2. Effect of acute icv administration of RXFP3-A2 (5 µg) versus aCSF (control) on the behaviour of adult, male Sprague-Dawley rats in the light-dark box. A. Entries into the light compartment, B. Time spent in the light compartment, C. Number of moves in the light compartment, D. Latency to enter the light compartment. Data represent mean ± SEM. Statistical significance evaluated using a Student's t-test, * P<0.05; ** P<0.01; *** P<0.001. RXFP3-A2, *n* = 19; control, *n* = 16.

Fig. 3. Effect of acute icv administration of RXFP3-A2 (5 µg) versus aCSF (control) on the behaviour of adult, male Sprague-Dawley rats in the large open field. A. Frequency to enter the centre, B. Time spent in the centre, C. Latency to exit centre, D. Locomotor activity. Data represent mean ± SEM. Statistical significance evaluated using a Student's t-test, ns, not significant. RXFP3-A2, *n* = 16; control, *n* = 25.

Fig. 4. Effect of acute icv administration of RXFP3-A2 (5 µg) versus aCSF (control) on the behaviour of adult, male Sprague-Dawley rats in the elevated plus maze. A. Entries into the open arms (as a percentage of the total number of arm entries), B. Time spent in the open arms (as a percentage of the total time on both open and closed arms), C. Total number of entries into the closed arms. Data represent mean ± SEM. Statistical significance evaluated using a Student's t-test, * P<0.05; ** P<0.01; *** P<0.001; ns, not significant. RXFP3-A2, *n* = 11; control, *n* = 13.

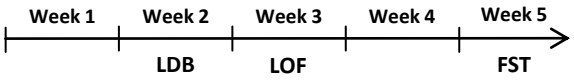
Fig. 5. A. Comparison of the effect of history (pre-testing vs naïve) and treatment (control vs RXFP3-A2) on the immobility time of adult, male Sprague-Dawley rats in the forced swim test. Data represent mean ± SEM. Statistical significance was evaluated using a two-way ANOVA, with a Bonferroni post-hoc test - ** P<0.01. B. Scatter plot of pretested rats demonstrating range of the immobility times for RXFP3-A2-treated and control rats. Naïve RXFP3-A2 (*n* = 5); naïve control (*n* = 8); pre-tested RXFP3-A2 (*n* = 9); pre-tested control (*n* = 18).

Fig. 6. Effect of previous anxiety testing ('pre-tested') versus no previous testing ('naïve') on the behaviour of adult, male Sprague-Dawley rats in the forced swim test. A. Bar graph. B.

Scatter plot. Data represent mean ± SEM of the total duration spent immobile in the 'Porsolt posture'. Statistical significance was evaluated using a Student's t-test. * P<0.05. Naïve (*n* = 9); pre-tested (*n* = 10).

Fig. 7. Effect of acute icv administration of RXFP3-A2 (5 μg) versus aCSF (control) on the behaviour of adult, male Sprague-Dawley rats in the locomotor cell. A. Total moves (novel environment), B. Total distance travelled (novel environment), C. Total moves (habituated), D. Total distance travelled (habituated). ns, not significant. RXFP3-A2, *n* = 10-15; control, *n* = 15.

Pre-tested rats



Naïve rats

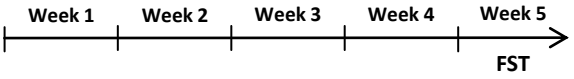


Figure 2

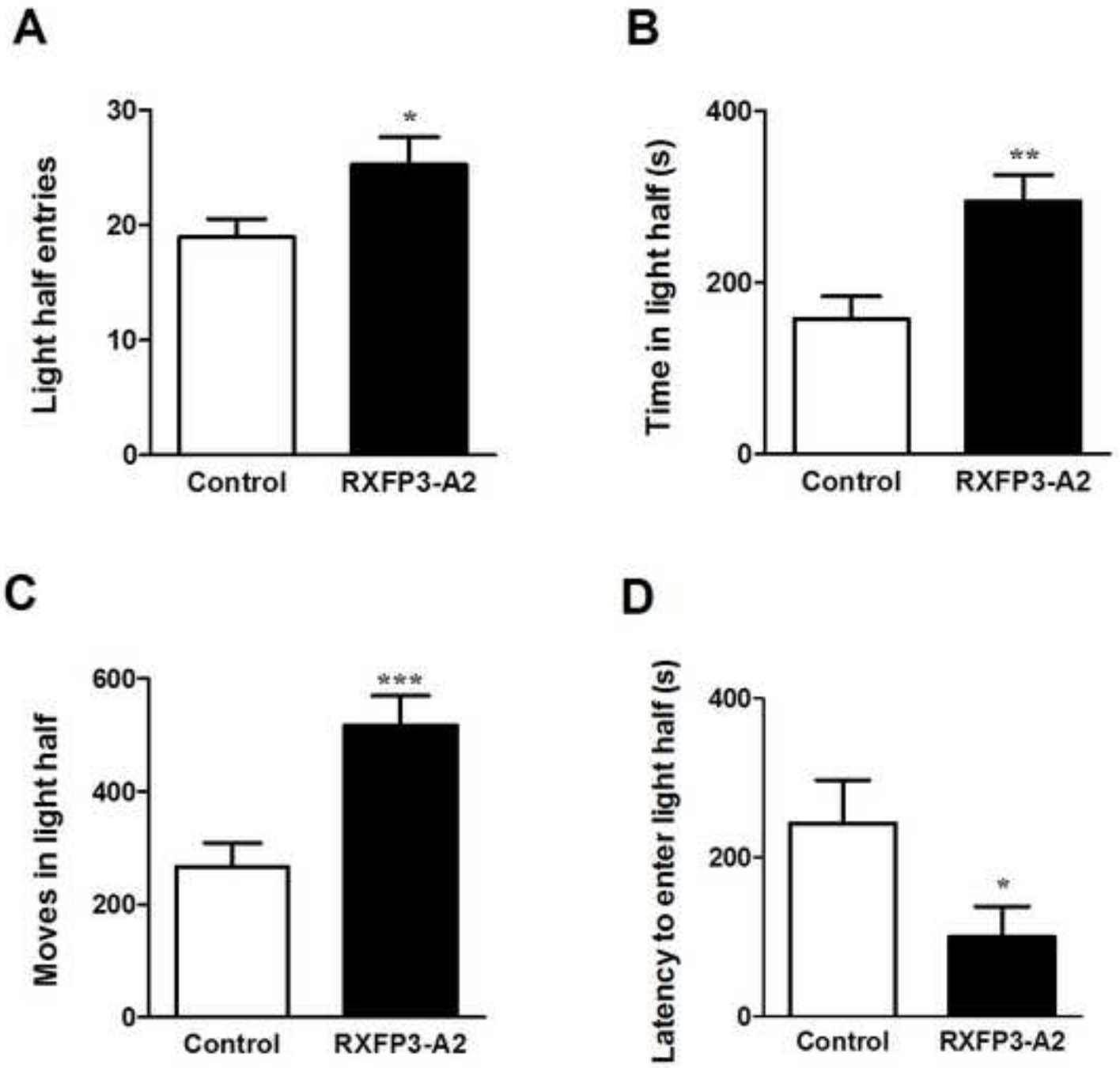

A
Light half entries
30
20
10
0
*
Control
RXFP3-A2
B
Time in light half (s)
400
200
0
**
Control
RXFP3-A2
C
Moves in light half
600
400
200
0
***
Control
RXFP3-A2
D
Latency to enter light half (s)
400
200
0
*
Control
RXFP3-A2

Figure 3

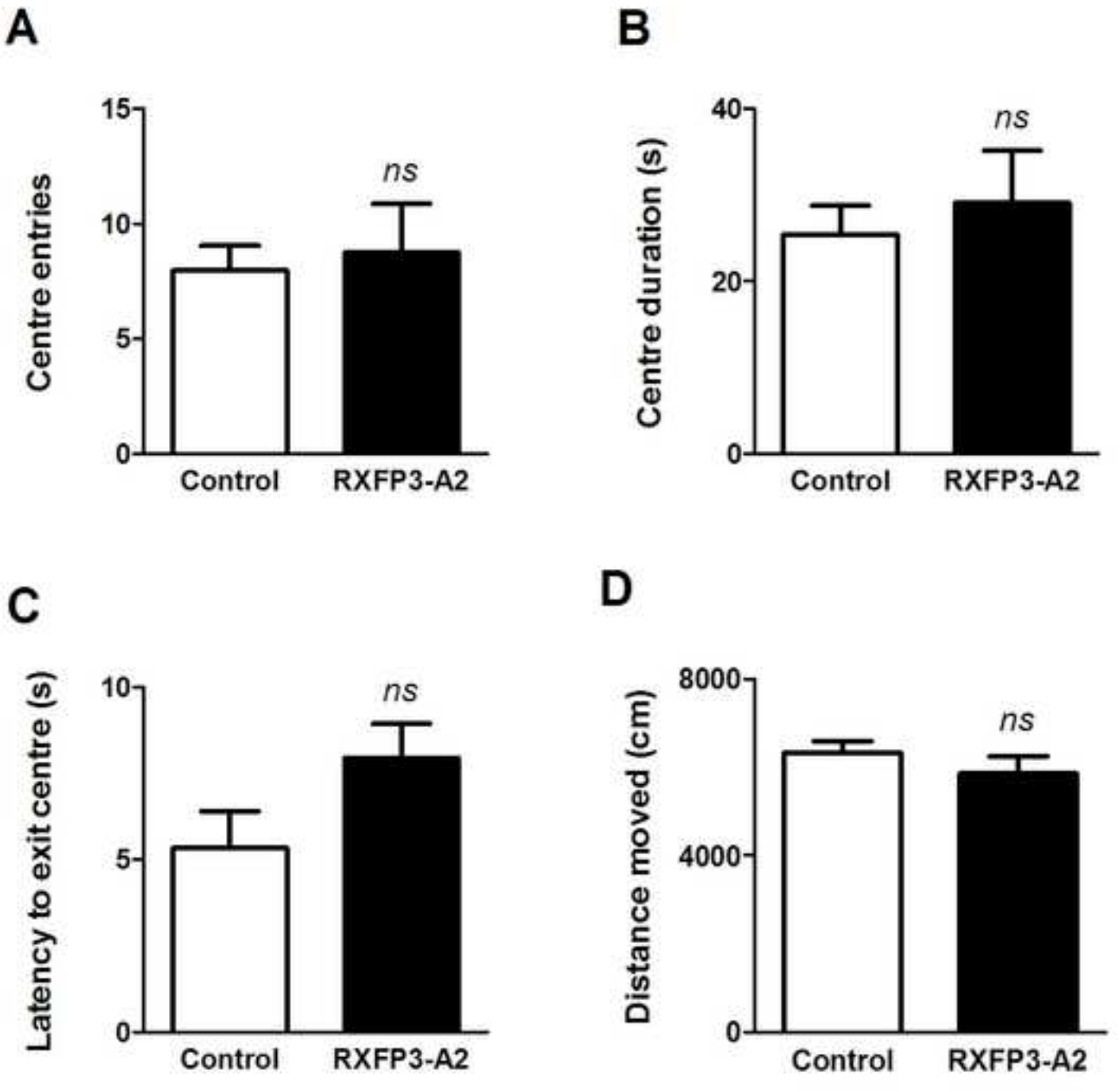

A
Centre entries
15
10
5
0
ns
Control
RXFP3-A2
B
Centre duration (s)
40
20
0
ns
Control
RXFP3-A2
C
Latency to exit centre (s)
10
5
0
ns
Control
RXFP3-A2
D
Distance moved (cm)
8000
4000
0
ns
Control
RXFP3-A2

Figure 4

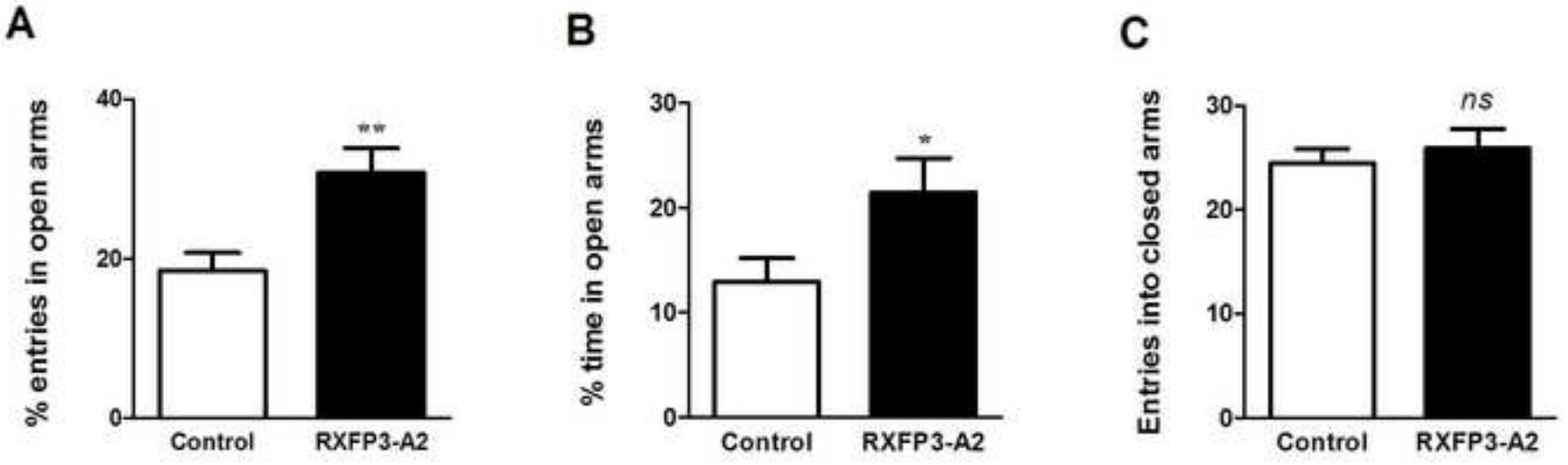


Figure 5

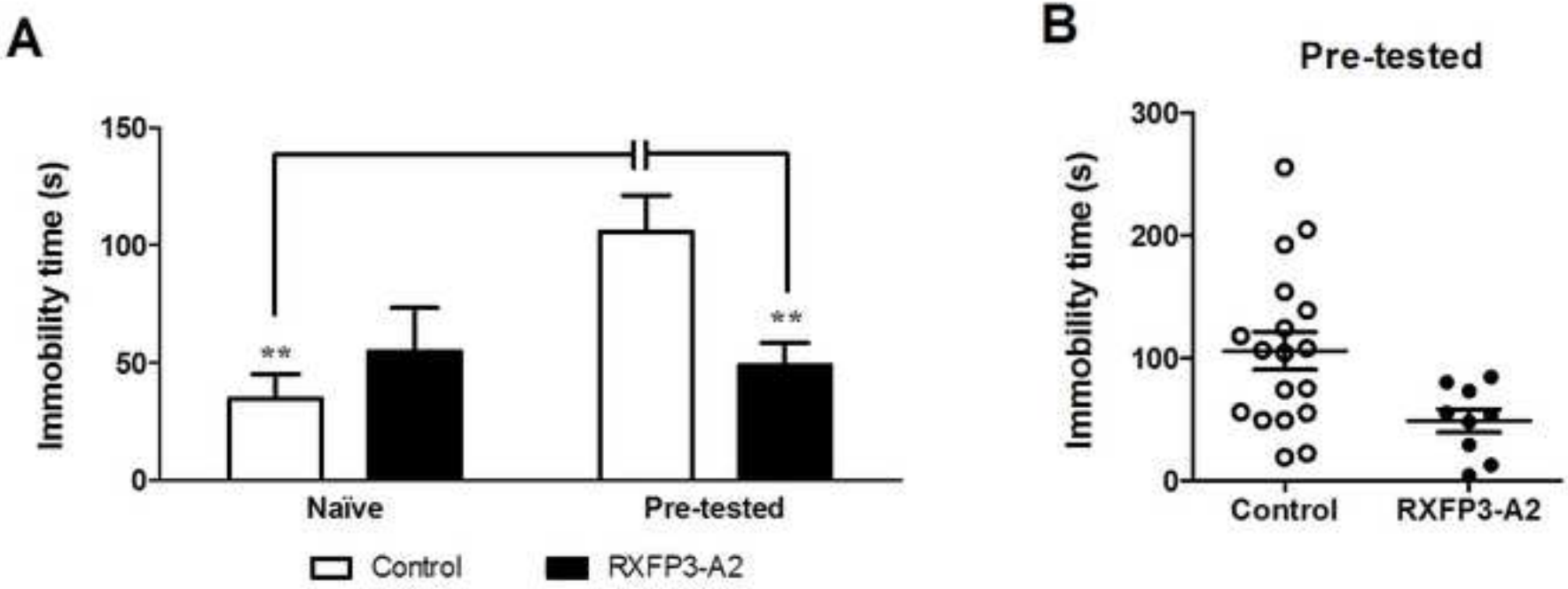

A
Immobility time (s)
150
100
50
0
**
**
Naïve
Pre-tested
Control
RXFP3-A2
B
Pre-tested
Immobility time (s)
300
200
100
0
Control
RXFP3-A2

Figure 6

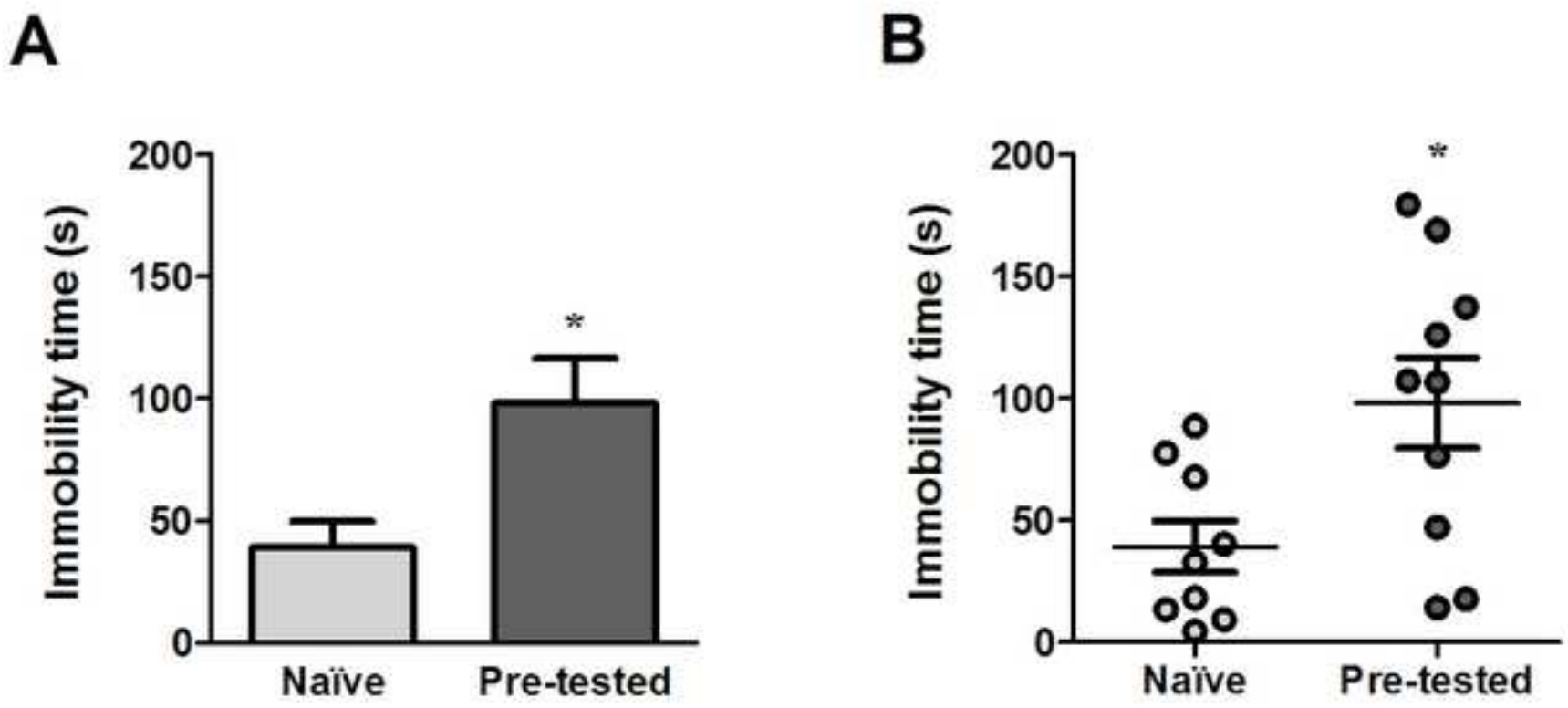
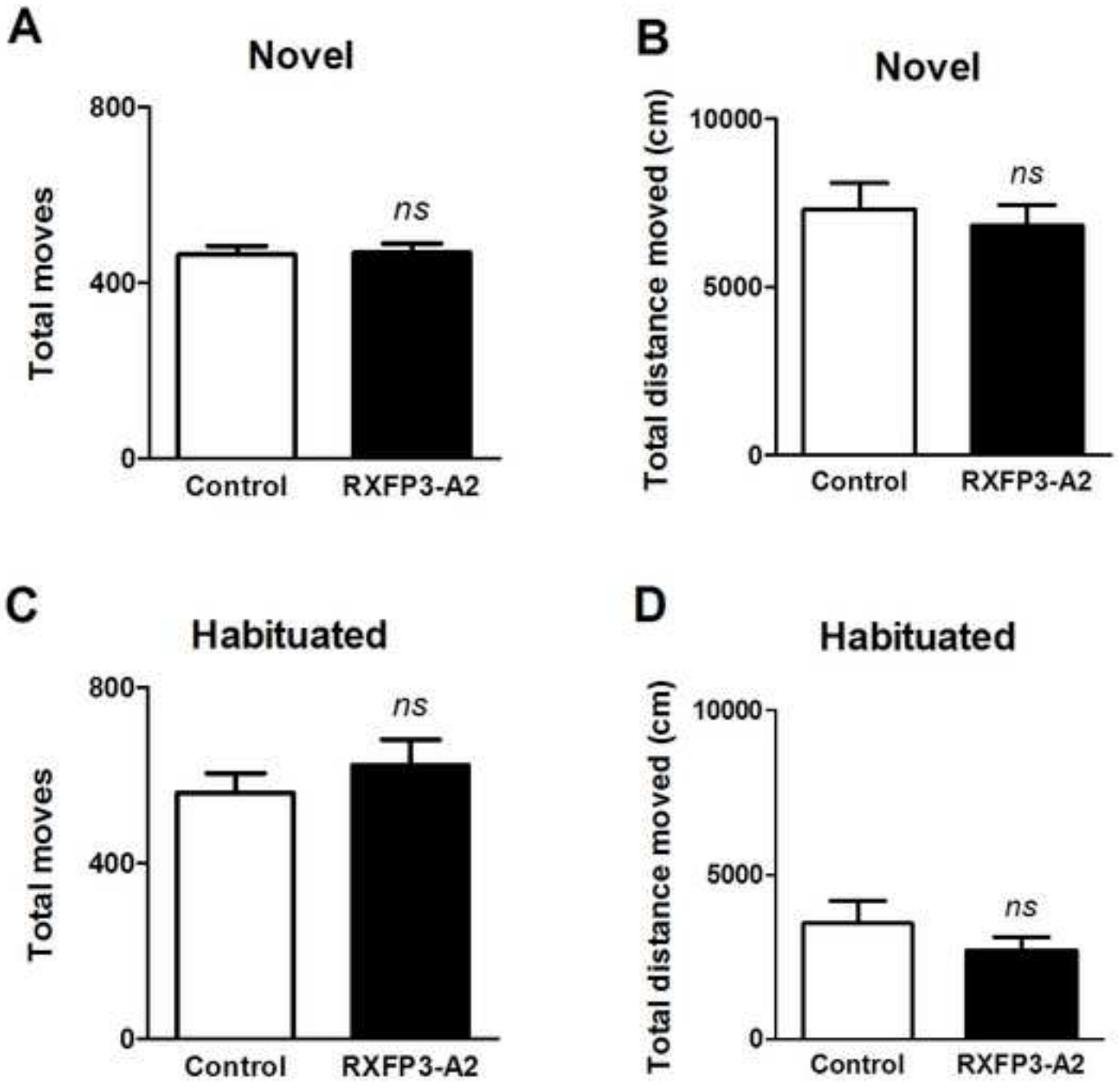

A
Immobility time (s)
200
150
100
50
0
*
Naïve
Pre-tested
B
Immobility time (s)
200
150
100
50
0
*
Naïve
Pre-tested

Figure 7


A
Novel
Total moves
800
400
0
ns
Control
RXFP3-A2
B
Novel
Total distance moved (cm)
10000
5000
0
ns
Control
RXFP3-A2
C
Habituated
Total moves
800
400
0
ns
Control
RXFP3-A2
D
Habituated
Total distance moved (cm)
10000
5000
0
ns
Control
RXFP3-A2

Author/s:
Ryan, PJ; Buechler, E; Shabanpoor, F; Hossain, MA; Wade, JD; Lawrence, AJ; Gundlach, AL

Title:
Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive-like behaviours in the rat

Date:
2013-05-01

Citation:
Ryan, P. J., Buechler, E., Shabanpoor, F., Hossain, M. A., Wade, J. D., Lawrence, A. J. & Gundlach, A. L. (2013). Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive-like behaviours in the rat. BEHAVIOURAL BRAIN RESEARCH, 244, pp.142-151. https://doi.org/10.1016/j.bbr.2013.01.034.