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Central relaxin-3 receptor (RXFP3) activation reduces elevated, but not basal, anxiety-like behaviour in C57BL/6J mice

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Highlights

Current treatments for anxiety disorders are inadequate
Relaxin-3/RXFP3 system has potential as a therapeutic target
Icv RXFP3 agonist did not reduce basal anxiety in mice
Icv RXFP3 agonist reduced pharmacologically-induced anxiety
Icv RXFP3 antagonist induced anxiety in the elevated plus maze test
Relaxin-3/RXFP3 effects on anxiety are conserved between mice and rats

Abstract

Anxiety disorders are among the most prevalent neuropsychiatric conditions, but their precise aetiology and underlying pathophysiological processes remain poorly understood. In light of putative anatomical and functional interactions of the relaxin-3/RXFP3 system with anxiety-related neural circuits, we assessed the ability of central administration of the RXFP3 agonist, RXFP3-A2, to alter anxiety-like behaviours in adult C57BL/6J mice. We assessed how RXFP3-A2 altered performance in tests measuring rodent anxiety-like behaviour (large open field (LOF), elevated plus maze (EPM), light/dark (L/D) box, social interaction). We examined effects of RXFP3-A2 on low ‘basal’ anxiety, and on elevated anxiety induced by the anxiogenic benzodiazepine, FG-7142; and explored endogenous relaxin-3/RXFP3 signalling modulation by testing effects of an RXFP3 antagonist, R3(B1-22)R, on these behaviours. Intracerebroventricular (icv) injection of RXFP3-A2 (1 nmol, 15 min pre-test)
did not alter anxiety-like behaviour under ‘basal’ conditions in the LOF, EPM or L/D box, but reduced elevated indices of FG-7142-induced (30 mg/kg, ip) anxiety-like behaviour in the L/D box and a single-chamber social interaction test. Furthermore, R3(B1-22)R (4 nmol, icv, 15 min pre-test) increased anxiety-like behaviour in the EPM (reflected by reduced entries into the open arms), but not consistently in the LOF, L/D box or social interaction tests, suggesting endogenous signalling only weakly participates in regulating ‘basal’ anxiety-like behaviour, in line with previous studies of relaxin-3 and RXFP3 gene knockout mice. Overall, these data suggest exogenous RXFP3 agonists can reduce elevated (FG-7142-induced) levels of anxiety in mice; data important for gauging how conserved such effects are, with a view to modelling human pathophysiology and the likely therapeutic potential of RXFP3-targeted drugs.

**Key words:** relaxin-3; RXFP3; neuropeptide receptor; arousal; anxiety; stress, FG-7142

1. **Introduction**

Anxiety disorders comprise a highly prevalent, heterogeneous group of clinically defined psychiatric conditions [1, 2], which stem from a wide range of potential causes [3, 4], and are often co-morbid with other psychiatric conditions such as addiction or mood disorders [5, 6]. Elevated levels of anxiety are often associated with heightened stress, linked to combined developmental and traumatic life-events and can be modelled experimentally in animals by subjecting them to acute or chronic stressors, such as predator exposure, neonatal isolation, social defeat, restraint, or chronic unpredictable stress [7]; or treatment with anxiogenic drugs, such as yohimbine or FG-7142 [8-10]. Using these animal models, it is possible to investigate the effectiveness of putative novel treatments and identify new molecular targets or signalling systems for improving existing therapeutics [11, 12].

Relaxin-3 is a highly conserved neuropeptide [13], which acts via a single cognate G_{i/o}-protein-coupled receptor, relaxin family peptide receptor 3 (RXFP3) [14]. The largest population of relaxin-3 expressing neurons is located within the tegmental area known as the nucleus incertus (NI), and these neurons project broadly throughout the brain [15-19]. The neuroanatomy of the relaxin-3/RXFP3 system suggests a broad role as an ascending neuromodulatory network [20, 21], akin to the monoamine systems including serotonin, and noradrenaline [22-25]. Anatomical and functional data [15-18] suggest that relaxin-3/RXFP3 systems may interact directly with monoamine [19, 26] and other peptide systems [27-29],
and/or act at shared downstream limbic and hypothalamic target areas to modulate ‘anxiety’ and other stress-related responses [30-34].

In rats and mice, high levels of RXFP3 mRNA are present within the amygdala, periventricular hypothalamus, ventral hippocampus, periaqueductal grey, and other brain areas/circuits [18, 35, 36] implicated in the modulation of innate anxiety and learned fear (see e.g. [6, 37-39]). Functionally, icv administration in adult Sprague-Dawley rats of the relaxin-3 agonist peptide, 'RXFP3-A2', which is selective for RXFP3 over RXFP1 [40], reduced anxiety-like behaviour [33]. The precise brain sites influenced by RXFP3 to produce these behavioural changes in the anxiety tests studied are not known, but it might involve activation of RXFP3 strongly expressed within areas involved in aversion-motivated exploration such as the extended amygdala, the periaqueductal grey and hippocampus [29, 41-43].

Relatively few experimental studies of the relaxin-3/RXFP3 system have been conducted in mice, but comparative studies with those in rats can indicate how functionally conserved this system is, and indicate the likelihood that findings are translatable to humans. For example, increased feeding and modest body weight gain following acute and chronic central RXFP3 activation is well characterised in the rat (see [31] for review), but icv or intra-hypothalamic injection of a specific RXFP3 agonist does not induce food intake in mice [44] and studies of relaxin-3 and RXFP3 knockout (KO) mice reveal no differences in body weight relative to their wildtype (WT) littermates [45, 46].

In this study we assessed the ability of centrally administered RXFP3 agonist to alter anxiety-like behaviour as reflected by performance in several tests - large open-field (LOF), elevated plus maze (EPM), light/dark (L/D) box and a social interaction test, which have been shown to be effective in detecting effects of new and existing anxiolytic and anxiogenic drugs [47]. We used the selective RXFP3 agonist, RXFP3-A2 [40], and assessed whether exogenous RXFP3 activation altered basal levels or 'elevated, stress-induced' levels of anxiety produced by administration of the partial inverse benzodiazepine agonist, FG-7142, an established anxiogenic drug (see [48]). We also examined the effects of centrally administrated R3(B1-22)R, an RXFP3 antagonist [49], on performance of mice in these same tests of anxiety-like behaviour, to determine whether endogenous relaxin-3/RXFP3 signalling modulates these outputs.

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, in compliance with the guidelines of the National Health and Medical Research Council of Australia. Adult male C57BL/6J mice were obtained from the Australian Research Centre (Canning Vale, WA, Australia). All mice were group-housed prior to guide cannulae implantation, and were then single-housed post-surgery. Mice were housed in a room with a 12 h light-dark cycle (light phase: 0700-1900) and had ad libitum access to standard mouse chow and water.

2.2 Surgery
Mice were anaesthetised with 5% isoflurane and maintained at 2% (flow rate 0.2 L/min) in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Analgesic (Meloxicam 20 mg/kg, sc; Troy Laboratories, Smithfield, NSW, Australia) was pre-administered and an incision was made to expose the skull, which was cleaned and dried with 6% hydrogen peroxide. Two or three small screws were implanted into holes drilled in the skull to anchor the cannula into place once implanted. Another small hole was drilled through the skull (relative to bregma, anterior-posterior: 0.46 mm; medial-lateral: 0.8 mm) and a stainless-steel guide cannula (11 mm, 24-gauge) was inserted such that its’ base was just dorsal to the left lateral ventricle (dorsal-ventral: -1.8 mm). Self-curing acrylic dental cement (Vertex-Dental, Zeist, The Netherlands) was applied to secure the cannula and screws in place. Mice were allowed to recover for at least 5 days.

2.3 Verification of cannula placement
After recovery, successful targeting of the cerebral ventricle was determined by injecting 24 pmol angiotensin II (Auspep, Melbourne, VIC, Australia) in 1 µL artificial cerebrospinal fluid (aCSF; 147 nM NaCl, 4 mM KCl, 0.85 mM MgCl₂, and 2.3 mM CaCl₂) using an 11.5 mm long stainless steel injector (0.3/0.15 mm outer/inner diameter; Small Parts Inc., Miramar, FL, USA) that protruded 0.5 mm below the guide cannula. Injectors were attached to polyethylene tubing (Microtube Extrusions, North Rocks, NSW, Australia) and connected to a Hamilton syringe (Harvard Apparatus, Holliston, MA, USA). A dipsogenic response (multiple drinking episodes totalling >20 s within 5 min) indicated correct targeting, while non-responsive mice were either re-tested with 12.0 mm and 12.5 mm injectors to allow for variation between mice, or excluded from analysis.

2.4 Peptide and drug injections
RXFP3-A2 and R3(B1-22)R were synthesised using solid phase peptide synthesis and purified using reverse phase HPLC, as described [40, 49]. Amino acid composition and purity were checked by reverse phase HPLC and MALDI-TOF mass spectrometry. Peptide stock solutions were made up to the same mass and molar dose by adjustment for purity, which ranged from 60-100%.

For injections, mice were gently restrained in a cloth with the guide cannula exposed. In order to reduce the stress associated with handling, mice were handled and restrained in the same cloth daily for 25 days prior to the first behavioural experiments. Injections (1 µL) were made via an injector protruding beyond the guide cannula (see above) and into the lateral ventricle 15 min before each behavioural test. Doses used and the timing of behavioural testing (see Results) were based on studies that indicated effective doses in rats and mice and the time course of activity of these peptides post-icv injection [29, 33, 44].

FG-7142 (N-methyl-β-carboline-3-carboxamide), a partial inverse agonist at the benzodiazepine allosteric site of the GABA_A receptor (Sigma-Aldrich, Castle Hill, NSW, Australia), was dissolved in 40% 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma-Aldrich)/0.9% saline solution. Vehicle (same as above) or FG-7142 (30 mg/kg) was administered intraperitoneally (ip) in a volume of 10 mL/kg, 30 min before each behavioural test involving induced anxiety (each test was 10 min in duration, Fig. 1A), as earlier reports indicate that 30-60 min post-injection is the period when anxiogenesis is highest [48]. At least 2 days were allowed between different behavioural tests (Fig. 1B) to allow for peptide/drug washout.

2.5 Behavioural testing
All behavioural testing was conducted under dim illumination (20-50 lux at the centre of the apparatus), unless otherwise stated. Behavioural apparatus were washed thoroughly with water between each mouse test, except for the social test, where the apparatus was first washed in a diluted detergent that is frequently used throughout the animal facility, followed by a thorough wash with water to eliminate social smells.

2.5.1 Elevated plus maze
The EPM consisted of an opaque grey acrylic material in a plus-sign shape, 30 cm above ground, with four 4 cm wide, 30 cm long arms. The ‘closed’ arms directly opposite each other had 20 cm high walls, while the other two arms were designated ‘open’. Each mouse was placed in the centre square of the plus maze at the commencement of recording. Each
trial ran for 10 min and movements, including duration and entries into the open and closed arms were tracked using EthoVision XT (Noldus Information Technology, The Netherlands).

2.5.2 Light/dark box
Mice were placed into a 27.5 × 27.5 cm locomotor cell (Med Associates, St. Albans, VT, USA) with a semi-opaque black box insert (which blocks visible light, but not detection photobeams) covering half of the cell. An opening in the box allowed mice to move between the light and dark compartments. The light half was illuminated to approximately 600 lx using an LED array. Mice were initially placed into the dark half of the box and allowed to freely explore for 10 min. Movement was tracked by an array of photobeams and analysed by Activity Monitor V6.02 software (Med Associates).

2.5.3 Single chamber social interaction test
For the FG-7142 studies, a single chamber social interaction test was used to measure social interaction. A single enclosed chamber (13 cm × 39 cm × 11 cm high walls) containing a wire mesh cage (13 cm x 12 cm x 11 cm high) placed at one end was used and two consecutive 10 min trials were conducted. Thirty minutes following FG-7142 injection, the ‘habituation’ trial commenced whereby the test mouse was placed into the chamber with the wire mesh cage empty. For the ‘test’ trial, the test mouse was briefly returned to its home cage while a novel conspecific was placed into the wire mesh cage. The test mouse was then immediately returned to the testing apparatus. Topscan Lite 2.0 (CleverSys) was used for automatic scoring of interaction time, which was defined by the amount of time the nose point of the mouse spent in a 1 cm zone adjacent to the wire mesh cage.

2.5.4 Large open field
The LOF was a circular arena measuring 1 m in diameter, with aluminium walls approximately 50 cm high. The arena was ‘subdivided’ into three zones: the centre (50 cm diameter), middle (17 cm wide ring) and outer (8 cm wide ring); and flood lights mounted on the ceiling which targeted the centre strongly illuminated the arena (800 lux in centre and 600 lux outer). Initially, with flood lights off, mice were placed in the very centre of the arena under an opaque cup connected to an overhead pulley system. The lights were turned on and the cup was lifted allowing the mouse to freely explore the whole arena for 10 min. Time spent in each of the zones was automatically scored by Topscan Lite (CleverSys, VA, USA).
2.6 Statistical analysis

All graphs and data were prepared and analysed using Prism 5.0 software (GraphPad, San Diego, CA, USA). All data in histograms are presented as mean ± SEM. Statistical comparisons were made using unpaired student t-tests in the basal anxiety cohort and the antagonist portion of the induced anxiety cohort. One-way ANOVA were used in FG-7142 experiments to determine significance. Differences between groups were considered statistically significant when p<0.05.

3. Results

Effects of changes in relaxin-3/RXFP3 signalling on anxiety-like behaviour in mice

The effects of icv RXFP3-A2 administration were initially examined in a series of behavioural tests under ‘basal’ or ‘low stress’ conditions, providing indices of basal/low innate anxiety. Based on previous preliminary studies, the effect of RXFP3 activation was also examined in mice displaying pharmacologically-induced higher general and social anxiety. Furthermore, in studies to assess the impact of endogenous relaxin-3/RXFP3 signalling, the effect of icv R3(B1-22)R administration on basal anxiety was examined.

3.1 Icv RXFP3 agonist did not alter low/basal levels of anxiety-like behaviour

We initially assessed whether icv administration of an RXFP3 agonist reduced 'basal' anxiety in mice, as reported in rats [33]. In the three tests of anxiety-like behaviour used (LOF, EPM, and L/D box), icv RXFP3-A2 (1 nmol) did not alter the indices measured (Table 1). For example, in the LOF, both treatment groups (vehicle, n=20; RXFP3-A2, n=12) spent approximately the same time (unpaired t-test, t(30)=0.210, p=0.835) and made a similar number of entries (unpaired t-test, t(30)=0.082, p=0.935) into the centre zone. In the EPM, the time spent in the open arms and the number of entries made into the open arms were not significantly different between treatment groups (unpaired t-test, t(35)=0.210, p=0.835) and made a similar number of entries (unpaired t-test, t(36)=0.082, p=0.935) into the centre zone. In the EPM, the time spent in the open arms and the number of entries made into the open arms were not significantly different between treatment groups (unpaired t-test, t(35)=1.61, p=0.116 and t(36)=1.22, p=0.229, respectively; vehicle, n=20; RXFP3-A2, n=17). Finally, in the L/D box, no significant differences were detected between groups in the time spent in and number of entries into the light side (unpaired t-test, t(36)=0.757, p=0.454 and t(36)=0.032, p=0.974, respectively; vehicle/RXFP3-A2, n=19).

3.2 Icv RXFP3 agonist reduced FG-7142-induced anxiety-like behaviour

Although central RXFP3-A2 administration did not alter the ‘basal’ levels of anxiety expressed by C57BL/6J mice in these studies, data from our rat studies [33] suggests icv
RXFP3-A2 treatment may alter heightened anxiety. The ‘control’ group of rats in this earlier study displayed relatively high levels of anxiety-like behaviour, whereby, on average control rats spent approximately half the time in the light side that control mice did in the current study, which is even more significant considering that 4 of 20 rats tested failed to enter the light half of the L/D box, and were subsequently excluded [33]. We therefore sought to determine whether RXFP3-A2 could alter levels of anxiety-like behaviour induced pharmacologically by FG-7142 [48]. In the L/D box, pre-administration of FG-7142 (30 mg/kg, ip) followed by an icv injection of aCSF (FG-7142/aCSF) significantly increased anxiety-like behaviour, and these mice spent ~50% less time in the light side (~130 s), compared to the previous basal anxiety cohort (~280 s, Fig. 2A, one-way ANOVA, Bonferroni post-test, p<0.001). Importantly, icv RXFP3-A2 reduced the anxiety-like behaviour induced by FG-7142 (FG-7142/RXFP3-A2, 1 nmol), as mice spent significantly more time in the light half (one-way ANOVA, Bonferroni post-test, p<0.01) and made an increased number of entries into the light half (Fig. 2B, unpaired t-test, t(20)=2.44, p=0.024). No difference in locomotor activity was observed (data not shown, unpaired t-test, t(20)=0.981, p=0.338).

However, in the EPM, FG-7142/aCSF (30 mg/kg, ip) did not significantly increase anxiety-like behaviour compared to a previous control cohort with both groups spending a similar amount of time in the open arms (~140 s; Fig. 2C). Consistent with the results obtained in the basal anxiety cohort (Table 1), RXFP3-A2 did not alter anxiety-like behaviour, reflected by time spent in the open arms (one-way ANOVA, p= 0.597) and the number of entries into open arms (Fig. 2D, unpaired t-test, t(27)=0.445, p=0.660).

Due to a lack of FG-7142-induced changes in performance in the EPM, a pilot cohort of mice was used to assess the effect of FG-7142 in the LOF. No change in anxiety-like behaviour was observed and therefore this test was not included in the study.

### 3.3 Effect of FG-7142 on social interaction and the influence of icv RXFP3-A2

In an effort to further investigate the nature of RXFP3 modulation of anxiety-like behaviour, we examined social anxiety using a single-chamber social interaction test. FG-7142/aCSF treated mice (30 mg/kg, ip) displayed significantly reduced levels of social interaction (defined as occurring when the test mouse’s nose point was within 1 cm of the novel mouse cage) compared to control (vehicle/aCSF) mice (Fig. 3, one-way ANOVA, p<0.05). The level of social interaction in FG-7142/RXFP3-A2 treated mice was not significantly different to vehicle/aCSF-treated mice, consistent with a reduction in social anxiety produced by
RXFP3-A2; although the difference between FG-7142/RXFP3-A2 and FG-7142/aCSF groups did not reach statistical significance.

3.4 Effect of icv RXFP3 antagonist on basal anxiety-like behaviour

In light of the data described, we assessed whether RXFP3 antagonism by R3(B1-22)R would alter (induce) innate anxiety-like behaviour under basal conditions. Analysis of innate anxiety in the behavioural tests used previously revealed that icv R3(B1-22)R (4 nmol; a dose shown to reduce motivated food seeking in mice [44]) increased anxiety-like behaviour in the EPM (Table 2). Mice treated with R3(B1-22)R spent ~50% less time in the open arms compared to controls (unpaired t-test, t(23)=2.74, p=0.012), and displayed a trend towards reduced entries into open arms (unpaired t-test, t(23)=1.905, p=0.069). In the LOF, R3(B1-22)R treated mice spent a similar time in the aversive centre zone (unpaired t-test, t(26)=1.498, p=0.146), but made more entries into the centre zone (unpaired t-test, t(26)=2.295, p=0.030). In the L/D box, R3(B1-22)R treated mice did not spend significantly different amounts of time in the light half than aCSF-treated mice (unpaired t-test, t(26)=1.244, p=0.225), and entries into the light half were similar in both groups (unpaired t-test, t(26)=1.208, p=0.238).

In the single chamber social interaction test, R3(B1-22)R treated mice did not spend more or less time investigating a novel mouse, as defined above (t(23)=0.306, p=0.763), or make a significantly different number of nose point entries into the interaction zone (t(23)=0.833, p=0.413), reflecting a lack of modulation of basal social anxiety (data not shown).

4. Discussion

These studies demonstrated that exogenous central RXFP3 activation in mice did not alter indices of basal anxiety, but reduced pharmacologically-induced anxiety-like behaviour, as icv administration of the RXFP3 agonist, RXFP3-A2, reduced anxiety induced by the anxiogenic drug, FG-7142. Furthermore, treatment of mice with the RXFP3 antagonist, R3(B1-22)R, increased an index of innate anxiety-like behaviour in the EPM test, but not in other standard tests (LOF and L/D box). These data suggest that endogenous relaxin-3/RXFP3 signalling plays a subtle role in anxiety-like behaviour under certain basal conditions, while previous studies in rats suggest endogenous relaxin-3/RXFP3 may play more profound roles in response to increased levels of stress and associated stimuli [27-29, 33]. Current findings indicate that effects of RXFP3 activation/inhibition depend on the ‘state
of anxiety’ in mice and that further studies of this possibility and associated sites and mechanisms of action are warranted.

In regard to the latter, RXFP3 mRNA is abundantly expressed in several brain areas associated with control of anxiety-like behaviours, including the well characterised extended amygdala [6, 50-52]. Relaxin-3 is primarily expressed in GABA neurons, as reflected by the co-expression of relaxin-3 and glutamate decarboxylase immunoreactivity in the rat nucleus incertus [18], which suggests RXFP3 activation may act to reinforce inhibitory signalling on target neurons. In line with such a role, in vitro studies have revealed activation of the G_{i/o}-protein-coupled RXFP3 by relaxin-3 and truncated analogues produces a dose-dependent inhibition of cellular cAMP levels [14, 53], and selective RXFP3 agonist peptides directly hyperpolarize neurons recorded in brain slices [54].

Currently it is not possible to precisely describe the role of relaxin-3/RXFP3 signalling in the regulation of basal or elevated stress-induced anxiety, but if mice have low levels of anxiety, the relevant circuits may be minimally activated, and hence central RXFP3 agonist treatment is unlikely to produce detectable reductions. However, if levels of anxiety are heightened, relevant neural circuits may be sufficiently activated such that exogenous RXFP3 agonist treatment can modulate their activity and reduce anxiety-like behavioural responses to aversive environments [55]. We tested this possibility using the anxiogenic partial inverse benzodiazepine agonist, FG-7142, to increase anxiety-like responses. Subsequently, icv RXFP3-A2 administration reduced the elevated levels of anxiety observed in the L/D box. In contrast, FG-7142 did not induce heightened anxiety in the EPM test in the same cohort, and RXFP3-A2 was without effect in this case, consistent with the finding that RXFP3 activation did not alter basal anxiety. FG-7142 has been reported to increase anxiety indices in mice of various strains in this test, but not in C57BL/6 mice, and often using a lower dose than in this study [56, 57].

Although there is a possibility that the doses of RXFP3-A2 or R3(B1-22)R were not sufficient to elicit a response in all assays, the 1 nmol dose of RXFP3-A2 was similar to that used in our rat study [33] and the 4 nmol dose of R3(B1-22)R was based on a study in mice in which a reduction in several measures of motivated food intake were observed [44], indicating that this is a physiologically active dose. Furthermore, our positive data appear comparable to findings in a study in which a cohort of rats spent an average of ~15% of the total test time in the light half of the L/D box [33], similar to the ~20% of total time spent in the light half by FG-7142/aCSF treated mice.
It should be noted, however, that it is possible that the effects we have observed may be specific to FG-7142-induced anxiety. RXFP3 activation may only block FG7142-induced anxiety, rather than more general anxiety. In order to extend our findings to more generalized elevations in innate anxiety in mice, studies using an alternate anxiogenic agent and/or behavioural manipulations to induce an anxious state might provide relevant insights [6-12].

In light of the effects of RXFP3 agonist treatment on ‘elevated’ general anxiety, we also examined the effects of RXFP3 activation on FG-7142-induced social anxiety in a single-chamber, social approach test. FG-7142 induced a significant level of social anxiety which was blunted by icv RXFP3-A2 treatment; but as this effect did not reach statistical significance, further studies are warranted to better determine the robustness of the involvement of relaxin-3/RXFP3 signalling in the control of social anxiety. In this regard, while a dissociation of actions on social and general anxiety has been reported for other neuropeptide/receptor systems such as neuropeptide S/NPSR [58] and in certain genetic disorders [59], high levels of RXFP3 mRNA are present in brain areas relevant to social behaviour control in mice including the olfactory bulb, medial amygdala, and ventral hypothalamus [36, 60-63] (see Allen Brain Atlas, <www.brain-map.org>).

Our studies with the RXFP3 antagonist, R3(B1-22)R, revealed that it increased anxiety-like behaviour in the EPM test only, which suggests endogenous RXFP3 signalling is only weakly involved in basal anxiety-like behaviours, and/or that the effects of RXFP3 signalling are only detectable under specific test conditions. Furthermore, the number of entries into the centre zone of the LOF was significantly increased after R3(B1-22)R, which is suggestive of an anxiolytic effect. However, it has been reported that the LOF is not a reliable method for screening anxiolytic or anxiogenic drugs, as results using similar drugs can yield opposing effects in this test [64]. Furthermore, the effects of neuropeptides on anxiety-like behaviours in the LOF have not been as extensively characterised as other drugs such as benzodiazepines [64] and therefore other anxiety tests may be more appropriate.

As icv injections presumably result in peptide reaching multiple areas in a gradient of concentrations, future studies should aim to identify brain sites where RXFP3 modulation influences anxiety-like behaviours. Although it is apparent from this study that the primary net effect of icv RXFP3 activation is anxiolytic, the contributions of particular brain regions/nuclei to this effect may differ in absolute terms or in magnitude. For example, such differences have been observed in the effects of the nonapeptide, oxytocin. Oxytocin receptor activation in the paraventricular hypothalamic nucleus (PVN) is anxiolytic [66], whereas activation of the same receptor in the lateral septum enhanced fear responses, suggesting an
anxiogenic role [67]. Interestingly, RXFP3 is expressed broadly throughout limbic centres and it is possible similar differential effects on neural outputs occur following RXFP3 activation. Therefore, future studies are warranted of local administration of RXFP3 agonist into candidate areas, such as regions of the extended amygdala or hypothalamus, similar to those of the effects of RXFP3 blockade on alcohol seeking and stress-induced reinstatement in rats [29]. Also, with the heterogeneous nature of neurons in these limbic structures [68, 69], studies examining the neurochemical identity of RXFP3-expressing neurons are equally important. Such studies will significantly increase our understanding of how relaxin-3/RXFP3 signalling modulates anxiety-like behaviours.

5. Conclusion

Although considerable clinical and preclinical research is focussed on anxiety disorders, current treatments are inadequate and these psychiatric conditions are highly prevalent and increasing globally [70]. Thus, RXFP3 may represent a new therapeutic target, as the current studies reveal that central administration of a relaxin-3 analogue was able to reduce elevated, pharmacologically-induced levels of anxiety. Furthermore, our studies have revealed that the modulation of anxiety-like behaviours via RXFP3 occurs in both rats and mice, suggesting this action is conserved and that both species are suitable for the investigation of relaxin-3/RXFP3 targeted therapeutics for anxiety disorders in humans. However, due to the extensive nature of this peptidergic system [18, 20, 36], further studies are needed to determine other major behavioural effects of RXFP3 signalling and the nature of interactions with other key transmitter systems under basal and pathological conditions, in order to gain a better understanding of the therapeutic potential of RXFP3-targeted drugs.

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References


Figures And Tables:
Figure 1. **Timing of behavioural testing.** (A) Schematic illustrating the timing of FG-7142 ip injections (for induced-anxiety studies) and RXFP3-A2 or R3(B1-22)R icv injections before behavioural testing. (B) Experimental time course illustrating the order of behaviour tests conducted on each cohort.

Figure 2. **Effect of RXFP3 activation on FG-7142-induced anxiety-like behaviour.** (A) Mice given an ip injection of FG-7142 displayed significantly reduced time spent in the light half compared to control mice from the previous basal anxiety cohort (see Table 1). RXFP3-A2 treatment significantly increased the time spent in the light zone in mice pre-treated with FG-7142. (B) Number of entries into the light half was significantly increased in FG-7142/RXFP3-A2 treated mice compared to mice treated with FG-7142/aCSF. (C) FG-7142 did not significantly alter the time spent in open arms of the elevated plus maze compared to control levels in the previous cohort, and subsequently, RXFP3-A2 did not alter this level of ‘basal’ anxiety. (D) FG-7142/RXFP3-A2 treated mice were not different to mice treated with FG-7142/aCSF in their open arm entries. Data are expressed as mean ± SEM, n = 10-15/group. *** p<0.001 compared to control in first cohort. **p<0.01 compared to FG-7142/aCSF treated mice. *p<0.05 compared to FG-7142 treated mice.

Figure 3. **Effect of RXFP3 activation on FG-7142-induced social anxiety.** Mice pre-treated with FG-7142 prior to the social interaction test spent less nose-point time in the interaction zone (i.e. displayed reduced social interaction) than vehicle-treated mice. Values for FG-7142/RXFP3-A2 treated mice were not significantly different to either vehicle/aCSF or FG-7142/aCSF groups. Entries into the interaction zone were not significantly different. Data are expressed as mean ± SEM, n = 10-15/group. *p<0.05 compared to vehicle/aCSF-treated mice.

Table 1. Effects of central RXFP3 activation by RXFP3-A2 on performance in tests of basal anxiety-like behaviour in C57BL/6J mice

<table>
<thead>
<tr>
<th>Test</th>
<th>Parameter</th>
<th>Vehicle</th>
<th>RXFP3-A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large open field</td>
<td>Time in centre (s)</td>
<td>68.4 ± 6.4</td>
<td>70.5 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>Entries into centre (#)</td>
<td>39.4 ± 3.2</td>
<td>38.9 ± 4.1</td>
</tr>
<tr>
<td>Elevated plus maze</td>
<td>Time in open arms (s)</td>
<td>125.7 ± 12.0</td>
<td>101.0 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>Entries into open arms (#)</td>
<td>20.1 ± 2.1</td>
<td>16.7 ± 1.7</td>
</tr>
<tr>
<td>Light/dark box</td>
<td>Time in light side (s)</td>
<td>281.0 ± 14.5</td>
<td>263.6 ± 17.8</td>
</tr>
<tr>
<td></td>
<td>Entries into light side (#)</td>
<td>22.2 ± 1.1</td>
<td>22.2 ± 1.2</td>
</tr>
</tbody>
</table>

No significant differences were detected between treatment groups in any of the tests. Data are expressed as mean ± SEM, n = 12-20/group.

Table 2. Effects of central RXFP3 inhibition by R3(B1-22)R on performance in tests of basal anxiety-like behaviour in C57BL/6J mice

<table>
<thead>
<tr>
<th>Test</th>
<th>Parameter</th>
<th>Vehicle</th>
<th>R3(B1-22)R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large open field</td>
<td>Time in centre (s)</td>
<td>86.5 ± 4.9</td>
<td>98.0 ± 5.94</td>
</tr>
<tr>
<td></td>
<td>Entries into centre (#)</td>
<td>27.3 ± 1.5</td>
<td>33.4 ± 2.2*</td>
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<tr>
<td>Elevated plus maze</td>
<td>Time in open arms (s)</td>
<td>150.9 ± 13.8</td>
<td>82.9 ± 21.9*</td>
</tr>
<tr>
<td></td>
<td>Entries into open arms (#)</td>
<td>20.6 ± 1.6</td>
<td>14.1 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>Time in light side (s)</td>
<td>200.7 ± 29.2</td>
<td>151.9 ± 25.4</td>
</tr>
</tbody>
</table>
Mice treated with R3(B1-22)R made an increased number of entries into the centre of the LOF and spent less time in the open arms of the EPM, but performed similarly to vehicle-treated mice in the light/dark box. Data are expressed as mean ± SEM, n = 12-15/group. *p<0.05 compared to vehicle-treated mice.

<table>
<thead>
<tr>
<th>Light/dark box</th>
<th>Time in light side (s)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>200.7 ± 29.2</td>
</tr>
<tr>
<td></td>
<td>151.9 ± 25.4</td>
</tr>
</tbody>
</table>
A  
FG-7142 injection (for induced anxiety)  
RXFP3-A2/R3(B1-22)R injection  
15 min  
15 min  
Behavioural testing

B  
Cohort 1: Effects of RXFP3-A2 on basal anxiety  
Elevated plus maze → Light/dark box → Large open field

Cohort 2: Effects of RXFP3-A2 on FG-7142-induced anxiety  
Elevated plus maze → Light/dark box → Social interaction

Cohort 3: Effects of R3(B1-22)R on basal anxiety  
Elevated plus maze → Light/dark box → Large open field → Social interaction

Zhang_BBR-D-15-00176R1_Figure 1
Zhang_BBR-D-15-00176R1_Figure 3